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Conversion of Carbon Dioxide to Metabolites by *Clostridium acetobutylicum* KCTC1037 Cultivated with Electrochemical Reducing Power

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ABSTRACT

In this research, metabolic fixation of CO_2 by growing cells of *C. acetobutylicum* cultivated with electrochemical reducing power was tested on the basis of the metabolites production and genes expression. In cyclic voltammetry, electrochemical oxidation and reduction reaction of neutral red (NR) immobilized in intact cells of *C. acetobutylicum* was stationarily repeated like the soluble one in the condition without CO_2 but the electrochemical reduction reaction was selectively increased by addition of CO_2. In electrochemical bioreactor, the modified graphite felt cathode with NR (NR-cathode) induced *C. acetobutylicum* to generate acetate, propionate, and butyrate from CO_2 in defined medium. When H_2 and CO_2 were used as an electron donor and an electron acceptor, respectively, *C. acetobutylicum* also produced the same metabolites in a defined medium. *C. acetobutylicum* was not grown in the defined medium without substituted electron donors (H_2 or electrochemical reducing power). *C. acetobutylicum* cultivated with electrochemical reducing power produced more butyrate than acetate in complex medium but produced more acetate than butyrate in defined medium. The genes of encoding the enzymes catalyzing acetyl-CoA in *C. acetobuylicum* electrochemically cultivated in defined medium than conventionally cultivated in complex medium. These results are a clue that *C. acetobutylicum* may metabolically convert CO_2 to metabolites and produce free energy from the electrochemical reducing power.

Keywords: *C. acetobutylicum*; CO_2-Assimilation; Electrochemical Reducing Power; Coupling Redox Reaction

1. Introduction

Chemoautotrophs that regenerate reducing power and produce free energy in coupling with oxidation of H_2 can more effectively fix CO_2 than ammonium, nitrite, and ferrous-oxidizing bacteria because redox potential of $H_2/2H^+$ (–0.42 V vs. NHE) is lower than NAD^+/NADH (–0.32 V vs. NHE) [1]. The reducing power generated in coupling with oxidation of ammonium, nitrite, and ferrous ion can't induce regeneration of NAD(P)H without a reverse electron transport system coupled to consumption of external energy [2-5]. Experimentally measured redox potential of NR is –0.325 V (vs. NHE), which is theoretically enough to mediate generation of electron-driving force from electrode to NADH [6]. Practically, electrochemically reduced NR catalyzes NADH regeneration by non-enzymatic catalysis [7]. Theoretically, electrochemically reduced NR may be more effective in reducing power than H_2 on the basis of non-enzymatic catalysis of NADH regeneration.

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C. acetobutylicum is a typical fermentation bacterium that produces acetate, propionate, and butyrate by the coupling redox reaction of reducing power and acetyl-CoA generated from metabolic oxidation of glucose, and also autotrophically produces acetyl-CoA from the coupling redox reaction of H_2 and CO_2 in defined medium with organic nitrogen nutrient [8,9]. The acetyl-CoA is metabolically oxidized to acetate coupled to regeneration of NADH or reduced to butyrate coupled to oxidation of NADH [10]. Biological production of organic polymers, fatty acids and alcohols from H_2 and CO_2 has been studied in order to decrease global warming and produce renewable energy and useful biomass [11,12]. H_2 is the most effective electron donor to bacteriologically fix CO_2 on the basis of its redox potential but is less practical to apply to biological system owing to very low solubility in water and possible explosiveness in the process of use, transport and storage. The cost for production of H_2 is not economical in comparison of glucose price. In order to solve the problem caused by the H_2 production cost, an alternative technology has been developed [13,14].

Electricity generated from the solar cell can be directly converted to biochemical reducing power in coupling with the redox reaction of NR immobilized in bacterial cell or graphite felt electrode [6]. Bacterial CO_2 fixation induced by biochemical reducing power electrochemically regenerated by the solar cell electricity may correspond to the photosynthesis because O_2 is generated from anode compartment and CO_2 is biochemically assimilated into biomass and converted to metabolites in cathode compartment [15]. Covalently immobilized NR in graphite felt electrode can function as a catalyst for NADH regeneration and a redox carrier for electron transfer from electrode to bacterial cell [16]. Redox potential of NR is –0.325 volt (vs. NHE), which is 0.05 volt lower than NAD^+. The electrochemical redox reaction of NR can be coupled to biochemical redox reaction as follows: [$NR_{ox} + 2e^- + 1H^+ \rightarrow NR_{red}$; $NR_{red} + NAD^+ \rightarrow NR_{ox}$ + NADH]. Commonly, NR_{ox} and NAD^+ are reduced to NR_{red} and NADH, respectively by accepting two electrons from electrode and NR_{red} (ox: oxidation; red: reduction).

In this study, electrochemical reducing power was charged to *C. acetobutylicum* culture using the NR-cathode to induce autotrophic production of acetate and butyrate from CO_2 in a defined medium and increase of butyrate production in a complex medium. The NR-cathode, to which –2 V of DC-electricity was charged, may be an optimized habitat for strict anaerobes because the lower oxidation-reduction potential than –300 mV (vs. NHE) is electrochemically generated and the electrochemically reduced NR may catalyze bacterial NADH regeneration.

2. Materials and Methods

2.1. Medium

Reinforced clostridial (RC) medium (Tryptone 10 g/L, Sodium chloride 5 g/L, Beef extract 10 g/L, Yeast extract 3 g/L, Glucose 20 g/L, Starch 1 g/L, L-cystein hydrochloride 0.5 g/L, Sodium acetate 3 g/L) was used as a complex medium and for successive cultivation of *C. acetobutylicum*. M9 mineral medium (Disodium phosphate 6.8 g/L, Monosodium phosphate 3 g/L, Ammonium chloride 5 g/L, Sodium chloride 0.5 g/L, Magnesium sulfate 0.246 g/L, Calcium chloride 0.0147 g/L) supplemented with sodium bicarbonate (25 mM) and yeast extract (3 g/L) was used as a defined medium. Fifty ml of RC or defined medium was prepared in anaerobic serum vials (total volume 165 ml) whose headspace was filled with 2 atmospheres of oxygen-free N_2 or H_2.

2.2. Electrochemical Bioreactor

An electrochemical bioreactor that was designed for continuous culture in previous research [16] was partially modified for cultivation of strict anaerobic bacterium in batch culture, as is shown in **Figure 1**. The electrochemical bioreactor (inner diameter, 80 mm; height, 200 mm; medium volume, 500 ml; electrode volume, 250 ml; total volume, 1000 ml; Pyrex, USA) with a built-in anode compartment was designed to equalize distance between anode and all round of cylindrical cathode. A sintered glass filter (diameter, 50 mm; thickness, 5 mm; pore, 1 - 1.6 µm, Duran, Germany) that was modified with cellulose acetate film (35 µm thickness, Electron Microscopy Sciences, USA) was fixed at the bottom end of the tube-type anode compartment (inner diameter 20 mm; height, 150 mm; working volume, 50 ml). Cellulose acetate film attached to the sintered glass functions as a semipermeable membrane capable of selectively transferring water, gas, and proton. Five hundred ml of the media was prepared in the electrochemical bioreactor (**Figure 1**) to which O_2-free CO_2 (50 ml·min^{-1}) was continuously supplied during cultivation. Inoculation ratio was adjusted to 5% (w/w) of medium volume. DC –2 V of electricity was charged to NR-graphite felt cathode to induce electrochemical reduction reaction of NR for *C. acetobutylicum*. The defined medium was used as anolyte to avoid generation of osmosis between anode and cathode compartment.

2.3. Electrode

Graphite felt (thickness, 10 mm; height, 200 mm; length,

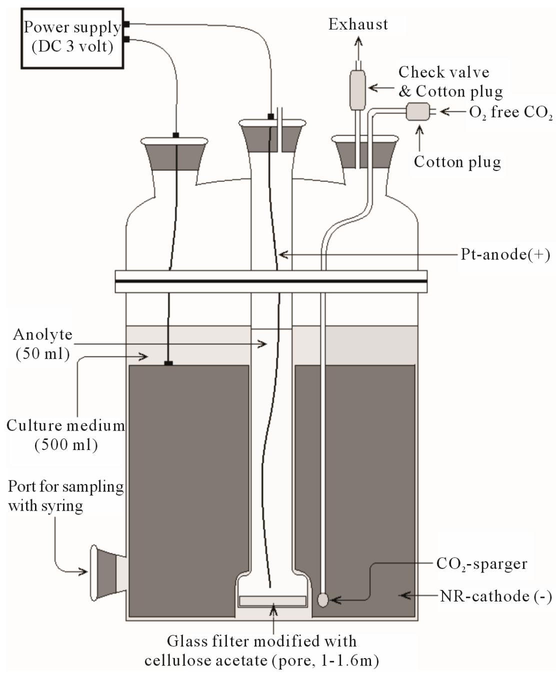

Figure 1. Electrochemical bioreactor composed of graphite felt modified with NR (NR-cathode), glass filter membrane modified with cellulose acetate film, and platinum anode.

500 mm; Electrosynthesis, USA) was rolled up to be a cylinder type (internal diameter, 40 mm; external diameter, 75 mm). Neutral red was immobilized to thegraphite felt (10 × 200 × 500 mm, Electrosynthesis, USA) by the covalent bond between neutral red and polyvinyl alcohol (mean molecular weight, 80,000, Sigma, USA) according to the technique used in previous research [16]. The graphite felt modified with NR was used as a cathode and a platinum wire (thickness 0.5 mm, length 150 mm) was employed as an anode. Electric potential charged to NR-cathode was precisely adjusted to –2 V.

2.4. Analysis of Electrochemical Reaction of *C. acetobutylicum*

The cyclic voltammetry was employed in order to analyze electrochemical redox reactions between electrode and intact cell of *C. acetobutylicum*. The cyclic voltammetry was conducted using a voltammetric potentiostat (BAS model CV50W, USA) linked to a data acquisition system. Aglassy carbon electrode (5mm diameter, Electrosynthesis, USA), a platinum wire, and an Ag/AgCl electrode (redox potential, +0.2 V vs. NHE, Electrosynthesis, USA) were utilized as a working electrode, counter-electrode, and reference electrode, respectively. The reactant was composed of 25 mM Tris-HCl buffer (pH 7.5) containing 5 mM NaCl and 100 μM NR. *C. acetobutylicum* that was anaerobically cultivated in the modified M9 medium for 48 hr under H_2 atmosphere was anaerobically centrifuged at 1500 × g and 4˚C for 60 min. The precipitated bacterial cells were suspended in 0.05 volume of the oxygen-free reaction mixture, in which NR was spontaneously immobilized in bacterial cells. Prior to and during the cyclic voltammetry measurement, argon (99.999%) was sparged into headspace of reaction beaker in order to protect contamination of the reaction mixture by oxygen. The scanning rate was 25 $mV \cdot s^{-1}$ over a range of 0 to –1200 mV. During cyclic voltammetry for NR dissolved in reactant or immobilized in *C. acetobutylicum*, the variations of upper voltammetric peaks (an indicator for electron transfer from electrode to bacterial cells through NR) and lower voltammetric peaks (an indicator for electron transfer from bacterial cells to electrode through NR) by addition of CO_2 were recorded.

2.5. Analysis of Metabolites

Bacterial metabolites were analyzed using a Gas Chromatography/Mass Spectrometry (Clarus 600 series + TurboMatrix HSS Trap, PerkinElmer, USA) equipped with Elite-FFAP column (ID 0.25 μm, OD 0.32 μm, length 30 m) and electron ionization system. Bacterial culture was centrifugation at 10,000 × g and 4˚C for 30 min and filtered with membrane filter (pore 0.22 μm), and then directly injected into the GC/MS injector. Concentration of metabolites was determined based on peak area of standard compounds and chemical species was determined based on mass profile database.

2.6. Microarray of mRNA

C. acetobutylicum was cultivated in the electrochemical bioreactor using the defined medium under strict anaerobic CO_2 atmosphere and in the complex medium under strict anaerobic N_2 atmosphere for 5 days. Total RNA was isolated and purified from harvested bacterial cells using a RNA purification kit (Total RNA, spin-column format, Oligotex mRNA mini kit, Qiagen Korea, Seoul). Microarray analysis of mRNA was conducted at Genomictree (Daejeon, Korea) using the systems, kits, DNA chips, and analysis software offered by Agillent Technologies (Korea branch, Seoul) via a turnkey-based analyzing order. The significantly expressed genes that are concerned with CO_2 fixation and energy metabolism were selectively analyzed to compare the relationship between metabolic pathway related with CO_2 fixation and cultivation conditions.

3. Results

3.1. Electrochemical Redox Reaction in Coupling with CO_2

Cyclic voltammetry is a useful technique to measure electrochemical coupling redox reaction of electron mediator immobilized in bacterial cells. In the cyclic voltammetry without bacterial cells, the electrochemical redox reaction of NR was measured to be –0.52 V (vs. Ag/AgCl), which is very similar to the experimental value –0.525 V (vs. Ag/AgCl) measured in standard condition. Both the upper and lower voltammetric peaks were not altered by addition of CO_2 as expected; in contrast, the upper voltammetric peak generated by NR immobilized in *C. acetobutylicum* was shifted upward from 2.4 to 2.9 μA and rightward from –0.52 to –0.55 V by addition of CO_2 as shown in **Figure 2**. Increase of 0.4 μA of current indicates that electrons are transferred from electrode to bacterial cells coupled to redox reaction of NR. Increase of –0.3 V of redox potential is a clue that electrons are transferred from electrode to NR by lower electrode (working electrode) potential than intrinsic redox potential of NR. Relatively higher electron-driving force (electrode potential) may be required for electrons to move through the electric resistance generated between electrode and NR immobilized in bacterial membrane. Meanwhile, other upper voltammetric peak (bold arrow mark) located at –0.9 V (vs. Ag/AgCl) was also shifted upward from 2.7 to 3.0 μA and rightward from –0.9 V to –0.93 V by addition of CO_2. It seems possible that electrons are transferred from the electrode via one of the electron carriers located in the bacterial membrane,

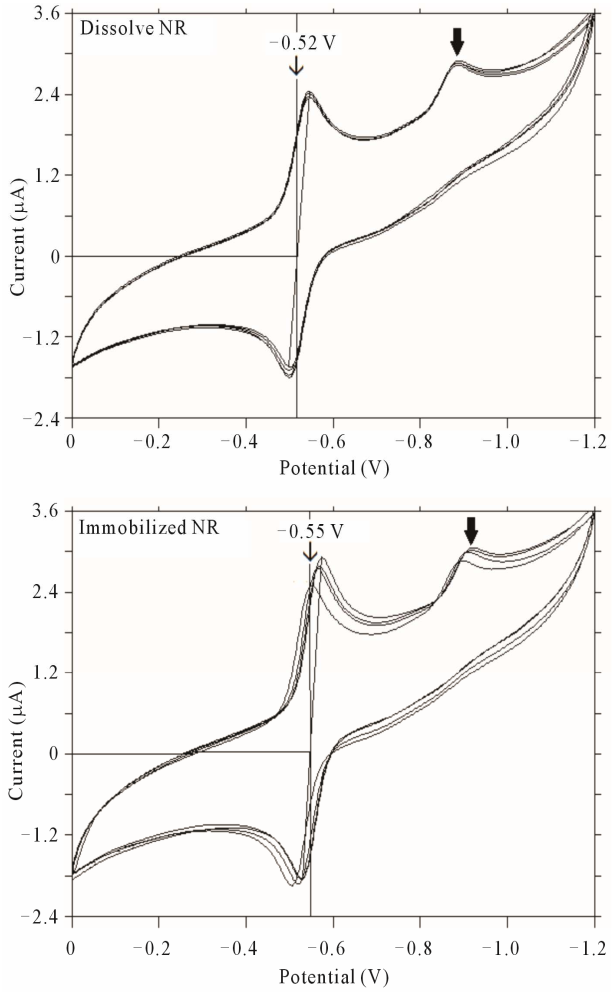

Figure 2. Cyclic voltammetry for NR dissolved in reactant and immobilized in *C. acetobutylicum* during cyclic potential scanning from 0 mV to –1200 mV. Upper and lower voltammetric peaks for dissolved NR were not altered but for immobilized NR were shifted upward and rightward, respectively, by addition of CO_2. Other upper peak (bold arrow mark) also was shifted upward and rightward by addition of CO_2.

allowing for current and potential increase by addition of CO_2. CO_2 could act as an electron acceptor to induce biochemical oxidation of NADH that may be electrochemically regenerated, by which electrons may be transferred from electrode to bacterial cells via the coupling redox reaction of NR and NAD^+.

3.2. Growth and Metabolite Production of *C. acetobutylicum*

C.acetobutylicum did not grow and didn't produce metabolites in the defined medium under N_2 atmosphere (DM-N_2) but grew and produced acetate, propionate, and butyrate under H_2 atmosphere (DM-H_2), as shown in **Table 1**. The metabolites detected in the chromatography for culture fluid of *C. acetobutylicum* cultivated in the DM-N_2 may have originated from the metabolites contained in the inoculum. The electrochemical reducing power generated from NR-cathode (reduced NR) is converted to the biochemical reducing power (NADH), which can be presumed on the basis of the growth and metabolite production of *C. acetobutylicum* cultivated in the DM-ER. *C. acetobutylicum* cultivated with electrochemical reducing power produced more acetate than butyrate in DM-ER but more butyrate than acetate in CM-ER. These are more clues that biochemical reducing power (NADH) may be regenerated by electrochemical reducing power generated from –2 V of NR-cathode and the high balance of NADH/NAD^+ may induce metabolic conversion of CO_2 to metabolites in coupling with free energy synthesis.

3.3. Quantitative and Qualitative Verification of Metabolites

Metabolites generated by *C. acetobutylicum* in different cultivation conditions were quantitatively and qualitatively analyzed by a specially trained expert, and found to be acetate, propionate, and butyrate as shown in **Figure 3**, as expected. This analytical process is absolutely

Table 1. Growth and metabolite production of *C. acetobutylicum* cultivated in complex medium (CM) and defined medium (DM) under 2 atm of N_2 atmosphere, 2 atm of H_2 atmosphere, and electrochemical reduction condition (ER) for 5 days.

Cultivation conditions	Growth at OD_{660} (initial-final)	Metabolites (mM)		
		Acetic acid	Propionic acid	Butyric acid
DM-N_2	0.08 - 0.06	0.6 ± 0.04	0.1 ± 0.01	0.5 ± 0.02
DM-ER	0.08 - 0.36	9.2 ± 0.2	0.8 ± 0.03	6.4 ± 0.3
DM-H_2	0.08 - 0.38	9.8 ± 0.3	0.9 ± 0.02	6.0 ± 0.3
CM-N_2	0.08 - 1.28	35.8 ± 1.4	2.8 ± 0.1	21.6 ± 1.1
CM-ER	0.08 - 1.06	19.6 ± 0.8	4.8 ± 0.2	38.4 ± 0.9
CM-H_2	0.08 - 1.21	24.4 ± 0.9	4.2 ± 0.2	36.6 ± 1.3

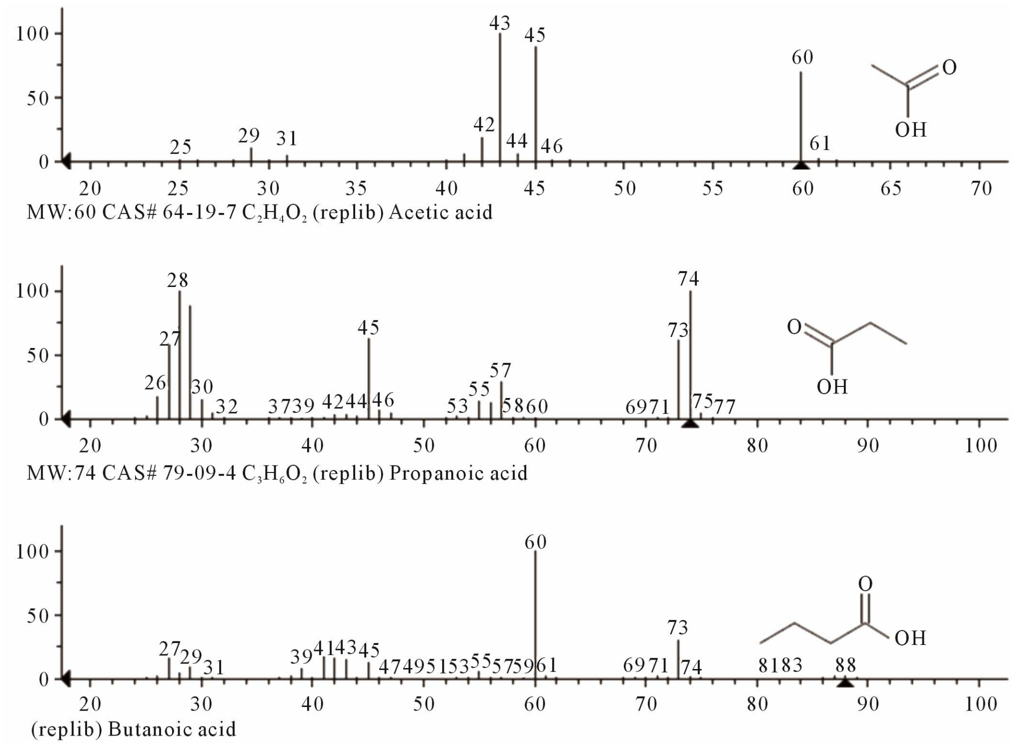

Figure 3. Mass spectrometer profiles of three major peaks detected in gas chromatography of volatiles contained in the culture fluid of *C. acetobutylicum*.

required because some metabolic intermediates derived from amino acids (yeast extract) may be produced by bacterial cells cultivated in the DM-H_2 and DM-ER condition.

3.4. Analysis of Genes Induced by Electrochemical Reducing Power

Significant genes (higher than twice the signal intensity) commonly expressed in *C. acetobutylicum* that was electrochemically cultivated in the defined medium under CO_2-atmosphere and in the complex medium under N_2-atmosphere numbered in 318. All of the fundamental genes related to CO_2-fixation were not detected; however, the genes of encoding the enzymes catalyzing acetyl-CoA synthesis from CO_2, ATP synthesis, and butyrate production were quantitatively analyzed and compared as shown in **Table 2**. The genes encoding the enzymes catalyzing acetyl-CoA generation from CO_2 and ATP synthesis in pathway from acetyl-CoA to acetate were more expressed but those catalyzing NADH regeneration coupled to oxidation of substrates (metabolic intermediates) and butyric acid production were less expressed in *C. acetobuylicum* cultivated in DM-ER condition than CM-N_2condition (**Table 1**).

4. Discussion

Electron transfer from electrode to bacterial cells can be generated by the simultaneous contact of an electron mediator with both electrode and bacterial cells. Contact of an electrode with the electron mediator (NR) immobilized in bacterial cell or contact of bacterial cells with the electron mediator immobilized in an electrode is a unique way to induce electron transfer between bacterial cells and electrode [17-19]. Patterns of cyclic voltammetry of NR immobilized in bacterial cells were identical to those of NR dissolved in reactant in the condition uncoupled to the external redox reaction, because the redox reaction is proportional to the concentration of NR contacted stably with the electrode (**Figure 2**). Some NRs immobilized in *C. acetobutylicum* are electrochemically reduced and biochemically oxidized coupled to NADH regeneration [6]. This electrochemical and biochemical coupling redox reaction of NR and NAD^+ may be continuously repeated in this condition with both electron donor and acceptor. The electrode may be an electron donor and CO_2 may be an electron acceptor in the cyclic voltammetry for the modified *C. acetobutylicum* with NR, considering that addition of CO_2 induced electron transfer from electrode to bacterial cells via NR immobilized in bacterial cells or other bacterial electron carrier (upper voltammetric peaks in **Figure 2**) can be quantitatively analyzed based on the increase of current (electron number) and variation of redox potential (electron-driving force). The NR immobilized in bacterial cells can temporarily function in proportion to physiological activity

Table 2. Comparison of genes expressed in *C. acetobutylicum* cultivated with electrochemical reducing power in the CO_2-saturated defined medium and cultivated with glucose in the complex medium.

Ratio of A/B	Gene products (Functions)	Signal intensity for specific genes expressed in *C. acetobutylicum*	
		Electrochemically cultivated (A)	Cultivated in complex medium (B)
4.87	Carbon monoxide dehydrogenase (CO formation from CO_2)	7764	1595
4.71	Biotin-acetyl-CoA-carboxylase (Malonyl-CoA production)	2846	604
31.7	Formyl-H_2folate synthase (Methyl-formation from CO_2)	317	10
2.89	Formyl-H_2folate cyclohydrolase (Methyl-formation from CO_2)	772	267
3.24	Putative methyltransferase (Methyl-formation from CO_2)	13,908	4287
244.8	Phosphotransacetylase (Formation of acetyl-Pi from acetyl-CoA)	2448	10
212.4	Acetate kinase (Acetate production coupled to ATP synthesis)	2124	10
0.74	NAD-dependent dehydrogenase (NADH regeneration coupled to substrate oxidation)	1856	2524
0.21	Acetyl-CoA acetyltransferase (Butyric acid production)	4179	19,492

of the bacteria modified with NR; however, NR immobilized in the graphite felt cathode can semipermanently function as long as it is contacting with intact cells of bacteria.

In the electrochemical bioreactor equipped with the NR-cathode, the electron transfer from electrode to bacterial cells can't be quantitatively analyzed because the number of electrons (current) transferred from power supply to NR-cathode is not proportional to the metabolic reduction reaction catalyzed by bacterial cells, and –2 V of electrode potential charged to the bioreactor was stronger than the redox potential of NR. This can generate electron-driving force to transfer electrons via NR immobilized in electrode to bacterial cells but may induce electrochemical reduction of medium ingredients and other organic compounds. The –2 V of electrode potential may be too strong to induce the electrochemical reduction of NR (–0.325 V vs. NHE) but is required to induce electron transfer from electrode to bacterial cells through the electron barrier (cytoplasmic membrane) because electric resistance may be generated by reactor membrane between anolyte and catholyte, connecting error between NR and bacterial cells, and structural mismatch between NR and NAD^+ [20].

It is unquestionable that the NR immobilized in graphite felt electrode mediated electron transfer from electrode to bacterial cells and catalyzed regeneration of biochemical reducing power on the basis of acetic and butyric acid production from CO_2 and production of more butyric acid than acetic acid from glucose. This result is very similar to that obtained from *C. acetobutylicum* culture using H_2 and CO_2 as an electron donor and acceptor. Metabolic production of acetic acid is coupled to NADH regeneration but that of butyric acid is coupled to NADH oxidation in *C. acetobuylicum* grown in glucose. Accordingly, higher production of butyric acid than acetic acid by *C. acetobutylicum* cultivated in CM-ER and CM-H_2 is another clue that H_2 and electrochemically reduced NR may be an additional reducing power to increase ratio of NADH/NAD^+ [21].

Metabolic conversion of CO_2 to metabolites is coupled to oxidation of biochemical reducing power regenerated by the electrochemically reduced NR or H_2; however, the metabolic pathways related to the autotrophic CO_2 fixation can be assumed only by the metabolites produced by *C. acetobutylicum* cultivated in different media and under different conditions. Microarray analysis of mRNA is effective to analyze variations of metabolic pathway and free energy production related to autotrophic CO_2fixation or heterotrophic growth. Practically, the specific genes related to the CO_2 fixation and energy metabolism expressed in *C. acetobutylicum* electrochemically or conventionally cultivated are a clue that the metabolic conversion of CO_2 to metabolites in coupling with the free energy production and redox reaction of reducing power may be generated by the electrochemical reducing power. Theoretically, –2 V of electricity charged to the NR-cathode located in culture medium may induce H_2 generation by electrolysis of H_2O. However, H_2 was not detected in the electrochemical bioreactor even by precision analysis. Accordingly, the NR-cathode may directly transfer electrons from electrode to intact cells of *C. acetobutylicum* and induce catalyzing of NADH regeneration in metabolism of *C. acetobutylicum*.

5. Conclusion

The electrochemical redox reaction of NR, the catalytic function of NR for NADH regeneration, and the immobilization technique of NR in the electrode permit *C. acetobutylicum* KCTC1037 to grow and produce metabolites using electrochemical reducing power. Mixed acid fermentation bacteria produced the relatively reduced metabolite (butyrate) or oxidized metabolite (acetate) depending on balance of NADH/NAD^+. In autotrophic microbes, CO_2 can be reduced to CO by catalysis of carbon monoxide dehydrogenase in coupling with oxidation of biochemical reducing power (NADH or NADPH). Practically, *C. acetobutylicum* produced more butyrate than acetate from glucose and more acetate than butyrate from CO_2, reasonable on the basis of metabolic pathway for ATP regenerations. *C. acetobutylicum* cultivated heterotrophically with glucose synthesizes ATP by substrate-level phosphorylation and regenerates NADH in both glycolysis and pathway from pyruvate to acetate but that cultivated autotrophically with electrochemical reducing power and CO_2 synthesizes ATP in the pathway from acetyl-CoA to acetate and regenerates NADH coupled to electrochemical redox reaction of NR.

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