Bioelectrochemical Mn(II) Leaching from Manganese Ore by *Lactococcus lactis* SK071115

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Received: July 27, 2010 / Revised: November 9, 2010 / Accepted: November 10, 2010

L. lactis **sk071115 has been shown to grow more actively and generate lower levels of lactate in glucose-defined medium with nitrate than in medium with Mn(IV). By adding Mn(IV) to a *L. lactis* culture, lactate production was relatively reduced in combination with Mn(II) production, but cell mass production levels did not increase. Both cell-free extract and intact *L. lactis* cells reacted electrochemically with Mn(IV) but did not react with Mn(II) upon cyclic voltammetry using neutral red (NR) as an electron mediator. A modified graphite felt cathode with NR (NR-cathode) was employed to induce electrochemical reducing equivalence for bacterial metabolism. Cell-free *L. lactis* extract catalyzed the reduction of Mn(IV) to Mn(II) under both control and electrochemical reduction conditions; however, the levels of Mn(II) generated under electrochemical reduction conditions were approximately 4 times those generated under control conditions. The levels of Mn(II) generated by the catalysis of *L. lactis* immobilized in the NR-cathode (L-NR-cathode) under electrochemical reduction conditions were more than 4 times that generated under control conditions. Mn(II) production levels were increased by approximately 2.5 and 4.5 times by the addition of citrate to the reactant under control and electrochemical reduction conditions, respectively. The cumulative Mn(II) produced from manganese ore by catalysis of the L-NR-cathode for 30 days reached levels of approximately 3,800 and 16,000 mg/l under control and electrochemical reduction conditions, respectively. In conclusion, the electrochemical reduction reaction generated by the NR-cathode activated the biochemical reduction of Mn(IV) to Mn(II) by *L. lactis*.**

Keywords: *Lactococcus lactis*, Mn leaching, electrochemical reduction, cyclic voltammetry, neutral red

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The reduction of insoluble manganese dioxide [Mn(IV)] to soluble manganese oxide [Mn(II)], which is required for the liquid extraction of manganese from ores, may be achieved biologically in the presence of metal-reducing bacteria [32, 33]. The metal-reducing bacteria capable of reducing Mn(IV) to Mn(II) in a dissimilative manner have proven useful for bacterial Mn leaching [2, 4, 18]. The dissimilative Mn(IV) reduction can be defined as the use of Mn(IV) as an external electron acceptor in bacterial respiration [17, 21]. Various bacteria that have typically been identified as dissimilative Mn(IV)-reducers are reported to accumulate Mn(II) in organically complex media under anaerobic conditions [22, 29]. Mn(IV) reduction has been identified as a minor pathway for electron flow in bacteria that reduce Mn(IV) to Mn(II) while metabolizing fermentable sugars or amino acids; this is consistent with the research results showing that certain fermentative Mn(IV)-reducing microorganisms consumed less than 5% of the reducing equivalents of their substrates for the metabolic reduction of Mn(IV) to Mn(II) [17, 18].

Lactococcus sp. belonging to the lactic acid-fermenting bacteria generate 2 moles of lactic acid from 1 mole of glucose, in which NADH regenerated *via* the metabolic oxidation of glucose is oxidized to NAD^+ coupled to the reduction of pyruvate to lactate. However, *L. garvieae* sk11 has been reported to generate less than 2 moles of lactate from 1 mole of glucose under conditions including Fe(III) [35], in which some of the NADH may be oxidized in combination with the reduction of Fe(III) to Fe(II), and Fe(III) may function as an external electron acceptor rather than an internal electron acceptor, which in this case is pyruvate. Meanwhile, another metal-reducing lactic acid bacterium (*L. lactis* sk071115) that is capable of selectively reducing Mn(IV) to Mn(II) also generated less than 2 moles of lactate from 1 mole of glucose under growth conditions with Mn(IV).

Bacterial Mn leaching by the metabolic reduction reaction of *Lactococcus* sp. differs from the bioleaching

process consisting of the leaching of copper from copper sulfides after oxidation, with enhancements by acidophilic bacteria such as *Thiobacillus ferroxidans*, *T. thiooxidans*, and *Leptospirillum ferrooxidans* [7, 9, 10]. Copper bioleaching involves the recovery of copper from a leaching solution that is subsequently returned after the replenishment of the sulfuric acid that reacted with salts in the ore; on the other hand, Mn bioleaching requires glucose rather than sulfuric acid. The metabolic reduction of Mn(IV) to Mn(II) has to be proportional to the reducing power generated from the metabolic oxidation of glucose. Mn(IV)-reducing bacteria have not been determined to gain energy to support growth from the reduction of Mn(IV). Mn(IV) appears to function as a minor electron sink in a primarily fermentative metabolism [18]. The bacterial reducing power generated, coupled to the metabolic oxidation of glucose, may be consumed in a limited fashion for the reduction of Mn(IV) to Mn(II). Accordingly, a special fermentation technique is required to enhance NADH regeneration in bacterial metabolism without depending on glucose.

The electrochemical oxidation-reduction reaction charged to bacterial culture is useful for the regulation of bacterial growth or metabolism [28]. Electrochemically reduced neutral red (NR) was determined to reduce NAD^+ to NADH directly without the need for enzyme catalysis [24], which is useful for the maintenance of $NADH/NAD^+$ balance during bacterial metabolism. Electrochemically reduced NR activated the enzymatic reduction of xylose to xylitol, catalyzed by cell-free extracts of *Candida peltata* [25]; additionally, *Zymomonas mobilis* immobilized in the NR-cathode generated profoundly higher levels of ethanol under electrochemical reduction conditions than under control conditions [11]. *Ochrobactrum* spp. immobilized in the NR-cathode were reported to reduce nitrate to nitrogen effectively under electrochemical reduction conditions [16].

Thus, the principal objective of this study was to measure the electrochemical reaction occurring between cell-free extract and Mn(IV) *via* cyclic voltammetry using NR in order to identify a specific function of *L. lactis* sk071115 for the catalysis of biochemical Mn(IV) reduction to Mn(II). The production of Mn(II) from Mn(IV) or manganese ore *via* catalysis of the L-NR-cathode under electrochemical reduction conditions was compared with that observed under control conditions in order to estimate the possibility of bioelectrochemical Mn(II) leaching using a lactic acid bacterium.

MATERIALS AND METHODS

Chemicals
All chemicals employed herein were purchased from the Korean branch of Sigma-Aldrich (Young In, Korea). Manganese ore was provided by the Korean Institute of Geoscience and Mineral Resources, and was ground with ball meal (Bestir, Poonglim, Korea) prior to use.

Microorganism
L. lactis sk071115 isolated from sediment in Jungrang-cheong (Seoul, Korea) using a glucose-defined medium (100 mM of glucose, 1 g/l of yeast extract, 100 mM of MnO_2, 25 mM of phosphate buffer, 2 ml/l of trace mineral stock solution, pH 7) was identified on the basis of 16S rDNA sequence homology. Mn(IV) was separately autoclaved and then mixed with other medium ingredients to protect the thermochemical reduction of Mn(IV) to Mn(II) during autoclaving. The trace mineral stock solution used herein contained 0.01 g/l of $MnSO_4$, 0.01 g/l of $MgSO_4$, 0.01 g/l of $CaCl_2$, 0.002 g/l of $NiCl_2$, 0.002 g/l of $CoCl_2$, 0.002 g/l of $SeSO_4$, 0.002 g/l of WSO_4, 0.002 g/l of $ZnSO_4$, 0.002 g/l of $Al_2(SO_4)_3$, 0.0001 g/l of $TiCl_3$, 0.002 g/l of $MoSO_4$, and 10 mM EDTA [12]. The glucose-defined medium (GDM) was used in all tests for bacterial culture, bacteriological Mn(IV) reduction, and bioelectrochemical Mn(II) leaching from manganese ore.

Identification of Isolate
The 16S ribosomal DNA was amplified *via* direct PCR using the chromosomal DNA template and the following 16S-rDNA-specific universal primers: forward 5'-GAGTTGGATCCTGGCTCAG-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The PCR reaction mixture (50 μl) consisted of 2.5 U of *Taq* polymerase, 250 μM of each dNTP, 10 mM of Tris-HCl (pH 9.0), 40 mM of KCl, 100 ng of template, 50 pM of primer, and 1.5 mM of $MgCl_2$. Amplification was conducted for 30 cycles of 1 min at $95^{o}C$, 1 min of annealing at $55^{o}C$, and 2 min of extension at $72^{o}C$ using a PCR machine (T Gradient model; Biometera, Germany). Bacterial identity was determined on the basis of 16S rDNA sequence homology with the GenBank database system.

Metabolic Characterization of *L. lactis*
L. lactis sk071115 was cultivated in the GDM with 100 mM of nitrate or Mn(IV) to estimate the effects of external electron acceptors on growth and lactate production. Eighty ml of medium was prepared in an anaerobic serum vial (total volume of 165 ml), the head space of which was filled with 99.99% nitrogen. Then 10% (v/v) of bacterial culture previously cultivated in GDM without Mn(IV) and nitrate was inoculated into the prepared medium in the serum vials, which were incubated for 48 h at $30^{o}C$ without shaking. Cell mass, substrate consumption, and metabolite [lactate, Mn(II) ion and nitrite] production were quantitatively analyzed. In order to measure the dry cell mass of *L. lactis*, 1 l of bacterial culture, the bacterial density of which was adjusted optically to 1.0, was filtered with a membrane filter (pore 0.22 μm, diameter 47 mm; Millipore, USA) that was previously dried for 24 h at $110^{o}C$ and washed with 200 ml of distilled water under vacuum. The washed membrane filter was dried for 24 h at $110^{o}C$. Dry cell mass was determined by the difference of weight between the previously dried plain membrane filter and the dried bacteria-filtrated membrane filter.

Cyclic Voltammetry
The cyclic voltammetry was conducted using a cyclovoltammetric potentiostat (BAS model 50W, USA) employing an Ag/AgCl reference electrode, a glassy carbon working electrode (3 mm diameter) and a platinum wire counter-electrode (0.5 mm diameter and 40 mm length)

into a reaction mixture containing 25 mM of Tris buffer (pH 7.5), 100 μM of NR, and intact cells (cell mass, 2.5 g/l) or cell-free extract (protein concentration, 10 mg/ml) of *L. lactis*. NR, which can be electrochemically oxidized (lower peak) and reduced (upper peak), was used as an electron mediator for electron transfer between the electrode and biocatalysts. The scan range was adjusted from –800 mV to 0 mV vs. Ag/AgCl, and the scan ratio was adjusted to 25 mV/s. In an effort to analyze the redox reaction between bacterial cells (cell extract) and substrate, Mn(IV) or Mn(II) was added to the reaction mixture at the moment of transition from the second to third cycles during 10 continuously repeated scan cycles.

Electrochemical Bioreactor

NR was immobilized in the graphite felt electrode, based on the technique developed by Jeon and Park [11], which was employed as a cathode (NR-cathode). A noncompartmented electrochemical bioreactor (working volume, 1 l) composed of the NR-cathode and a three-layered carbon anode was prepared in accordance with the technique developed by Lee and Park [16] as shown in Fig. 1. Two volts of DC electricity was charged between the NR-cathode (reduction electrode) and the carbon anode (oxidation electrode) to induce electrochemical reduction reactions of NR, by which a

Fig. 1. Schematic structure of noncompartmented electrochemical bioreactor (NCEB) (**A**), mechanism of anodic reaction (**B**), and three-layered anode (**C**).
Water transferred from the cathode compartment to porous carbon anode through the cellulose acetate film may be electrolyzed to protons, electrons, and oxygen. The cellulose acetate film can selectively penetrate water or gas but not solutes.

reducing equivalence for bacterial metabolism can be generated; however, the DC electricity was not charged between the NR-cathode and the carbon anode for control experiments. The electrochemical bioreactor used for control experiments was named the control bioreactor.

Preparation of Biocatalyst

Cell-free extract or intact cells of *L. lactis* sk071115 were used as a biocatalyst for biochemical Mn(IV) reduction to Mn(II). Cell-free extract was obtained from disrupted cells of *L. lactis* sk071115 cultivated for 48 h and then harvested and washed *via* 40 min of centrifugation at 5,000 ×g and 4°C. Bacterial cells were disrupted by pulsed ultrasonication (Sonic & Materials, VCX-400W) at 30 s intervals for 40 min at 4°C. Intact cells were prepared by the centrifugation of bacterial cells cultivated for 48 h at 3,000 ×g and 4°C for 30 min under aseptic conditions. Ten ml of harvested cells was mixed with 10 ml of 25 mM phosphate buffer (pH 7.0) containing 4% alginate, and then absorbed into the NR-cathode. The NR-graphite felt containing a mixture of alginate and bacterial cells was soaked in a 100 mM CaCl$_2$ solution to induce calcium alginate coagulation for 30 min, and then washed with 25 mM of phosphate buffer, which was employed as the biocatalyst for batch and continuous cultures to skip over the lag phase. The final cell mass immobilized in a NR-cathode (30×60×210 mm) was adjusted to a dry weight of 2 g.

Biochemical Reduction of Mn(IV) to Mn(II)

Cell-free extract of *L. lactis* sk071115 was used as a biocatalyst to identify the existence of an enzyme catalyzing the reduction of Mn(IV) to Mn(II). The reaction mixture composed of 100 mM of Mn(IV), 25 mM of phosphate buffer (pH 7.5), 10 μM of NAD$^+$, and cell-free extract (protein, 0.6 mg/ml) was prepared in an electrochemical bioreactor (working volume, 50 ml) composed solely of the NR-cathode. The GDM with 100 mM Mn(IV) was prepared in the electrochemical bioreactor composed of the L-NR-cathode to estimate the reduction of Mn(IV) to Mn(II) by intact *L. lactis* cells. The reaction was initiated by charging the electrochemical bioreactor with electricity. The control bioreactor was not charged with electricity.

Mn(II) Production from Manganese Ore

GDM with 20 mM of citrate and 50 g/l of manganese ore was prepared in the control and the electrochemical bioreactor composed of L-NR-cathode. The reaction was initiated by charging electricity between the L-NR-cathode and carbon anode. The fresh reaction mixture without manganese ore was flowed continuously in the bioreactors at a rate of 330 ml/day based on the growth rates of *L. lactis*, and the reactant flowed out from the bioreactor was analyzed to determine the concentration of Mn(II). The concentration of Mn(II) generated from the manganese ore was totaled up every 3 days to calculate the amount of cumulative Mn(II) produced from manganese ore for 30 days.

Analysis of Glucose and Lactic Acid

Glucose and lactic acid contained in the bacterial culture were analyzed by HPLC using an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, USA) and a refractive index detector. The column and detector were adjusted to a temperature of 35°C. Sulfuric acid (0.008N) was used as a mobile phase, at a flow rate of 0.6 ml/min. The samples prepared *via* 30 min of centrifugation at 12,000 ×g and
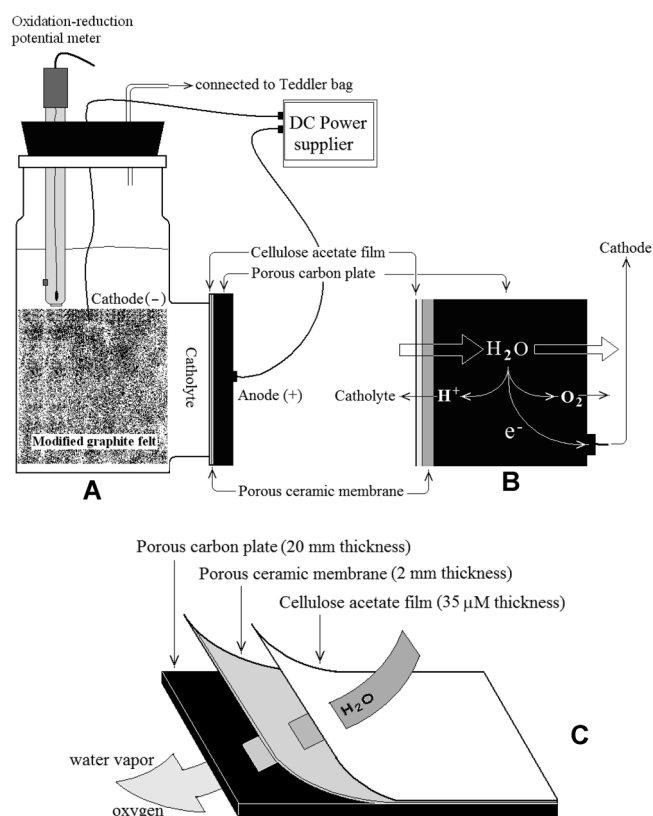
Table 1. Growth and metabolism of *L. lactis* sk071115 on glucose-defined medium containing Mn(IV) or nitrate for 72 h.

Substrates (mM)	Metabolite (lactate, mM)	Cell mass (dry weight, g/l)	e^--acceptor reduced (mM)	Lactate/glucose[a]
Glucose	195±9	0.61±0.02	-	1.9±0.2
Glucose-NO$_3^-$	157±6	0.73±0.05	NO$_2^-$ 4.7±0.23	1.5±0.1
Glucose-Mn(IV)	171±8	0.62±0.02	Mn(II) 1.9±0.03	1.7±0.2

[a]Lactate/glucose indicates the ratio of lactate production to glucose consumption.

4°C were subsequently filtered with a 0.22-μm-pored membrane filter. Twenty μl of filtrate was then injected into the HPLC injector. The concentrations of glucose and lactic acid were calculated based on the peak area in the chromatograms obtained using standard materials.

Analysis of Mn(II)
Water-soluble Mn(II) was determined using a technique adapted from *Standard Methods for the Examination of Water and Wastewater* [8]. Bacterial culture, enzyme reaction mixture, and bacterial reactant containing water-insoluble Mn(IV) were centrifuged for 30 min at 5,000 ×*g* and 4°C. The centrifugal supernatant containing Mn(II) was filtrated with a 0.22-μm-pored membrane filter and diluted appropriately to near the detection limit. The diluted supernatant was reacted with coloring reagent (citrate buffer and sodium periodate; HACH, Germany) and then measured with a spectrophotometer (DR2500; HACH, Germany) in accordance with the manufacturer's recommended procedures. The concentration of Mn(II) in the reactant was calculated using a program provided by the manufacturer.

Analysis of Nitrite
Nitrates and nitrites were analyzed using an ion chromatograph (IC, Dionex DX-500, USA) equipped with an anion exchange column (IonPac, Dionex AS14A, 4×250 mm). 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate solutions were used as a mobile phase at a flow rate of 2.0 ml/min. The column temperature was adjusted to 30°C. Samples were prepared *via* the centrifugation of bacterial cultures at 12,000 ×*g* and 4°C for 30 min and then filtered

with a membrane filter with a pore size of 0.22 μm. The filtrate was diluted appropriately to near the detection limit and then injected into an injector; the injection volume was adjusted to 50 μl with a sample loop.

RESULTS

Metabolic Characterization of *L. lactis* sk071115
Lactate generated by *L. lactis* (GenBank Accession No. EU284135) grown under conditions with nitrate and Mn(IV) was measured at approximately 52% and 74%, respectively, of that measured under conditions without nitrate and Mn(IV). The cell mass of *L. lactis* grown under conditions with nitrate was increased by approximately 19%; on the other hand, the cell mass of *L. lactis* under conditions with Mn(IV) did not vary greatly, as demonstrated in Table 1. *L. lactis* is expected not to produce free energy coupled to metabolic Mn(IV) reduction in comparison with the cell mass generated in the GDM; however, it may produce free energy in combination with the reduction of metabolic nitrate. Growing cells of *L. lactis* generated 4.7 mM of nitrite and 1.9 mM of Mn(II) from nitrate and Mn(IV), respectively, during the metabolic oxidation of 100 mM glucose for 72 h.
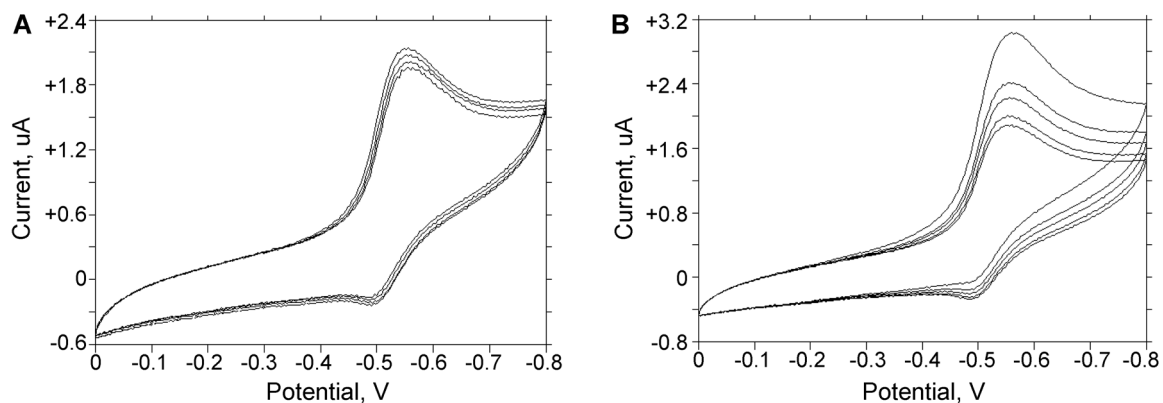

Fig. 2. Variation of cyclic voltammogram for cell-free extract of *L. lactis* by addition of Mn(II) (**A**) and Mn(IV) (**B**).
One hundred μl of 100 mM Mn(II) or Mn(IV) was added to the reactant (5 ml) at the moment converting from the second cycle to the third cycle. The protein concentration of cell-free extract was adjusted to 10 mg/ml in the reactant composed of 25 mM Tris buffer (pH 7.5) and 100 mM neutral red.
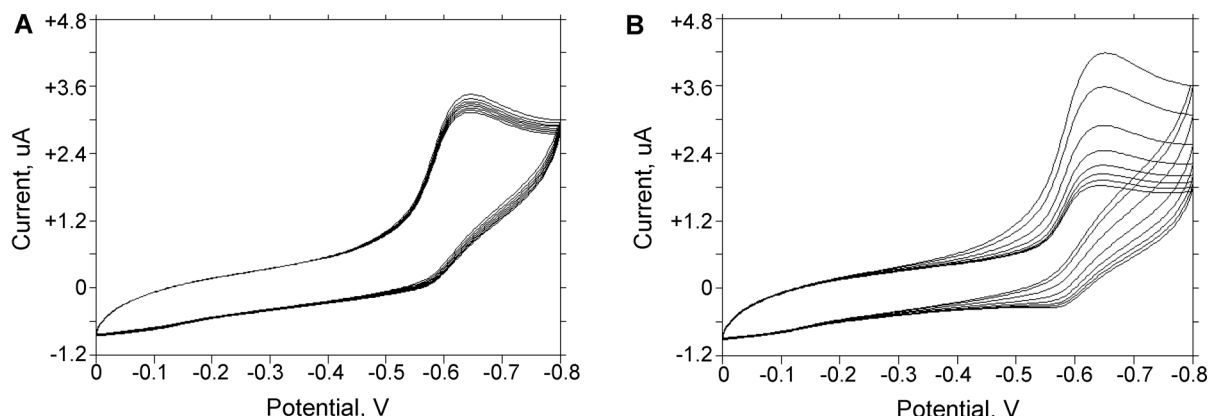
Fig. 3. Variation of cyclic voltammogram for modified intact cells of *L. lactis* with NR by addition of Mn(II) (**A**) and Mn(IV) (**B**). One hundred μl of 100 mM Mn(II) or Mn(IV) was added to the reactant (5 ml) at the moment converting from the second cycle to the third cycle. The biomass was adjusted to 8.0 based on the optical density in the reactant composed of 25 mM Tris buffer (pH 7.5).

Electrochemical Reaction of Biocatalysts with Manganese Ions

Cyclic voltammetry is a useful technique for observing the electrochemical reaction of biocatalysts with manganese ions. The biochemical or metabolic redox reaction of manganese ions catalyzed by cell-free extract or intact *L. lactis* cells was measured by cyclic voltammetry (CV) and NR. As shown in Fig. 2 and 3, the upper peak height in the CV for cell-free extract (crude enzyme) and intact *L. lactis* cells was increased as the result of the addition of Mn(IV), but was not increased by the addition of Mn(II). This phenomenon can occur when electrons are transferred unidirectionally from the electrode to Mn(IV) in combination with the electrochemical redox reaction of NR *via* the catalysis of intact cells or cell-free *L. lactis* extracts.

Mn(IV) Reduction by Cell-Free Extract

A cell-free extract of *L. lactis* catalyzed the reduction of Mn(IV) to Mn(II) under both control and electrochemical reduction conditions. As is shown in Fig. 4, more than 4
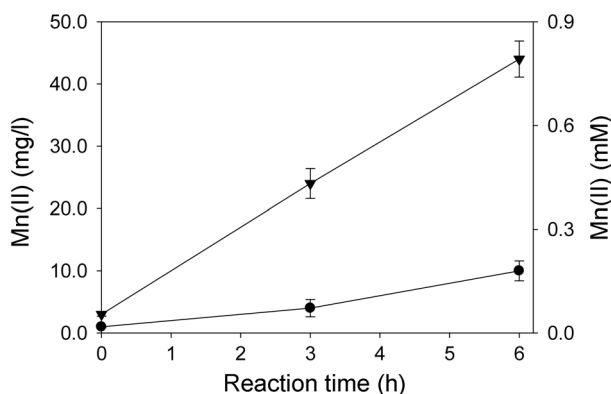
times the amount of Mn(II) was produced by the catalysis of cell-free extract under the electrochemical reduction conditions generated by the NR-cathode than under control conditions. It is possible that the high NADH/ NAD$^+$ balance maintained in combination with the electrochemical reduction reaction of NR may induce the specific enzyme contained in the cell-free extract to reduce Mn(IV) actively to Mn(II). The NR-cathode did not catalyze the reduction of Mn(IV) to Mn(II) under the reaction conditions without cell-free extract (data not shown). These results present a clear clue that a specific enzyme of *L. lactis* that catalyzes the metabolic Mn(IV) reduction may be activated by using the NR-cathode under electrochemical reduction conditions.

Mn(IV) Reduction by Intact Cells

The L-NR-cathode was employed as a biocatalyst for the analysis of the bioelectrochemical reduction of Mn(IV) to Mn(II) by intact *L. lactis* cells. As shown in Fig. 5, the L-
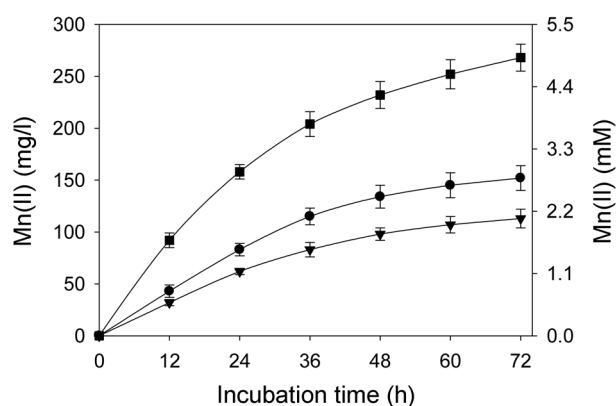

Fig. 4. Mn(IV) reduction to Mn(II) by cell-free extract of *L. lactis* under the control condition (●) and the electrochemical reduction condition (▼) composed of the NR-cathode.

Fig. 5. Mn(IV) reduction to Mn(II) by L-NR-cathode in the control condition (●), the electrochemical reduction condition (▼) without glucose, and in the electrochemical reduction condition (■) with glucose.
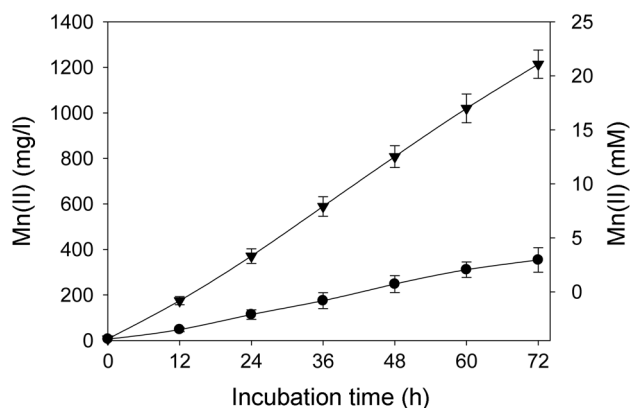
Fig. 6. Effect of citrate on production of Mn(II) from Mn(IV) by the L-NR-cathode in the control condition (●) and electrochemical reduction condition (▼).

Fig. 7. Mn(II) production from manganese ores by bacterium–NR-graphite in the control condition (●) and the electrochemical reduction condition (▼).

NR-cathode catalyzed the production of approximately 2.1 mM of Mn(II) from Mn(IV) under electrochemical reduction conditions without glucose; meanwhile, approximately 2.7 mM and 5.0 mM of Mn(II) were produced from Mn(IV) under control and electrochemical reduction conditions, respectively, during the metabolic oxidation of 100 mM glucose for 72 h. This is a critical clue, suggesting that the NR-cathode may catalyze electrochemical NADH regeneration in the cytoplasm of *L. lactis*, and Mn(IV) may be metabolically reduced to Mn(II) in combination with the oxidation of NADH to NAD^+. The electrochemically regenerated NADH may be reoxidized to NAD^+ in combination with the reduction of Mn(IV) to Mn(II), because the internal electron acceptor (pyruvate) is not generated under conditions without glucose; meanwhile, NADH regenerated in combination with the metabolic oxidation of glucose may be oxidized largely in coupling with the reduction of pyruvate to lactate. Accordingly, the metabolic production of Mn(II) by the L-NR-cathode must be effectively increased under reaction conditions with both the electrochemical reducing power and glucose; however, the rate of Mn(II) production was slowed with increasing incubation time. This may be induced by the adhesion of Mn(II) to solid compounds such as bacterial cells, electrodes, or Mn(IV).

Effect of Citrate on Mn(II) Production by Intact Cells
The adhesion of Mn(II) to solid compounds is not likely to be leached. In order to solve this problem, a biologically generated chelating agent was introduced to the bioreactor. The rate of Mn(II) production tended to be increased in a straightforward fashion in proportion to the incubation time *via* the addition of citrate, as shown in Fig. 6. The direct proportional relationship between Mn(II) production and incubation time is a critical factor that is absolutely required for the leaching of Mn(II) from manganese ore. Practically, the production of Mn(II) *via* catalysis of the L-
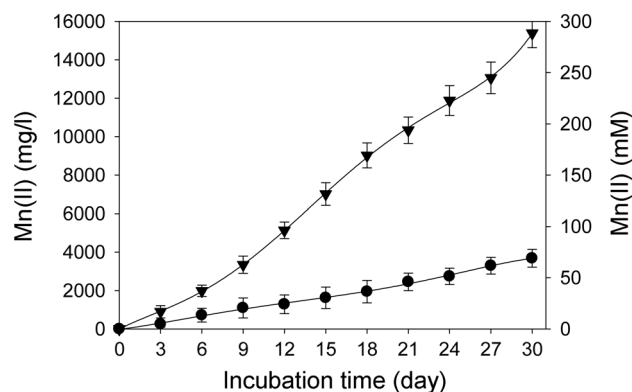
NR-cathode in the electrochemical bioreactor was 4 times higher than that measured in the control bioreactor.

Mn(II) Leaching from Manganese Ore by Intact Cells
The L-NR-cathode was employed as a biocatalyst to leach Mn(II) from manganese ore, which exists as a water-insoluble black powder under both control and electrochemical reduction conditions. As shown in Fig. 7, the cumulative concentration of Mn(II) under electrochemical reduction conditions reached a level of approximately 16,000 mg/l for 30 days; this is approximately 4.5 times that measured under control conditions. The maintenance of catalytic activity of *L. lactis* for Mn(II) leaching for 30 days indicates that the metabolic function of *L. lactis* can be maintained without any loss of its physiological activity for at least 30 days.

DISCUSSION

The electrochemically reduced NR was already demonstrated to regenerate NADH without enzyme catalysis [11, 16, 25]. NR must make simultaneous contact with both the electrode and the bacterial cells to mediate electron transfer from the electrodes to the bacterial cells. NR was covalently immobilized in the graphite felt cathode to maintain electrochemical reducing equivalence for bacterial metabolism. *L. lactis* was immobilized in the NR-cathode by entrapment to maintain the regeneration (growth) of the bacterial cells while in contact with the NR-cathode [34]. This may be a useful technique for the maintenance of physiological activity and growth of *L. lactis* in a specific reactant for Mn(IV) reduction.

In order to leach Mn(II) from manganese ore biologically, the metabolic reduction of Mn(IV) to Mn(II) by non-pathogenic or non-hazardous bacteria is required. *L. lactis* is a typical lactic-acid-producing bacterium without associated pathogenicity or hazards [31]; however, metabolic activity

for the reduction of Mn(IV) to Mn(II) is not sufficient for Mn(II) leaching. The metabolic function of *L. lactis* sk071115 catalyzing the reduction of Mn(IV) to Mn(II) is similar to that of metal-reducing bacteria, but physiologically different from those of *Bacillus* sp. [5, 6], such as *Acinetobacter calcoaceticus* [13], *Shewanella putrefaciens* [22], and *Thiobacillus ferrooxidans* [31]. The majority of metal-reducing bacteria catabolize hydrogen, hydrogen sulfide, lactate, pyruvate, formate, or acetate in combination with the reduction of Mn(IV) or Fe(III) under anoxic or anaerobic conditions [1, 20, 21, 29]; meanwhile, it is very difficult to catabolize glucose *via* metal-reducing bacterial species under anoxic or anaerobic conditions [19]. This may be a limiting factor of the metal-reducing bacteria for the microbial leaching of Mn(II) from manganese ore. Glucose, which can be cheaply obtained from starch *via* microbial or enzymatic hydrolysis, is a more plentiful and stable organic compound than hydrogen, hydrogen sulfide, lactate, or formate. Glucose, which can be fermented by *L. lactis*, may be metabolically converted to lactic acid in a bioreactor for Mn(IV) leaching, by which the reactant pH is reduced to below 7. Generally, metal ions are water-soluble and more stable at lower than neutral pH [30].

The microbial reduction of the Mn(IV) to Mn(II) in terrestrial or marine sediment containing manganese dioxides results in the release of dissolved manganese, but existing microbial leaching methods are too slow to be competitive with chemical processes [27]. In *L. lactis* metabolism, the majority of reducing power (NADH) regenerated from NAD^+ in combination with the catabolic oxidation of glucose is reoxidized for the reduction of pyruvate to lactate, but a very small portion of this is oxidized for the reduction of Mn(IV) to Mn(II). *L. lactis* is capable of metabolically reducing Mn(IV) to modulate $NADH/NAD^+$ (redox) balance (Table 1). Accordingly, the production of Mn(II) from Mn(IV) or manganese ore must be increased in proportion to the $NADH/NAD^+$ balance [34]. *L. lactis* may generate free energy by both substrate-level phosphorylation (SLP) and electron transport phosphorylation in the culture with nitrate, but generates only SLP in the culture containing Mn(IV). It is quite possible that Mn(IV) may function as an electron sink but not an electron acceptor in the energy metabolism of *L. lactis* [18, 23]. No free energy is generated by the metabolic reduction of an electron sink in combination with NADH oxidation; however, the reduced electron sinker, Mn(II), may accumulate on the surfaces of the bacterial cells.

Mn(II) may be leached from manganese ore *via* catalysis of the L-NR-cathode under electrochemical reduction conditions without glucose; however, the cell mass immobilized in the NR-cathode may be diminished with advancing reaction time in the absence of glucose. The electrons may be transferred from the electrochemically regenerated NADH to Mn(IV) *via* the catalysis of *L. lactis*

sk071115 without the generation of any free energy, owing to the potential electron sink functions of Mn(IV). Accordingly, glucose is an absolute requirement as an energy source for the generation of free energy and as a carbon source for the regeneration and maintenance of cell mass. Theoretically, 200 mM of ATP is generated from 100 mM of glucose by SLP in the metabolism of lactic acid-producing bacteria, by which 4.3 g of cell mass per 100 mM of glucose (1.43 g of cell mass per 33 mM glucose) may be generated according to the growth yield and efficiency of fermentative microorganisms [14, 26]. The initial cell mass immobilized in the NR-graphite felt electrode was 2.5 g/l, and glucose was controlled at a supply rate of 33 mM per day based on the glucose consumption (100 mM for 72 h) of *L. lactis* (Table 1). We were unable to determine with precision how long the cell mass of *L. lactis* was maintained in the GDM, but could estimate that the physiological activity of *L. lactis* for the reduction of Mn(IV) may be maintained for more than 30 days based on the experimental record showing that the production of Mn(II) from manganese ore was maintained *via* the catalysis of the L-NR-cathode (Fig. 6).

In conclusion, Mn(II) was bioelectrochemically produced from manganese ore *via* the catalysis of *L. lactis,* which was immobilized in the modified graphite felt electrode with NR. The immobilized NR in the graphite felt electrode by covalent bonding may function as both a solid electron mediator for electron transfer from the electrode to bacterial cells, and a catalyst for the electrochemical reduction of NAD^+ to NADH. The metabolic stability of *L. lactis* for Mn(IV) reduction cannot be stronger than the chemical stability of the NR-cathode; however, the biocatalyst can be continuously regenerated within the NR-cathode. Glucose is an organic, biologically produced compound, and the electricity employed for the bioelectrochemical reduction of Mn(IV) can be generated using solar cells. Accordingly, the bioelectrochemical Mn(II) leaching induced by *L. lactis* may prove to be a required green technology in the low-carbon age.

Acknowledgement

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