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Electrochemical and Biochemical Analysis of Ethanol Fermentation of *Zymomonas mobilis* KCCM11336

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An electrochemical bioreactor (ECB) composed of a cathode compartment and an air anode was used in this study to characterize the ethanol fermentation of *Zymomonas mobilis*. The cathode and air anode were constructed of modified graphite felt with neutral red (NR) and a modified porous carbon plate with cellulose acetate and porous ceramic membrane, respectively. The air anode operates as a catalyst to generate protons and electrons from water. The growth and ethanol production of *Z. mobilis* were 50% higher in the ECB than were observed under anoxic nitrogen conditions. Ethanol production by growing cells and the crude enzyme of *Z. mobilis* were significantly lower under aerobic conditions than under other conditions. The growing cells and crude enzyme of *Z. mobilis* did not catalyze ethanol production from pyruvate and acetaldehyde. The membrane fraction of crude enzyme catalyzed ethanol production from glucose, but the soluble fraction did not. NADH was oxidized to NAD^+ in association with H_2O_2 reduction, *via* the catalysis of crude enzyme. Our results suggested that NADH/NAD^+ balance may be a critical factor for ethanol production from glucose in the metabolism of *Z. mobilis*, and that the metabolic activity of both growing cells and crude enzyme for ethanol fermentation may be induced in the presence of glucose.

Keywords: *Zymomonas mobilis*, electrochemical bioreactor, neutral red, ethanol fermentation, hydrogen peroxidase

Zymomonas mobilis is anticipated to be a useful organism for biological ethanol production and industrial applications, because it is quite well adapted to a great variety of plant juices and saps [1, 4, 16, 18, 21, 28]. *Z. mobilis* anaerobically ferments sugar *via* the Entner-Doudoroff pathway, yielding ethanol and CO_2 [3, 7, 27, 30]. The cell-free extract of *Z. mobilis* has been shown to catalyze ethanol production from glucose [31], which is a very specific feature that is not evidenced by the cell extract of *Saccharomyces cerevisiae*. However, the reason why the crude enzyme of *Z. mobilis* can catalyze the multistep pathway from glucose to ethanol, as well as the mechanisms underlying this phenomenon, have yet to be determined. *Z. mobilis* harbors NADH-dependent membrane-bound oxidase and superoxide dismutase, by which oxygen can be reduced to water in association with the oxidation of NADH to NAD^+ [5, 19]. NADH oxidation, coupled with oxygen reduction, exerts a deleterious influence on ethanol fermentation, because alcohol dehydrogenase is dependent upon NADH [10].

A higher NADH/NAD^+ balance may prove helpful in maintaining ethanol production in the presence of oxygen. The electrochemically reduced neutral red (NR) can catalyze NADH-regeneration without enzyme catalysis, which is useful for the control of NADH-dependent enzyme reactions [11, 20, 26]. Theoretically, NADH/NAD^+ may be balanced in all types of fermentation metabolisms, as the NADH regenerated in association with substrate oxidation is oxidized in association with the final product reduction. However, a variety of intracellular factors, including radicals and noxious materials, may cause a reduction in the NADH/NAD^+ balance [8, 9, 13].

In this study, we assessed ethanol production from glucose, pyruvate, and acetaldehyde *via* the catalysis of the growing cells, crude enzyme, soluble fraction, and membrane fraction of *Z. mobilis*, in order to characterize the fermentation metabolism using an electrochemical bioreactor (ECB) specifically designed to control ethanol fermentation metabolism.

Materials and Methods

Chemicals

All chemicals and coenzymes utilized in this research were purchased from the Korean branch of Sigma-Aldrich Co. (Yongin, Kyunggi-do, Korea), except for the medium ingredients and Bradford reagent (Bio-Rad, U.S.A.).

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Electrochemical Bioreactor

An ECB (working volume, 300 ml) was newly designed in this study to induce an electrochemical cathodic (reduction) reaction without anolyte and anode compartments, as shown in Fig. 1. A three-layered electrode composed of cellulose acetate film (35 μM thickness; Electron Microscopy Science, U.S.A.), porous ceramic membrane (2 mm thickness), and porous carbon plate (20 mm thickness) was substituted for the anolyte and anode compartment. The porous ceramic membrane and porous carbon plate were constructed on the basis of the method developed by Park and Zeikus [21, 22]. The modified graphite felt (100 mm×40 mm, thickness 10 mm; Electrosynthesis, U.S.A.) with NR was employed as a cathode [21, 22]. NR can catalyze the electrochemical reduction of NAD^+ to NADH without enzyme catalysis [26]. Water contained in the catholyte was transferred to the porous carbon plate (anode) through the semipermeable cellulose acetate film, and electrolyzed into protons, electrons, and oxygen inside the anode as shown in Fig. 1B. Water vapor diffused from the surface of the anode was confirmed qualitatively with a cooling device, and the oxygen generated from the anode was detected with a GC (Acme 6000; Young Lin, Korea) equipped with a Carboxen 1000 column (Supelco, U.S.A.). Column temperature and carrier gas (Ar) flow rates were adjusted to 30 ml and 35°C, respectively. DC -3 volts of electricity were charged to the cathode in order to induce a reduction reaction. The DC power supply drives electron flow from the anode to cathode, by which proton translocation from the anode to the catholyte can be activated (Fig. 1B). The oxidation-reduction potential (ORP) of the catholyte was measured continuously to monitor the function of the ECB.

Microorganisms

Zymomonas mobilis KCCM11336, which was used as a biocatalyst, was cultivated in a medium containing 5 g/l of yeast extract and 1.0 M glucose (YG medium) at 30°C. The fresh *Z. mobilis* cultivated for 24 h in the YG medium was employed as a seed.

Preparation of Biocatalysts

The fresh culture of *Z. mobilis* was harvested *via* 30 min of centrifugation at 5,000 ×*g* and 4°C, and then washed twice in 25 mM phosphate buffer (pH 7.0). The washed cells were then disrupted *via* 10 min of 5 second-pulsed ultrasonication (400 W) at 4°C or by 120 min of lysozyme treatment (100,000; units, Sigma,

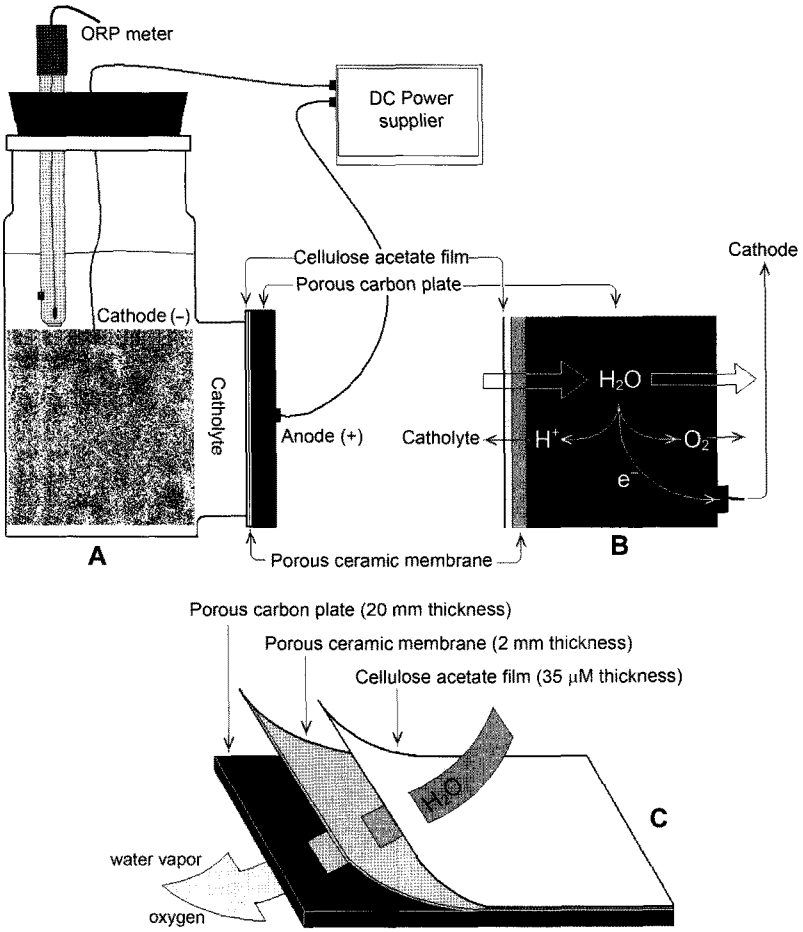

Fig. 1. Schematic structure of the electrochemical bioreactor (**A**), mechanism of anodic reaction (**B**), and schematic structure of the three-layered anode (**C**).

Water transferred from the cathode compartment to the porous carbon anode through the cellulose acetate film may be electrolyzed to proton, electron, and oxygen. Water or gas can penetrate the cellulose acetate film but solutes cannot.

U.S.A.) at 20°C and pH 6.5. The cell-free extract was prepared from the disrupted cells *via* 50 min of centrifugation at 4°C and 15,000 ×*g*; this was the crude enzyme. To prepare the membrane and soluble fractions, the cell-free extract was fractionated *via* 90 min of ultracentrifugation at 4°C and 150,000 ×*g*. The supernatant was decanted and saved as the soluble fraction (membrane-free extract), and the clear brown precipitant was washed twice in 25 mM phosphate buffer (pH 7.0) and resuspended in the same buffer *via* homogenization, and then saved as the membrane fraction [20].

Ethanol Production by Growing Cells

The bacterial cells were cultivated in the ECB to induce the electrochemical reduction reaction, under anoxic nitrogen conditions to induce a neutral redox reaction, under anaerobic hydrogen conditions to induce a chemical reduction reaction, and under aerobic conditions to induce the oxidation reaction. To prepare the anaerobic hydrogen conditions, the bacterial cells were cultivated in a 500-ml gas-tight bottle sealed with a butyl rubber stopper. After autoclaving, dissolved oxygen was purged from the medium (250 ml) *via* 30 min of 99.99% hydrogen gassing, after which the hydrogen-filled headspace (250 ml) was connected to a Teddler bag (10 l; SKC, U.S.A.) containing 2 l of hydrogen to release the pressure of the carbon dioxide generated by the bacterial culture. The anoxic nitrogen conditions were prepared *via* the same method used to achieve the anaerobic hydrogen condition, except that 99.99% nitrogen was used. The bacterial culture was incubated at 120 rpm in a rotary shaking incubator in order to maintain the aerobic conditions.

Ethanol Production by Cell-Free Extract

The cell-free extract, membrane fraction, and soluble fraction were obtained *via* lysozyme treatment, but some of them were obtained *via* ultrasonic treatment for a specific purpose. Ethanol production from glucose, pyruvate, and acetaldehyde was assessed and compared in the ECB, under aerobic, anoxic nitrogen, and anaerobic hydrogen conditions. The basal reaction mixture was composed of 50 μM ADP and 100 mM substrates in 50 mM Tris-HCl buffer (pH 7.5). The NADH concentration added to the basal reaction mixture was adjusted to 2 mM; however, NAD^+ was at 100 μM, thereby allowing for the electrochemical regeneration of NADH from NAD^+. TritonX-100 (100 μM) was added to a reactant catalyzed by the crude enzyme in order to separate the membrane-binding proteins from the membrane. The protein concentration of the crude enzyme was adjusted to 10 mg/ml, which was confirmed with Bradford reagent (BioRad, U.S.A.). Ethanol production from glucose, pyruvate, and acetaldehyde by the crude enzyme extracted from *Saccharomyces cerevisiae* ATCC26603 was tested and compared under reaction conditions identical to those utilized with *Z. mobilis*.

Assay of NADH-Dependent Hydrogen Peroxidase

The hydrogen peroxidase in the cell extract was assayed spectrophotometrically at 340 nm by measuring the oxidation of NADH to NAD^+ coupled to the reduction of hydrogen peroxide to water. The protein concentration of the crude enzyme was 17.4 mg/ml, and 50 mM of hydrogen peroxide diluted in 50 mM Tris-HCl buffer (pH 7.5) was utilized as a substrate. Samples isolated from the reactor at 5-min intervals were refrigerated for 10 min at 0°C in order to halt the enzyme reaction, and then centrifuged for 10 min at 12,000 ×*g* and 4°C to remove oxygen bubbles generated by the catalase. The samples were then subjected to spectral analysis by scanning in a range of wavelengths from 400 nm to 250 nm. The specific activity was defined as the μM of NADH consumed coupled to the reduction rates of hydrogen peroxide per minute and milligram protein. The NADH concentration was calculated using the millimolar extinction coefficient of NADH ($\varepsilon 340=6.23\ mM^{-1}cm^{-1}$) and the Beer-Lambert equation ($Ab=\varepsilon lc$).

Cyclic Voltammetry

The cyclic voltammetry was conducted using a glassy carbon electrode (5 mm diameter) as a working electrode, platinum wire as a counter-electrode, and Ag/AgCl as a reference electrode. The reaction mixture was composed of 50 mM Tris-HCl buffer (pH 7.5), 100 μM NR, 5 mM NaCl, and cell extract (12 mg/ml protein) or intact cells ($OD_{660}=3.0$). Cyclic voltammetry was conducted using a cyclovoltammetric potentiostat (BAS model CV50W, U.S.A.) linked to a data acquisition system. Prior to use, the electrodes were cleaned with an ultrasonic cleaner, and dissolved oxygen in the reaction mixture was purged *via* nitrogen (99.999%) gassing. The scanning rate was 25 mV/s over a range of 0.0 volts to −0.8 volts. In order to observe the electrochemical reaction for the ethanol fermentation metabolism of intact cells or cell extracts, 10 mM of glucose, pyruvate, or acetaldehyde was injected into the reactor with a microsyringe at the moment the second cycle was completed. Additionally, variations were observed in the heights of the upper peak (reduction reaction) and the lower peak (oxidation reaction).

SDS-PAGE

The SDS-PAGE technique utilized in the present study was adapted from the method developed by Laemmli [12]. The cell-free extract, membrane fraction, and soluble fraction prepared as the biocatalyst were used as the samples for SDS-PAGE. The protein concentration was determined with Bradford reagent (BioRad) as a coloring agent and bovine serum albumin as a protein standard.

Analysis

The glucose and ethanol contained in the bacterial culture or enzyme reactant were analyzed *via* HPLC with an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, U.S.A.) and a refractive index detector. The column and detector were adjusted to a temperature of 35°C. The mobile phase was sulfuric acid (0.008 N) and the flow rate was 0.6 ml/min. The samples prepared *via* 30 min of centrifugation at 12,000 ×*g* and 4°C were filtrated with a membrane filter with a pore size of 0.22 μm in order to remove the microparticles. The filtrate was then injected into the HPLC injector, and the injection volume was controlled automatically with a 20-μl loop. The concentrations of glucose and ethanol were calculated on the basis of the peak area in the chromatograms obtained with standard materials.

RESULTS

Ethanol Production by Growing Cell

Ethanol production by the growing cells of *Z. mobilis* under the ECB (electrochemically induced reduction condition), anoxic nitrogen, anaerobic hydrogen, and aerobic conditions was compared among groups to estimate the effect of oxidation-reduction potential on bacterial metabolism and select the optimal ethanol fermentation conditions. As

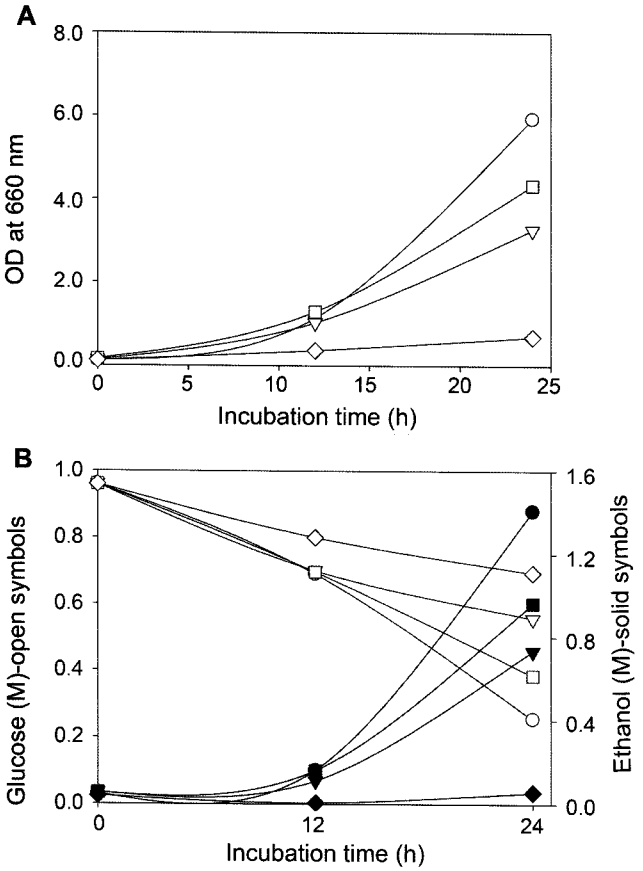

Fig. 2. Growth (**A**) of *Z. mobilis* KCCM11336 in the electrochemical bioreactor (○), and under anaerobic hydrogen condition (▽), anoxic nitrogen condition (□), and aerobic condition (◇); and ethanol production in coupling with glucose consumption (**B**) by catalysis of growing cells of *Z. mobilis* in the electrochemical bioreactor (○, ●), and under anaerobic hydrogen condition (▽, ▼), anoxic nitrogen condition (□, ■), and aerobic condition (◇, ◆).

is shown in Fig. 2, growth and ethanol production were highest in the ECB but were lowest under aerobic conditions, and ethanol production was directly proportional to the growth. Hydrogen can function as a reducing agent like the electrochemical cathodic reaction, but activated ethanol production at a lesser degree than was noted under the anoxic nitrogen conditions. The ratio of ethanol production/glucose consumption by *Z. mobilis* was 1.97 in the ECB condition, 1.66 in the anoxic nitrogen condition, 1.74 in the anaerobic hydrogen condition, and 0.23 in the aerobic

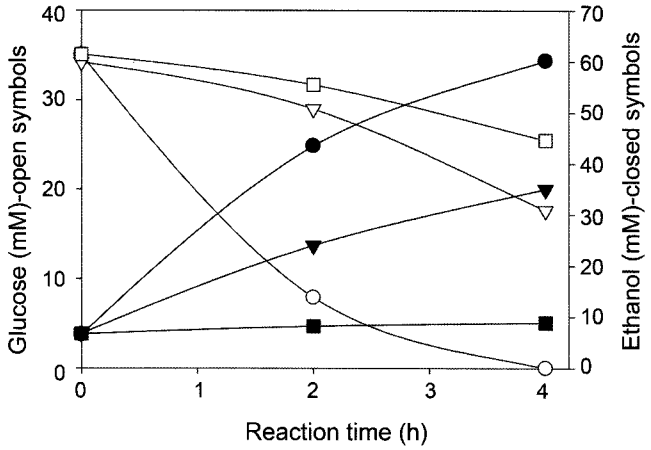

Fig. 3. Ethanol production in coupling with glucose consumption by catalysis of crude enzyme extracted from *Z. mobilis* in the electrochemical bioreactor (○, ●), and under anoxic nitrogen condition (▽, ▼) and aerobic condition (□, ■).

conditions. Ethanol productivity was 1.42 M in the ECB condition, 0.96 M in the anoxic nitrogen condition, 0.73 M in the anaerobic hydrogen condition, and 0.06 M in the aerobic condition, as is shown in Table 1. No ethanol was generated from pyruvate by the growing *Z. mobilis* cells under any of the experimental conditions (data not shown).

Ethanol Production by Crude Enzyme

The patterns of ethanol production by the crude enzyme of *Z. mobilis* under the different experimental reaction conditions were quite similar to those observed with the growing cells, as is shown in Fig. 3. In particular, ethanol production from glucose *via* the catalysis of the crude enzyme is a very peculiar phenomenon, as the metabolic pathway from glucose to ethanol is composed of 7 to 11 steps, which are catalyzed by different enzymes [29]. In identical experiments using pyruvate and acetaldehyde rather than glucose, no ethanol was produced (Table 2).

Ethanol Production by Fractionated Crude Enzyme

In an effort to gain insight into the manner in which the crude enzyme can catalyze ethanol production from glucose in the serial pathway requiring 7 to 11 enzymes, the crude enzyme was prepared physically *via* sonication, or biochemically *via* lysozyme treatment, and then simply

Table 1. Correlation between growth and ethanol production, and glucose consumption and ethanol production of *Z. mobilis* grown in the electrochemical bioreactor, and under anoxic nitrogen condition, anaerobic hydrogen condition, and aerobic condition, for 24 h.

Growth conditions	Growth (OD_{660})	Ethanol production (M)	Glucose consumption (M)	Ethanol/Glucose (ratio)	Ethanol/OD (ratio)
Electrochemical reduction	6.0	1.42	0.72	1.97	0.24
Anoxic nitrogen	4.3	0.96	0.58	1.66	0.22
Anaerobic hydrogen	3.2	0.73	0.42	1.74	0.23
Aerobic	0.7	0.06	0.26	0.23	0.09

Table 2. Ethanol production from glucose, pyruvate, and acetaldehyde by catalysis of crude enzyme extracted from *Z. mobilis* KTCC11336 in different reaction conditions in the electrochemical bioreactor (ECB) and conventional bioreactor (CB).

Reactors	Reaction conditions	Ethanol production (mM/h/mg protein) from		
		Glucose	Pyruvate	Acetaldehyde
CB	CE+2 mM NADH	1.6	0	0
	CE+2 mM NADH+Triton X-100	0.2	0	0
	CE*+2 mM NADH	0.1	0	0
	CE*+2 mM NADH+Triton X-100	0.1	0	0
ECB	CE+100 μM NAD^+	8.8	0	0
	CE+100 μM NAD^++Triton X-100	0.4	0	0
	CE*+100 μM NAD^+	0.1	0	0
	CE*+100 μM NAD^++Triton X-100	0.2	0	0
ECB	MF+100 μM NAD^+	6.9	0	0
	SF+100 μM NAD^+	0.2	0	0
	MF*+100 μM NAD^+	0.1	0	0
	SF*+100 μM NAD^+	0	0	0

*These were physically prepared by ultrasonication.
Crude enzyme (CE), membrane fraction (MF), and soluble fraction (SF) were used as a biocatalyst.

fractionated into the membrane and soluble fractions. As is shown in Table 2, ethanol was generated from glucose *via* the catalysis of the enzymatically prepared crude enzyme and the membrane fraction, but no ethanol was generated from glucose *via* the physically prepared crude enzyme. The crude enzyme treated with Triton X-100 did not catalyze ethanol production from glucose. Meanwhile, the biochemically prepared crude enzyme and membrane fraction did not catalyze ethanol production from pyruvate and acetaldehyde. The crude enzyme of *Saccharomyces cerevisiae* did not catalyze ethanol production from glucose, but did catalyze ethanol production from pyruvate and acetaldehyde (Table 3).

NADH-Dependent Hydrogen Peroxidase

Dissolved oxygen in the bacterial culture or enzyme reactant may be the impetus behind the generation of hydrogen peroxide, in association with the oxidation of some reducing powers. The hydrogen peroxide can be reduced to water by catalase and hydrogen peroxidase. Catalase activity was analyzed qualitatively *via* the observation of oxygen bubbles generated from the reactant (data not shown). The oxygen generated from hydrogen peroxide by catalase may constitute a potential source for the generation of another radical. Accordingly, the complete removal of radical potential by hydrogen peroxidase was required. Specific activity calculated with the difference in absorbance between the initial value and the value at 5 min after initial reaction was 1.198 μM NADH/min/mg protein (see Fig. 4).

Cyclic Voltammetry

To determine why ethanol was produced from glucose only, and not from pyruvate and acetaldehyde by the catalysis of the crude enzyme and intact cells, the cyclic voltammetry technique was applied to the modified intact cells or to the crude enzyme with NR. As shown in Fig. 5, the downward peak height of the cyclic voltammogram was increased by the addition of glucose, but not by pyruvate or acetaldehyde.

SDS-PAGE Pattern of Cell-Free Extract

Proteins contained in the biochemically prepared crude enzyme, membrane fraction, or the soluble fraction appeared to be more stable or less damaged than the physically prepared ones, as shown in Fig. 6. In the SDS-PAGE pattern of the physically prepared crude enzyme, soluble, and membrane fraction, some proteins appear to be dispersed, and a big band indicating low-molecular weight clearly appeared. In particular, the big protein bands showing in the SDS-PAGE pattern of the enzymatically prepared

Table 3. Ethanol production from glucose and pyruvate by catalysis of crude enzyme (cell-free extract) extracted from *Saccharomyces cerevisiae* ATCC26603 in the electrochemical bioreactor (ECB) and conventional bioreactor (CB).

Reactors	Reaction conditions	Ethanol production (mM/h/mg protein) from		
		Glucose	Pyruvate	Acetaldehyde
CB	CFE+2 mM NADH	0	0.6	1.3
ECB	CFE+100 μM NAD^+	0	1.8	4.2

Protein concentration of crude enzyme was adjusted to 10 mg/ml.

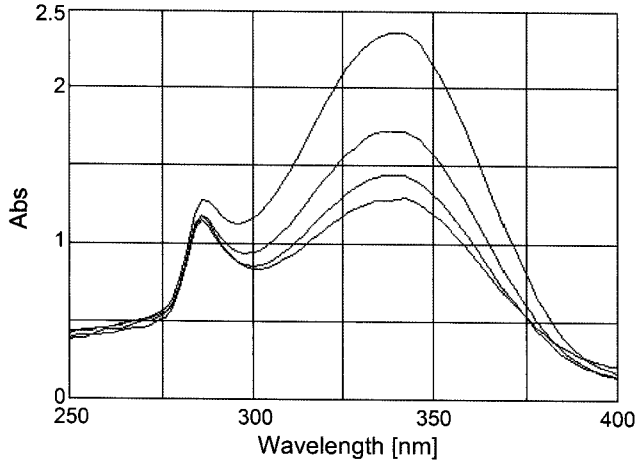

Fig. 4. Oxidation of NADH to NAD^+ in coupling with reduction of hydrogen peroxide by catalysis of cell-free extract of *Z. mobilis* KCCM11336.
Each spectrum was obtained by scanning at the intervals of 5 min in the range of wavelength from 400 nm to 250 nm. From top, the first, second, third, and fourth spectra were obtained before the reaction, and at 5 min, 10 min, and 15 min after reaction, respectively. Specific activity calculated with the absorbance variation between the initial and 5 min reactions was 1.198 μM NADH/min/mg protein.

membrane fractions disappeared from those of the physically prepared one. Some protein bands observed in the SDS-PAGE pattern of the enzymatically prepared crude enzyme and membrane fraction were dimmer than in the soluble fraction or had disappeared completely; these bands may have represented the specific membrane-binding proteins.

DISCUSSION

The ethanol productivity may be proportional to the efficiency of the NADH/NAD^+ redox reaction, and the ratio of ethanol production/glucose consumption (ethanol/glucose ratio) must be dependent on NADH/NAD^+ balance in the obligate fermentative metabolism [17]. The NADH generated *via* the pathway from glucose to pyruvate is re-oxidized through the pyruvate-to-ethanol pathway in the Entner-Doudoroff pathway, by which NADH/NAD^+ may be balanced at a level of 1.0 in the growing cells of *Z. mobilis* [29]. However, the physiological NADH/NAD^+ balance based on the fermentation metabolism may be lower under conventional growth conditions than electrochemical reduction condition, because some of the NADH may be oxidized to NAD^+ in association with the reduction of oxygen radicals or the biosynthesis of cell structures [5, 19, 33]. Theoretically, the ethanol/glucose ratio must be 2.0 or close to 2.0 at the balanced NADH/NAD^+ ratio in the fermentation metabolism of *Z. mobilis*. The ethanol/glucose ratio and ethanol productivity calculated on the basis of glucose consumption and ethanol production by *Z. mobilis* grown under electrochemical reduction condition were close to the theoretical value (see Table 1). The ethanol productivity, ethanol/glucose ratio, and ethanol/biomass ratio were significantly higher in the ECB than those observed in the anoxic nitrogen condition. These results demonstrate that the NADH/NAD^+ balance may be increased in association with the electrochemical reduction reaction of NR [22–25], but reduced dramatically in association with the reduction reaction of hydrogen peroxide generated from oxygen in the aerobic condition [14, 15, 32], as shown in the proposed mechanism of Fig. 7.

Hydrogen may function as a reducing power or reducing agent, similarly to the electrochemical reduction reaction or the cathodic reaction, because the redox potential (−0.4 volts vs. NHE) of hydrogen is lower than that (−0.32 volt vs. NHE) of NADH [6]. However, hydrogen was not shown to function as a reducing power for NADH regeneration, but may function as a reducing agent that inhibits radical effects in the metabolism of *Z. mobilis* on the basis of the lower growth, lower ethanol production, and higher ethanol/glucose ratio observed under the anaerobic hydrogen conditions, as compared with the anoxic nitrogen condition.

We applied an electrochemical technique to determine why *Z. mobilis* generated ethanol from glucose only; this technique was premised on the electrochemical activation of biochemical ethanol production or metabolic ethanol fermentation. Cyclic voltammetry may prove to be a very effective technique for the analysis of a serial biochemical reaction from the primary substrate (glucose) to the final product (ethanol) in association with the redox reaction of NAD^+, because the cyclic voltammogram can show the oxidation (downward shift) or reduction (upward shift) reaction generated by the addition of substrate and metabolic intermediates. The increase in the downward peak height indicates a serial electron transfer from glucose to NR as the result of the biochemical oxidation of the substrate, coupled with the electrochemical redox reaction of NADH/NAD^+ [12, 20]. Meanwhile, the increase in the upward peak height is indicative of a serial electron transfer from the electrode to NR, coupled to the electrochemical redox reaction of NADH/NAD^+, which can, however, never be generated between the glucose and electrode. The oxidation of biochemical glucose *via* the catalysis of the crude enzyme or the metabolism of the intact cells is coupled to NADH regeneration; NADH may then be oxidized in association with the chemical reduction of NR without enzyme catalysis; finally, the reduced NR can be electrochemically reoxidized on the surface of the electrode. The electrochemical oxidation of the reduced NR induces an increase in the downward peak height [23]. Neither pyruvate nor acetaldehyde induced an increase in the downward peak height. These results can be a clue that glucose may be a signal to induce the catalytic activity of the enzymes operating in the fermentation metabolism of *Z. mobilis*.

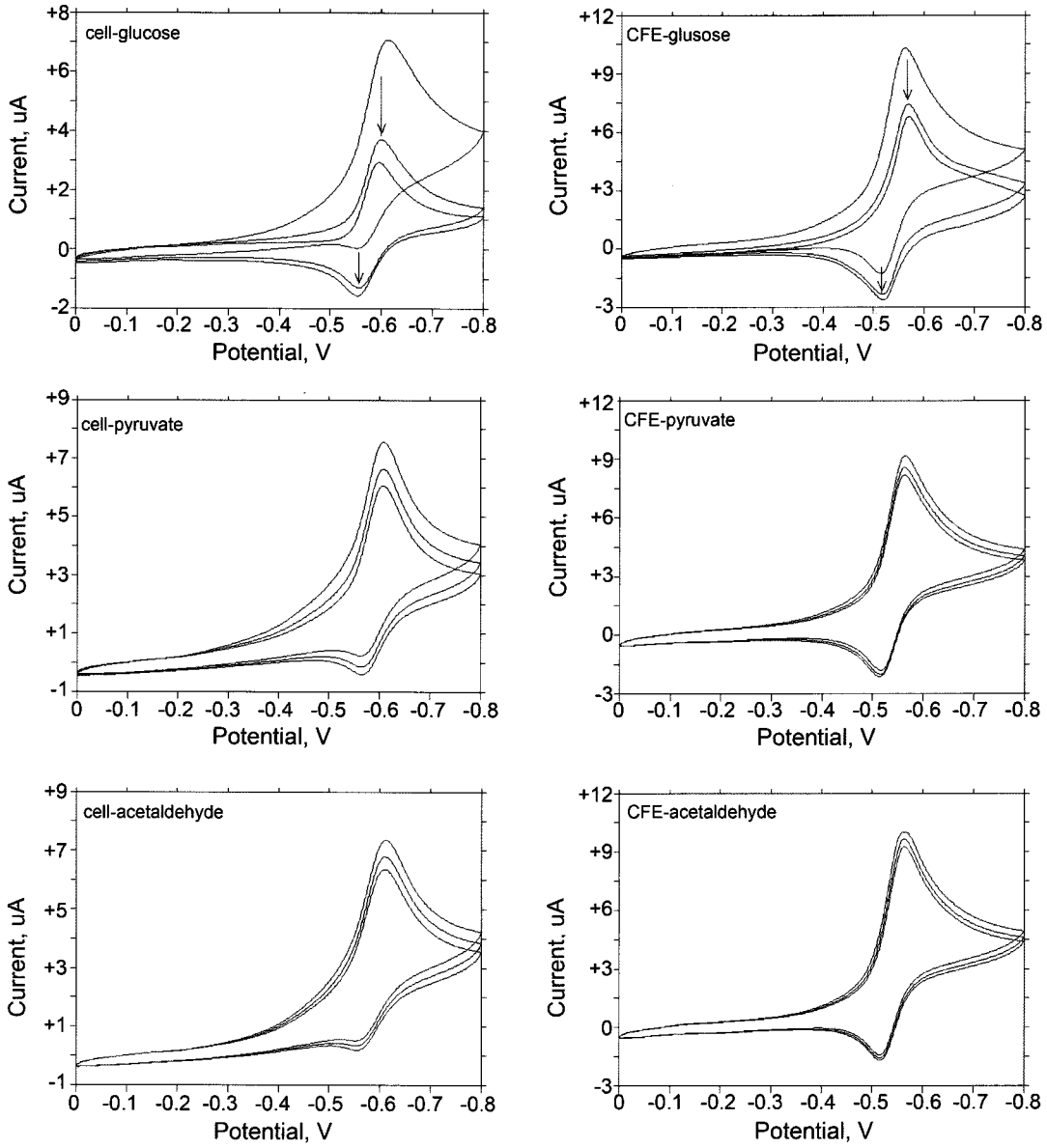

Fig. 5. Cyclic voltammograms obtained from intact cells and cell-free extract (CFE) of *Z. mobilis* modified with neutral red during reaction with glucose, pyruvate, and acetaldehyde.
At the point the 3rd cycle was started, 20 mM glucose, pyruvate, or acetaldehyde was added to the reactant to induce metabolic reaction of intact cell or CFE. The lower peak variation indicates that reducing power may be produced by intact cells or CFE in coupling with metabolic oxidation of the substrate.

The production of ethanol from glucose *via* the catalysis of both growing cells and crude enzyme of *Z. mobilis* is a very peculiar phenomenon, because the pathway from glucose to ethanol is dependent on many more enzymes than are involved in the pathway from pyruvate or acetaldehyde to ethanol. Theoretically, the enzymes that catalyze a serial metabolism from glucose to ethanol in the cytoplasm of bacterial cells may be scattered or separated from each other as the result of the cell disruption. However, the crude enzyme extracted from *Z. mobilis* was shown in this study to catalyze the serial metabolic reaction from glucose to ethanol. The crude enzyme extracted from *S. cerevisiae* catalyzed the production of ethanol from pyruvate or acetaldehyde, but not from glucose, as we had expected. Ethanol production from glucose occurring as the result of the catalysis of the biochemically prepared crude enzyme or membrane fraction may suggest that the enzymes that catalyze the pathway from glucose to ethanol may constitute a cluster of membrane-binding proteins. The fact that the physically prepared crude enzyme and membrane fraction did not catalyze the production of ethanol from glucose may be a paradoxical clue to support the notion that the enzymes of *Z. mobilis* may be a cluster of membrane-binding enzymes.

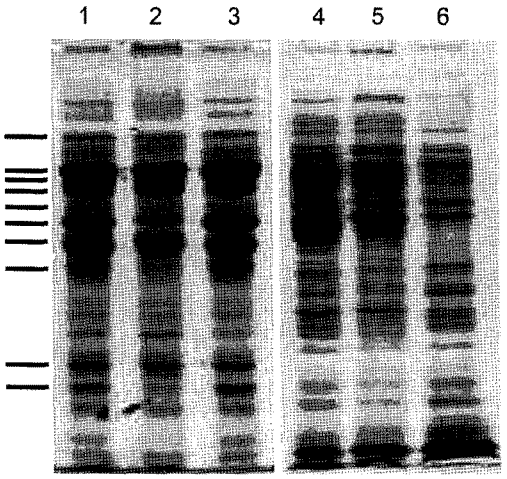

Fig. 6. SDS-PAGE patterns of cell-free extract (1, 4), soluble fraction (2, 5), and membrane fraction (3, 6) extracted from *Z. mobilis* KCCM11336, which were prepared by lysozyme (1, 2, 3) and ultrasonic (4, 5, 6) treatments.
The ladder-marked bars on the left side of the figure indicates phosphogluconate dehydrogenase, pyruvate decarboxylase, pyruvate kinase, glucose dehydrogenase, enolase, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase II, 6-phosphogluconolactonase, and aldehyde dehydrogenase from the upper, which was extracted from the paper reported by An *et al.* [2].

The differences in the results of SDS-PAGE between the physically and enzymatically prepared crude enzymes and the enzymatically prepared membrane and soluble fraction may be another clue supporting the notion that the enzymes that catalyze ethanol fermentation constitute a membrane-binding protein cluster. The protein bands on the SDS-PAGE gels of the enzymatically prepared membrane fraction of *Z. mobilis* KCCM11336 were completely identical to those of the soluble proteins extracted from another strain of *Z. mobilis* by French press [2]. According to the data previously reported by An *et al.* [2], the ladder-marked proteins of the membrane fraction are phosphogluconate dehydrogenase, pyruvate decarboxylase, pyruvate kinase, glucose dehydrogenase, enolase, 3-phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, alcohol dehydrogenase II, 6-phosphogluconolactonase, and aldehyde dehydrogenase from the upper, all of which either disappeared or were altered upon the SDS-PAGE of the physically prepared membrane fraction (lane 6 of Fig. 6).

In conclusion, we did not explain in this study why the growing cells and crude enzyme of *Z. mobilis* catalyzed ethanol production solely from glucose; however, we did propose a possible mechanism by which the crude enzyme or membrane fraction could catalyze ethanol production from glucose only. We also analyzed the physiological characteristics of *Z. mobilis* with regard to ethanol fermentation, using an electrochemical technique and the fractionated crude enzyme. The specific phenomenon observed in the crude enzyme of *Z. mobilis* may prove to be a good tool for the development of a system to produce ethanol from glucose without a bacterial culture and an expensive complex medium.

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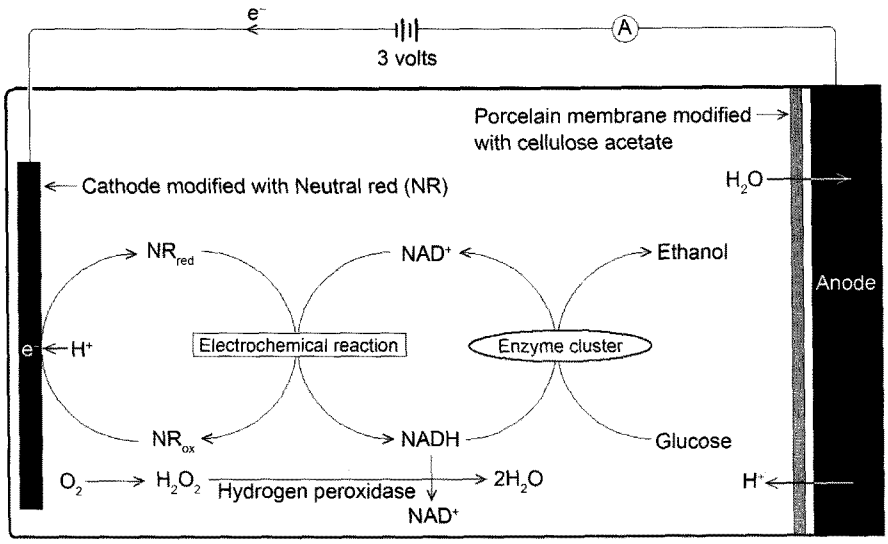

Fig. 7. Proposed mechanism for ethanol production from glucose by catalysis of crude enzyme in coupling with the electrochemical redox reaction of NADH/NAD^+ and neutral red (NR).
The modified graphite felt electrode with immobilized NR functions as a reducing power to regenerate NADH and an electron mediator from electrode to bacterial cells. The electrochemically regenerated NADH may function as a reducing power for enzymatic reduction of acetaldehyde to ethanol and a reducing agent for enzymatic reduction of hydrogen peroxide to water.

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