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Improvement of Ethanol Production by Electrochemical Redox Combination of *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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***Zymomonas mobilis* was immobilized in a modified graphite felt cathode with neutral red (NR-cathode) and *Saccharomyces cerevisiae* was cultivated on a platinum plate anode. An electrochemical redox reaction was induced by 3 volts of electric potential charged to the cathode and anode. The *Z. mobilis* produced 1.3–1.5 M of ethanol in the cathode compartment, whereas the *S. cerevisiae* produced 1.7–1.9 M in the anode compartment after 96 h. The ethanol produced by the *Z. mobilis* immobilized in the NR-cathode and *S. cerevisiae* cultivated on the platinum plate was 1.5–1.6 times higher than that produced under conventional conditions. The electrochemical oxidation potential inhibited *Z. mobilis*, but activated *S. cerevisiae*. The SDS–PAGE pattern of the total soluble proteins extracted from the *Z. mobilis* cultivated under the electrochemical oxidation conditions was gradually simplified in proportion to the potential intensity. *Z. mobilis* and *S. cerevisiae* were cultivated in the cathode and anode compartments, respectively, of an electrochemical redox combination system. The *Z. mobilis* culture cultivated in the cathode compartment for 24 h was continuously transferred to the *S. cerevisiae* culture in the anode compartment at a rate of 300 ml/day. Approx. 1.0–1.2 M of ethanol was produced by the *Z. mobilis* in the cathode compartment within 24 h, and an additional 0.8–0.9 M produced by the *S. cerevisiae* in the anode compartment within another 24 h. Thus, a total of 2.0–2.1 M of ethanol was produced by the electrochemical redox combination of *Z. mobilis* and *S. cerevisiae* within 48 h.**

Keywords: *Saccharomyces cerevisiae*, *Zymomonas mobilis*, ethanol fermentation, redox combination, electrochemical bioreactor

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Zymomonas mobilis is used for biological ethanol fermentation in various industrial fields [2, 6]. The metabolic pathway of *Z. mobilis* for ethanol fermentation is partially different from that of *S. cerevisiae*; however, the fermentation metabolism of *Z. mobilis* is extremely sensitive to oxygen and inhibited by the oxidation potential [5]. *Z. mobilis* scavenges oxygen radicals or controls the oxidation potential in association with the oxidation of NADH to NAD^+ [7]. A decrease in the NADH/NAD^+ balance exerts a deleterious influence on ethanol fermentation [13]. The metabolically regenerated NADH is re-oxidized coupled to the metabolite production, through which the NADH/NAD^+ balance is maintained in all types of fermentation metabolisms. Various intracellular factors, including radicals and noxious materials, cause the NADH/NAD^+ balance to be less than 1.0 [12]. Electrochemically reduced NR can catalyze NADH regeneration without enzyme catalysis, which is useful to maintain the NADH/NAD^+ balance in bacterial cytoplasm [20, 23]. Some specific coenzymes (electron carriers) located in the cytoplasmic membrane are known to mediate the direct electron transfer from bacterial cell to electrode [4]. The coenzymes catalyzing this electron transfer are bound to the mitochondrial membrane in *S. cerevisiae* [10]. As mitochondria cannot come into direct contact with an electrode, this is a barrier for electron transfer between electrodes and yeast coenzymes. Theoretically, the metabolic activation of *S. cerevisiae* is not effective based on the electrochemical control of the NADH/NAD^+ balance, as yeast can efficiently control the NADH/NAD^+ balance based on the respiratory metabolism, even under aerobic conditions [3].

A direct electric current between two electrodes installed in a bacterial culture has been reported to induce an alteration of the bacterial shape and increase the hydrophobicity of the cell surface [18]. The effects of an electric field, electric pulse, and electric shock on bacterial physiology, morphology, and biofilm structure have already been studied in various research fields [14].

The oxidation potential or oxygenic environment has been reported to be an essential factor for yeast growth [1], which can be electrochemically induced. Rosenfeld *et al.* [25] reported that the addition of oxygen to a lipid-depleted medium resulted in the synthesis of sterols and unsaturated fatty acids, whereas the addition of oxygen to a medium containing excess ergosterol and oleic acid increased the fermentation rate and cell viability and shortened the fermentation period. In particular, fatty acids and molecular oxygen have been demonstrated to be required for lipid biosynthesis, plasma membrane integrity, and the maintenance of ethanol production rates [9, 15].

Accordingly, the objective of the present study was to increase the ethanol production rates and shorten the cultivation period by combining *Z. mobilis* immobilized in an NR-graphite felt cathode and *S. cerevisiae* cultivated on a platinum anode surface. Whereas the NR-graphite felt functions as an electron donor for NADH regeneration in the cathode compartment, the platinum plate functions as an oxygen generator in the anode compartment, thereby significantly activating the fermentative metabolism of both organisms. This study also introduces the first electrochemical redox-combined cultivation system.

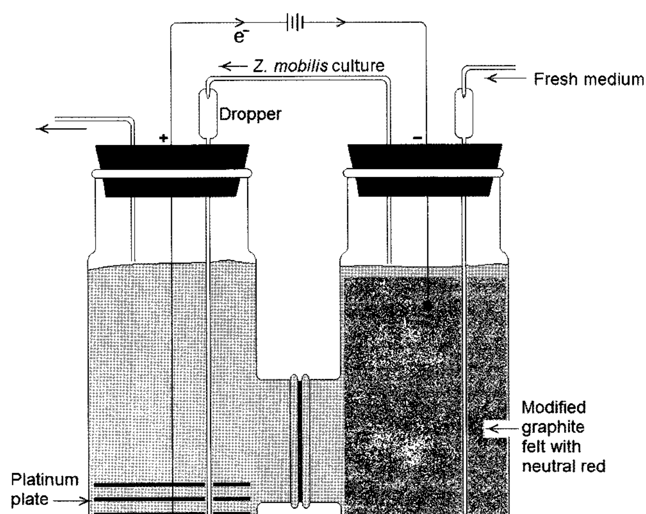

Fig. 1. Schematic structure of the two-compartment electrochemical bioreactor separated by a modified ceramic membrane (5 mm thickness) with cellulose acetate film (35 μm thickness).
Z. mobilis was immobilized in the modified graphite felt electrode (NR-graphite felt; 40×120×30 mm) with neutral red and *S. cerevisiae* was grown on the platinum plate (diameter 50 mm, thickness 0.1 mm). The *Z. mobilis* culture was designed to transfer from the cathode to the anode compartment in proportion to the feeding of the fresh medium in the continuous culture system, but not in the batch and fed-batch culture system.

MATERIALS AND METHODS

Chemicals

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

Electrochemical Bioreactor

A two-compartment electrochemical bioreactor (TCECB) was designed to induce electrochemical oxidation and a reduction reaction simultaneously. As shown in Fig. 1, the anode and cathode compartments were separated by a modified porcelain membrane with a cellulose acetate film (35 μM thickness; Electron Microscopy Science, Hatfield, PA, U.S.A.). The working volume of each compartment was adjusted to 300 ml. The porcelain membrane (5 mm thickness) was used as a matrix to fix the cellulose acetate film that functioned as a semipermeable membrane. The porcelain membrane was prepared based on the method developed by Park and Zeikus [21]. NR-graphite felt (20×40×100 mm, 80 ml) and a platinum plate (diameter 50 mm, thickness 0.1 mm) were used as the cathode and anode, respectively.

Immobilization of NR in Graphite Felt

The neutral red was immobilized in the graphite felt by a covalent bond between the neutral red and polyvinyl alcohol (mean molecular weight, 80,000; Sigma) in accordance with the following procedures. The graphite felt (10×100×40 mm) was soaked in a 1% (w/v) polyvinyl alcohol solution (100 ml) at 60°C under a vacuum for 3 h, and then dried for 12 h at 100°C. The completely dried graphite felt was then soaked in pure chloroform containing 10% thionylchloride and 0.1% neutral red to induce a covalent bond between the -OH of the polyvinyl alcohol and the $-NH_2$ of the neutral red for 6 h, after which the graphite felt was washed with methanol to remove any unbound neutral red. During this reaction, the water-soluble polyvinyl alcohol was converted into water-insoluble polyvinyl neutral red and the graphite felt electrode hardened, like a plastic sponge. The resulting polyvinyl neutral red-graphite felt complex (NR-graphite felt) was very stable and unchanged after autoclaving more than 10 times, as shown in Fig. 2.

Scanning Electron Microscopy

After being used more than 10 times in the electrochemical bioreactor for *Z. mobilis* cultures, a piece (10×10 mm) of the NR-graphite felt was cut, autoclaved in double-distilled water for cleaning, and dried at 100°C for 24 h. This piece was then directly used as a sample for the scanning electron microscopy. Native graphite felt was used to

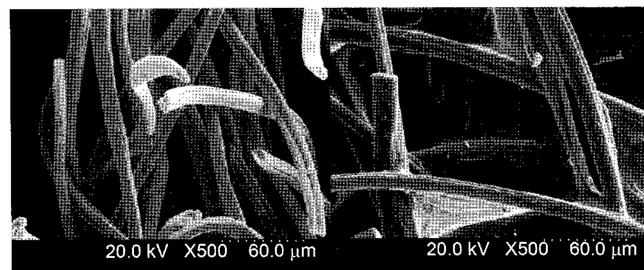

Fig. 2. Native graphite felt (left) and modified graphite felt with neutral red (right).
The thin film-like materials around the graphite felt networks (right) are a polyvinyl–neutral red complex, which was generated by a covalent bond between polyvinyl alcohol and neutral red.

compare the structural difference of the NR-graphite felt. The Daegu Center of Korea Basic Science Institute (Daegu Metropolitan City) were asked to take SEM pictures of the samples.

Microorganisms

The *Z. mobilis* KCCM11336 and *S. cerevisiae* ATCC26603 were commonly cultivated in a medium (500 ml) containing 3 g/l of yeast extract and 1.2 M of glucose (YG medium) at 30°C.

Immobilization of *Z. mobilis* in Graphite Felt

All the procedures were performed under complete aseptic conditions. Ten ml of the harvested cells was mixed with 10 ml of a 25 mM phosphate buffer (pH 7.0) containing 4% alginate and absorbed into the NR-graphite felt. The NR-graphite felt containing the alginate and bacterial cells was then soaked in a 100 mM $CaCl_2$ solution for 30 min to induce calcium alginate coagulation and washed with a 25 mM phosphate buffer, which was used as a biocatalyst for the batch, fed-batch, and continuous cultures to skip over the lag phase. The final cell mass immobilized in the NR-graphite felt (20×40×100 mm) was adjusted to 1.5 g/l as the dry weight.

Preparation of *S. cerevisiae* Inoculum

The *S. cerevisiae* culture 1,000 ml cultivated for 24 h was harvested by centrifugation at 5,000 ×*g* for 30 min and then suspended in 200 ml of a fresh medium, thereby concentrating the initial cell mass 5 times. A concentrated yeast culture (2.5 g/l of dry weight) was used as a biocatalyst for the batch, fed-batch, and continuous cultures to skip over the lag phase.

Ethanol Production by Batch Culture

The *Z. mobilis* immobilized in the NR-graphite felt and the concentrated *S. cerevisiae* culture were cultivated under oxidation and reduction conditions generated by 1, 2, 3, or 4 volts of electrochemical potential charged to the anode and cathode. The ethanol produced by the *Z. mobilis* and *S. cerevisiae* was analyzed daily for 4 days to determine the stationary phase for ethanol production.

Ethanol Production by Fed-Batch Culture

After cultivating the batch culture for 4 days, the *Z. mobilis* and *S. cerevisiae* cultures were both replaced with the same value of a fresh YG medium on a daily basis under complete aseptic conditions. Although the suspended microorganisms were washed away, the immobilized *Z. mobilis* cells in the NR-graphite cathode and the *S. cerevisiae* settled on the platinum plate anode remained when the culture was exchanged with a fresh medium. Most of the *S. cerevisiae* cells sank spontaneously and piled up on the platinum plate anode.

Ethanol Production by Continuous Culture

The *Z. mobilis* culture continuously and automatically flowed into the *S. cerevisiae* culture, while the *S. cerevisiae* culture flowed out in proportion to the volume of fresh medium fed into the *Z. mobilis* culture at a speed of 300 ml/day, which was precisely controlled with an error of less than 2%. The hydraulic retention time was 2 days based on the total volume of the anode and cathode compartments (600 ml) and the feeding volume (300 ml).

SDS–PAGE

The SDS–PAGE technique utilized in this study was adapted from the method developed by Laemmli [17]. The harvesting and washing of the microorganisms were performed using centrifugation at 5,000 ×*g* and 4°C for 40 min, followed by washing with double-distilled water. The protein concentration was then determined using the Bradford reagent (BioRad) as a coloring agent and bovine serum albumin as the protein standard. The total soluble proteins were obtained from disrupted cells of *Z. mobilis* and *S. cerevisiae* cultivated for 36 h and 48 h, respectively. The cell disruption was performed using a mini-beadbeater (Biospec, Seoulin Bioscience, Seoul, Korea) at 2,500 strokes and 4°C for 10–20 min. Beads of 0.1 mm and 0.5 mm were used to disrupt the *Z. mobilis* and *S. cerevisiae* cells, respectively.

Analysis

The ethanol contained in the bacterial or yeast culture was analyzed *via* HPLC using an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, U.S.A.) and 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 0.22-μm-pore membrane filter. Twenty μl of the filtrate was then injected into the HPLC injector. The concentrations of glucose and ethanol were calculated based on the peak area in chromatograms obtained with standard materials.

RESULTS

Ethanol Production in Batch Culture

The ethanol production by *Z. mobilis* gradually increased in proportion to the potential intensity under the electrochemical reduction conditions, but was significantly inhibited in proportion to the potential intensity from 1 to 4 volts under the electrochemical oxidation conditions, as shown in Table 1. Meanwhile, the ethanol production by *S. cerevisiae* increased in proportion to the potential intensity from 1 to 3 volts under the electrochemical oxidation conditions, but was not influenced by the potential intensity under the electrochemical reduction conditions, as shown in Table 2. The ethanol produced by *Z. mobilis* and *S.*

Table 1. Influence of electrochemical reduction (–) and oxidation potential (+) on ethanol fermentation of *Z. mobilis* immobilized in NR-graphite felt charged with –1 to –4 volts or 1 to 4 volts of DC electricity.

Potential (Volts)	Ethanol production (mM)/Incubation time (h)			
	24	48	72	96
0	417±36	686±49	893±85	988±78
–1	422±29	712±38	928±56	1,057±82
–2	639±56	963±69	1,152±91	1,203±93
–3	1,085±169	1,359±118	1,596±102	1,782±119
–4	1,105±98	1,336±135	1,657±96	1,817±152
1	385±42	498±65	485±52	471±32
2	268±53	381±64	387±35	376±26
3	211±29	326±24	281±28	279±39
4	215±41	337±47	288±48	275±29

Table 2. Influence of electrochemical reduction (-) and oxidation potential (+) on ethanol fermentation of *S. cerevisiae* cultivated on platinum plate charge with 1 to 4 volts or -1 to -4 volts of DC electricity.

Potential (Volts)	Ethanol production (mM)/Incubation time (h)			
	24	48	72	96
0	696±54	965±83	1,088±63	1,150±47
-1	691±48	971±85	1,075±97	1,163±54
-2	695±72	966±75	1,120±75	1,144±72
-3	688±39	978±92	1,103±93	1,125±78
-4	690±65	975±72	1,090±82	1,139±89
1	740±84	985±105	1,120±72	1,220±98
2	854±59	1,085±91	1,190±92	1,340±86
3	1,201±71	1,531±94	1,813±139	1,927±129
4	1,172±98	1,506±107	1,824±132	1,949±108

cerevisiae with a potential intensity of 4 volts was not very different from that produced with a potential intensity of 3 volts. Thus, 3 volts appeared to be the maximal potential intensity to activate ethanol fermentation by *Z. mobilis* or *S. cerevisiae*. Whereas the ethanol produced by *Z. mobilis* and *S. cerevisiae* was about 1 M after 96 h under conventional conditions, it almost reached 2 M after 96 h under the electrochemical reduction or oxidation conditions.

Influence of Redox Potential on Bacterial Physiology

The metabolic inhibition of *Z. mobilis* under electrochemical oxidation conditions may have been caused by physiological damage, as represented by the SDS–PAGE pattern shown in Fig. 3. Some proteins were absent in the cell extract of the *Z. mobilis* cultivated under electrochemical oxidation conditions, and the number of absent protein bands was proportional to the intensity of the electric potential. However, the SDS–PAGE profile of the cell extract of the *Z. mobilis* cultivated under electrochemical reduction conditions was only marginally different from that cultivated under conventional conditions. Meanwhile, the SDS–PAGE pattern of the soluble proteins extracted from the *S. cerevisiae* cultivated under both electrochemical oxidation and reduction conditions was the same as that cultivated under conventional conditions (data not shown), which correlated with the ethanol produced by the *S. cerevisiae* under electrochemical oxidation and reduction conditions (Table 2). These results show that the primary metabolism of *Z. mobilis* for ATP generation may be inhibited or bacterial cell structures may be destroyed by the oxygen radicals generated from the anode.

Ethanol Production in Fed-Batch Culture

The NR-graphite felt cathode and platinum plate anode were charged with a reduction and oxidation potential of 3 volts. The ethanol produced by the *Z. mobilis* immobilized in the NR-graphite felt reached about 1.0–1.1 M/day after 10 days of incubation time, whereas that produced by the *S. cerevisiae* grown on the platinum plate reached about 1.1–1.2 M/day after 6 days of incubation time, as shown in Fig. 4.

Ethanol Production in Continuous Culture

The *Z. mobilis* culture (cathode compartment) was continuously fed a fresh YG medium at a speed of 300 ml/day, thereby continuously transferring the *Z. mobilis* culture to the *S.*

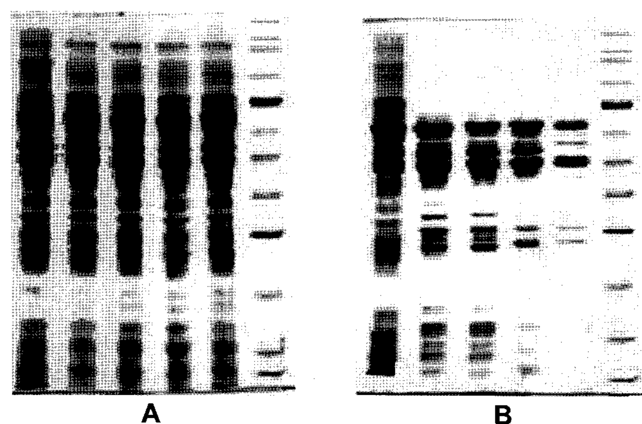

Fig. 3. SDS–PAGE patterns of total soluble proteins extracted from *Z. mobilis* cultivated in electrochemical reduction (**A**) and oxidation condition (**B**) after 36 h.

The electric potential was not charged for the control test (lane 1). The DC electric potential charged to the anode and cathode was adjusted to 1 volt (lane 2), 2 volts (lane 3), 3 volts (lane 4), and 4 volts (lane 5). Lane 6 indicates the protein markers that are 212, 158, 116, 97.2, 66.4, 55.6, 42.7, 34.6, 27, 20, 14.3, 6.5, 3.4, and 2.3 kDa from the top.

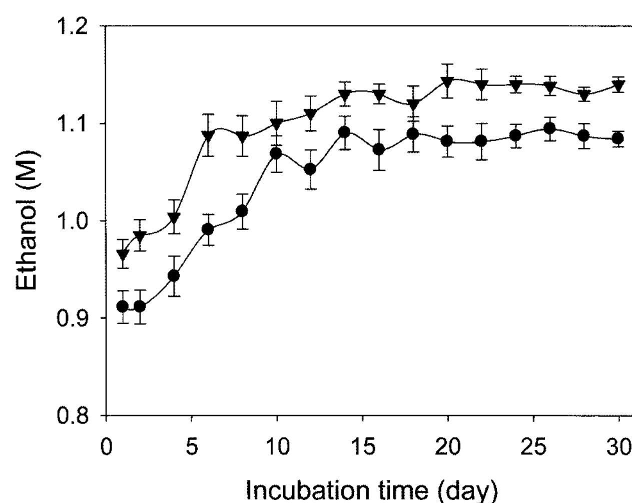

Fig. 4. Ethanol production by *Z. mobilis* KCCM11336 immobilized in NR-graphite felt (●) under electrochemically reduced conditions and by *S. cerevisiae* ATCC26603 grown on electrically oxidized platinum plate (▼).

The bacterial or yeast culture (reactant) was replaced daily with a fresh YG medium.

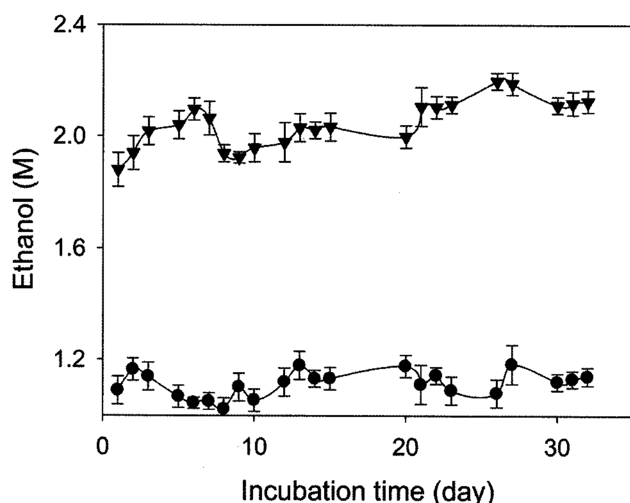

Fig. 5. Ethanol production by *Z. mobilis* (●) immobilized in NR-graphite felt in the cathode compartment (reduction conditions) and by *S. cerevisiae* (▼) cultivated on platinum plate in the anode compartment (oxidation conditions).

Three hundred ml of the *Z. mobilis* culture was transferred from the cathode to the anode compartment in proportion to the feeding of the fresh medium.

cerevisiae culture (anode compartment) in proportion to the feeding speed of the fresh medium (Fig. 1). Although the *Z. mobilis* cells transferred to the anode compartment could not grow, the *S. cerevisiae* produced ethanol from the residual glucose contained in the *Z. mobilis* culture. As shown in Fig. 5, the *Z. mobilis* produced about 1.0–1.1 M of ethanol in the cathode compartment within 1 day, whereas the *S. cerevisiae* produced an additional 0.9–1.0 M of ethanol in the anode compartment within 1 more day. Accordingly, the total ethanol produced by the combined culture of *Z. mobilis* and *S. cerevisiae* was about 2.0–2.1 M, which is the maximal possible productivity generated from 1.2 M of glucose within 2 days.

Discussion

NR-graphite felt functions as a solid electron mediator that catalyzes the regeneration of NADH [19, 22], and the $NADH/NAD^+$ ratio is the major factor shifting the metabolic flux in the fermentative metabolism [11]. Although the $NADH/NAD^+$ ratio is balanced in the fermentative metabolism by the redox coupling between substrate oxidation and metabolite reduction, the $NADH/NAD^+$ ratio can be lower than the metabolic demand for biosynthesis based on a NADH oxidation reaction coupled to radical scavenging and noxious material removal. Eventhough the electrochemical NADH regeneration cannot be biochemically measured *in vivo*, it has been verified *via* the electrochemical control of the metabolic flux shift for fermentation bacteria [20, 23].

The $NADH/NAD^+$ balance in the *Z. mobilis* cultivated in the NR-graphite cathode was estimated to be higher than the metabolic demand, thereby shifting the metabolic flux in the direction to decrease the $NADH/NAD^+$ balance in coupling with an increase of metabolite (ethanol) production. The ethanol production by the *Z. mobilis* cultivated in the NR-graphite felt cathode was significantly higher than that produced under conventional conditions, as the NADH was continuously regenerated by the NR-graphite felt cathode [16].

The *Z. mobilis* continuously growing inside the NR-graphite felt was dispersed from the electrode into the culture medium, which was then transferred to the anode compartment through the culture flow in the continuous electrochemical redox combination system (Fig. 1). The bacterial cells transferred to the anode compartment lost their physiological function owing to the radicals induced by the continuously generated oxygen from the platinum anode. The platinum plate allowed the yeast culture conditions to be oxidized and functioned as an oxygen generator, which are the essential factors to activate the *S. cerevisiae* metabolism. The oxidation potential capable of maintaining the critical oxygenation level was lower than 4 volts yet higher than 3 volts for *S. cerevisiae*, which was the optimal potential for a high ethanol yield but low cell yield, as the ethanol production at 3 volts was nearly the same as that at 4 volts [24, 28, 29]. Anaerobic conditions have been reported to cause the $NADH/NAD^+$ ratio to be imbalanced and limit the sugar transport in the fermentative metabolism of *Saccharomyces* sp. [8, 27]. This indicates that oxygen is absolutely required for the normal growth and fermentative metabolism of *S. cerevisiae* [26]. The ethanol tolerance of *S. cerevisiae* has been reported to depend on the oleic acid content of the cellular membrane [30]. The lipid biosynthesis of the cellular membrane is also known to be activated under oxygenic conditions [9, 15, 25].

The continuously generated oxygen from the platinum plate in the anode compartment induced radical generation in the *Z. mobilis* cytoplasm as a reaction to the reducing power (NADH and $FADH_2$). Oxygen radicals are enzymatically reduced to water in coupling with NADH oxidation. Thus, the consumption of NADH for radical generation and scavenging may have caused the $NADH/NAD^+$ balance to be continuously decreased and finally exhausted. The primary metabolism of *Z. mobilis* stopped with an extremely low balance of $NADH/NAD^+$. This metabolism and growth stop may have then caused the bacterial structure to be spontaneously disrupted. The radical generation must be proportional to the oxygen generation, which in turn must be proportional to the potential intensity. Although the maintenance or disappearance of bacterial structures, including DNA, proteins, cytoplasmic membrane, and peptidoglycan, is difficult to estimate using biochemical methods, proteins can be easily estimated by electrophoresis.

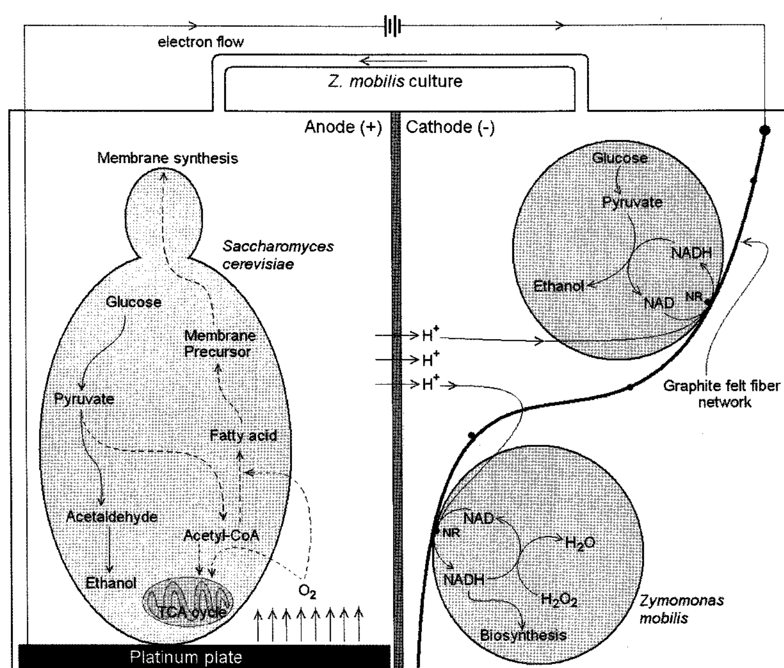

Fig. 6. Proposed mechanism for ethanol production by combined culture of *Z. mobilis* in the cathode compartment (right) and *S. cerevisiae* in the anode compartment (left).
Dotted lines indicate the oxygen-dependent metabolic pathway.

Thus, the number or pattern of protein bands on the SDS–PAGE gel was a marker indicating the difference between the intact and damaged cells of *Z. mobilis*. In contrast, it is difficult to compare other structural compounds, as the DNA, cytoplasmic membrane, and peptidoglycan are hard to separate analytically using electrophoresis and other techniques.

The combined electrochemical reduction and oxidation of *Z. mobilis* and *S. cerevisiae* was demonstrated as an optimized technique for improving biological ethanol production, as the ethanol production by a separated or mixed culture of *S. cerevisiae* and *Z. mobilis* was less than 1.5 M for 2 days. In particular, both compartments of an electrochemical bioreactor can be effectively utilized by the combined technique. Conclusively, ethanol fermentation by *Z. mobilis* was activated in the cathode compartment electrochemically connected to the anode compartment, in which *S. cerevisiae* grew actively and produced ethanol using the oxygen generated from the platinum plate, as shown by the proposed mechanism in Fig. 6. The biochemical regeneration of NADH in the cathode compartment was electrochemically coupled to the oxygen generation in the anode compartment. In order to produce about 2.0 M of ethanol when using a separated or mixed cultivation of *Z. mobilis* and *S. cerevisiae*, 4 days were required, yet 2 days were enough when using the electrochemical combined cultivation system. In the two-compartment electrochemical bioreactor composed of an anode and cathode, the former functions as the working part, and the latter functions as a counterpart that is simply required to maintain the redox balance of the working part without any productivity. The anode and cathode compartments can both be effectively utilized in the combined technique for *Z. mobilis* and *S. cerevisiae*, representing a green technique applicable to industry as regards saving electric energy and cultivation time. However, techniques are still needed for designing and manufacturing industrial-scale electrochemical bioreactors. The high pressure of an industrial volume on the porcelain membrane may also be solved by balancing the anode and cathode compartment volumes. The manufacture of an industrial-sized NR-electrode could also be composed of multiple connections of small-sized electrodes.

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