Extraction of electrons by magnetite and ferrihydrite from hydrogen-producing *Clostridium bifermentans* by strengthening the acetate production pathway

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Conductive mineral nanoparticles, such as magnetite, can promote interspecies electron transfer between syntrophic partners. However, the effect of magnetite has only been inferred in intraspecific electron output. Herein, a hydrogen-producing strain, namely, *Clostridium bifermentans*, which holds several electron output pathways, was used to study the effect of magnetite on the intraspecific electron output manner. Additionally, insulated amorphous ferrihydrite, which was used as an extracellular electron acceptor, was selected to compare with magnetite. Electrons, which were originally used to generate hydrogen, were shunted with the addition of magnetite and ferrihydrite, which resulted in the reduction of hydrogen production and accumulation of Fe(II). Interestingly, more electrons (39.7% and 53.5%) were extracted by magnetite and ferrihydrite, respectively, which led to less production of butyrate and more acetate. More importantly, the increased electron extraction efficiency suggested that electroactive microorganisms can switch metabolic pathways to adapt to electron budget pressure in intraspecific systems. This work broadens the understanding of the interaction between iron oxides and fermentative hydrogen-producing microbes that hold the capacity of Fe(III) reduction.

magnetite, ferrihydrite, Fe(III) reduction, electron extraction, increased acetate production, *Clostridium bifermentans*

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1 Introduction

Hydrogen, as a vital reducing power, plays an important role in the biogeochemical processes [1,2]. Hydrogen-producing microorganisms, such as *Clostridium*, can release intracellular excess reducing equivalents by producing molecular hydrogen, and hydrogen can act as an important energy resource for other microbes [2–4]. It should be noted that the production and utilization of hydrogen often simultaneously occurs between syntrophic partners in anaerobic environments.

Iron-bearing minerals, which are widespread in natural environments, make significant contributions to the biogeochemical cycle [5–7]. Iron is an important factor for the growth of hydrogen-producing bacteria. Moreover, electrons can be extracted from Fe(II)-bearing minerals by iron-oxidizing bacteria to fix CO₂ for the production of organic matter [8,9]. In contrast, anaerobic dissimilatory iron-reducing bacteria (DIRB) are found to donate electrons to exogenous Fe(III)-bearing minerals accompanied by their

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respiration processes [3,10]. DIRB can be divided into respiratory iron reducing and fermentative iron reducing bacteria. For fermentative iron reducing bacteria, the reducing equivalents that are transferred to ferric iron are typically less than 5% and no energy is offered for microbial growth [1].

Iron-bearing minerals, such as magnetite, have been extensively used to enhance hydrogen production because iron is the key metal active center of the hydrogenase enzyme [11–14]. Furthermore, the high surface area and unique physiochemical properties of nanoparticles may support bacterial growth [15]. Many fermentative hydrogen-producing bacteria have the ability to reduce extracellular iron oxide minerals [16–18]. In addition, Fe(III) reduction is an electron-consuming process. Therefore, it is logical that the existence of iron-bearing minerals can decrease hydrogen production for fermentative hydrogen-producing microbes with the capacity of dissimilarly Fe(III) reduction. However, limited research on this has been reported.

In our previous work, a fermentative dissipatory iron-reducing bacterium, C. bifermentans, was isolated from the Yellow River Delta and identified as a type of electroactive microbe [19,20]. Namely, C. bifermentans presents several electron output manners such as hydrogen evolution, Fe(III) reduction and electricity production. Herein, to study the influence of Fe(III) reduction on hydrogen production, crystalline magnetite (conductive) and amorphous ferrihydrite (insulative) were selected with crystalline and conductivity as standards. Furthermore, the total extracellular electron output efficiency, metabolic product distribution and the electrochemical activity of C. bifermentans were evaluated.

2 Materials and methods

2.1 Microorganism and culture medium

C. bifermentans EZ-1 was isolated from the Yellow River Delta in our previous study and stored at −80°C. The recipes of the modified PYG medium (1 L) were briefly depicted as follows: peptone, 0.5 g; yeast extract, 0.5 g; NaCl, 5 g; glucose, 5 g [21]. The initial pH was adjusted to 7.0±0.1. The prepared medium was dispensed into 25-mL serum vials with 10 mL of working capacity. Serum vials were flushed with N2 for 5 min and sealed with butyl rubber plugs and aluminum caps, and then sterilized by autoclaving at 121°C for 20 min.

2.2 Preparation of magnetite and ferrihydride

Ferrihydrite was prepared as described elsewhere [22]. In brief, ferrihydrite was formed by neutralizing a 0.4 mol/L solution of FeCl3 to a pH of 7 with NaOH. Magnetite was synthesized by slowly adding Fe(II)/Fe(III) acidic solution (0.8 mol/L FeCl3 and 0.4 mol/L FeCl2 in 0.4 mol/L HCl) into a vigorously mixed NaOH solution (1.5 mol/L), purified by centrifugation and suspended in deoxygenated water [23]. The synthesized magnetite and ferrihydrite were identified by X-ray diffraction (XRD-6100, JPN) and a transmission electron microscope (TEM -1400, JPN). The diameter of the nanoparticle magnetite was about 50 nm, and the length and width of the nanorod ferrihydrite were 20–30 and 2–3 nm, respectively.

2.3 Experimental procedures

Batch experiments were conducted in 25-mL serum vials with a working volume of 10 mL. The final concentrations of the magnetite and ferrihydrite were at 50, 100 and 200 mmol/L. The control treatment was mineral-free. C. bifermentans with an inoculum of approximately 1% (v/v) was used in this study. To test the effect of magnetite and ferrihydrite on hydrogen absorption, three treatments (magnetite or ferrihydrite or mineral-free) were conducted with an initial hydrogen content of approximately 200 μmol. All batch experiments were cultured at 30°C without light and shaking.

2.4 Chemical analysis

The Fe(II) concentration in the batch groups was analyzed at the initial and 30th hour of cultivation by the ferrozine method as previously reported [24,25]. Headspace gas (200 μL) and suspension (100 μL) were sampled by degassed syringes at a 2 h interval. The gas was analyzed by gas chromatography (GC, Agilent 7280, USA) equipped with a thermal conductivity detector (TCD). The temperature of the column, the injector and the thermal conductivity were 80°C, 250°C and 250°C, respectively. Glucose, acetate and butyrate were tested using high-performance liquid chromatography (HPLC, Agilent 1260, USA) equipped with a refractive index detector (RID). A Hi-plex H column (7.7 × 300 mm) was used to separate glucose and volatile organic acid (VFA) with 5 mmol/L H2SO4 as the eluent. The temperature of the column and RID were 60°C and 55°C, respectively. To verify the effect of magnetite and ferrihydrite on the growth of C. bifermentans, the biomass was detected at the 20th hour using the bicinchoninic acid (BCA) protein quantification kit (Solarbio, CN) [26]. pH is an important parameter for the metabolism of fermentative microorganisms and the effect of ferrihydrite and magnetite addition on the pH was examined.

2.5 Electrochemical characterization

To monitor the effect of magnetite and ferrihydrite on the electrochemical activity of C. bifermentans, cyclic voltam-
metry (CV) was conducted using an electrochemical station (model-660E, Chenhua, CN) equipped with a three-electrode system containing a graphite sheet (30 mm×25 mm×3 mm) as the working electrode, whereas a platinum sheet and Ag/AgCl were used as the counter electrode and reference electrode, respectively [27]. The CV was conducted between –1.0 and 1.0 V with a 10 mV/s scan rate. Three batch groups were conducted as follows: (1) 80 mL PYG medium plus 100 mmol/L magnetite/ferrihydrite; (2) 80 mL PYG medium plus 5% C. bifermentans; and (3) 80 mL PYG medium plus 100 mmol/L magnetite or ferrihydrite and 5% C. bifermentans.

2.6 Statistical analysis

All of the statistical analyses were performed with SPSS 19.0 (SPSS Inc., Chicago, USA) and Origin 8.0 (Origin Lab Corporation, USA) software. A one-way analysis of variance with LSD’s test was used to analyze the significance of the effect of magnetite and ferrihydrite on the metabolites of C. bifermentans.

3 Results and discussion

3.1 The adverse effect of magnetite and ferrihydrite on hydrogen production

The effect of magnetite and ferrihydrite on hydrogen production was explored. As the experimental results showed, hydrogen started to accumulate after a short lag phase (approximately 2 h) in all batch experiments (Figure 1). However, all batch groups with magnetite and ferrihydrite presented a lower hydrogen production rate and production compared with the blank control. With the addition of 50, 100 and 200 mmol/L ferrihydrite, respectively (Figure 1(b)). These results indicate that both crystalline magnetite and amorphous ferrihydrite can exert an obvious negative effect on hydrogen production.

These results are contrary to many studies where iron oxide minerals were supplied to enhance the hydrogen-producing activity of Clostridium or a mixed consortia dominated by Clostridium spp. [28,29]. The basic principle is that iron is a key cofactor of hydrogenase and iron oxide can increase the hydrogenase activity [30]. However, it should be noted that hydrogen production and Fe(III) reduction were both electron output pathways, and many hydrogen-producing bacteria can reduce Fe(III) in iron oxide minerals. Park et al. [18] reported that Clostridium butyricum, a representative hydrogen-producing bacterium, was capable of reducing ferrihydrite. Herein, the Fe(III) reduction capacity of C. bifermentans may be the underlying reason for the adverse effect of magnetite and ferrihydrite on hydrogen production. Magnetite and ferrihydrite may act as an electron sink and redirect the electron flow to Fe(III) reduction, which results in a decrement of electrons for hydrogen production. In agreement with our results, a related study showed decreased hydrogen production accompanied with Fe(II) accumulation due to the reduction of hematite by Orenia metallireducens [31].

To exclude the absorption of magnetite and ferrihydrite on hydrogen, batch experiments (only magnetite or ferrihydrite and mineral-free treatments) were conducted. Hydrogen slightly decreased with the increment of sampling numbers over time. However, no distinct changes were observed among the batch groups that were supplemented or not supplemented with magnetite and ferrihydrite (Figure 2(a)). These results indicate that magnetite and ferrihydrite did not adsorb hydrogen. Moreover, the effect of magnetite and ferrihydride on bacterial growth was conducted. As the data of biomass show, the influence of magnetite and ferrihydrite on cell growth could be ignored because of no significant difference between the control and the experimental groups amended with magnetite and ferrihydrite (Figure 2(b)).

![Figure 1](Color online) The effect of magnetite (a) and ferrihydride (b) on hydrogen production.
These results disagree with a previous report that metal oxide nanoparticles could disrupt the cell member and kill bacteria [26]. These different results could be attributed to the various object strain and properties of nanoparticles. pH is a primary factor for fermentative metabolic distribution; therefore, the effect of ferrihydrite and magnetite addition on the pH of the fermentative systems was monitored. As Figure 2(c) illustrates, the pH in the three batch experiments all decreased with the accumulation of fermentative products (acetate and butyrate). However, in comparison with the blank control, no distinct difference was observed in the presence or absence of magnetite/ferrihydrite.

### 3.2 The stimulation of electron output efficiency by magnetite and ferrihydrite

To uncover the reason for decreased hydrogen production, we speculated and verified that electrons originally used for hydrogen production were shunted by Fe(III) reduction. The Fe(II) concentration significantly increased in the batch groups supplied with magnetite and ferrihydrite after 30 h of cultivation (Figure 3), which suggests that Fe(III) in magnetite and ferrihydrite was reduced to Fe(II). These results are consistent with the hypothesis that the decrease of hydrogen production was due to the reduction of magnetite and ferrihydrite. Additionally, these results confirm that electrons derived from glucose oxidation could be simultaneously shunted by hydrogen production and Fe(III) reduction.

Interestingly, our results show that the amount of electrons used to reduce Fe(III) were more than that of decreased hydrogen production. For example, with the addition of 200 mmol/L magnetite, approximately 524 μmol Fe(II) was accumulated, i.e., 524 μmol electrons were used to reduce Fe(III) (Figure 3(a)). In parallel, decreased hydrogen production was 153 μmol (Figure 3(b)). An extra harvest of approximately 218 μmol (524–2×153) of electrons occurred (Figure 3 and Table 1). This phenomenon also appeared in the batch groups containing ferrihydrite. Furthermore, the total extracellular electron output efficiency was calculated (Table 1). The maximum total extracellular electron output efficiency in the presence of 100 mmol/L ferrihydrite was elevated by 53.5% compared to the blank control. These results indicate that magnetite and ferrihydrite can extract more electrons from *C. bifermentans*.

### 3.3 The enhancement of the acetate production pathway

To clarify the source of the extra electrons, the metabolic product distribution of *C. bifermentans* was analyzed. Figure 4 shows that substrate glucose (278 μmol) was completely consumed after 30 h of cultivation, except for the
batch groups with a high concentration of ferrihydrite (200 mmol/L). The hydrogen yield depends on the metabolic pathway [32].

\[
\begin{align*}
C_6H_{12}O_6 + 2H_2O & \rightarrow 2C_2H_4O_2 + 2CO_2 + 4H_2 & \text{(1)} \\
C_6H_{12}O_6 & \rightarrow C_4H_6O_2 + 2CO_2 + 2H_2 & \text{(2)}
\end{align*}
\]

In this study, acetate production was significantly increased with the addition of magnetite compared to the blank control \((p<0.01)\) and it was positively correlated with the magnetite concentration (Figure 4(a)). Butyrate production slightly declined compared to the blank control. These results indicate that magnetite directed the metabolic pathways of \(C. bifermentans\), i.e., the high concentration of magnetite redirected the carbon and electron fluxes to the acetate production pathway. Similarly, ferrihydrite enhanced acetate production and lowered butyrate production (Figure 4(b)).

Given that the acetate production pathway can generate more electrons than that of the butyrate production pathway (eqs. (1) and (2)), extra electrons could be extracted by iron oxide minerals and by redirecting the glucose fermentation pathways.

The acetate production pathway was accompanied by more electrons (more hydrogen) production than that of the butyrate production pathway, and a higher electron output efficiency was obtained by promoting the acetate production pathway with the addition of both magnetite and ferrihydrite. This indicates that minerals could squeeze more electrons from \(C. bifermentans\). These results are consistent with several previous studies that iron oxide could redirect the carbon flux to less-reduced fermentation products [16,33].

### 3.4 Electrochemical activity influenced by magnetite and ferrihydrite

\(C. bifermentans\) was defined as an electrochemical active \(G^+\) bacterium, and a maximum current density of 6.5 mA/m² was detected in a double chamber MFC in our previous study [19]. To explore the effect of magnetite and ferrihydrite on the electrochemical activity of \(C. bifermentans\), CV tests were conducted. The redox reaction was not detected when only magnetite or ferrihydrite was added into the PYG medium (Figure 5). An oxidation peak and reduction peak appeared at 0.6 and –0.4 V, respectively, which indicates that \(C. bifermentans\) could release a redox mediator and this was consistent with our previous results. Oxidation peaks shifted right in the presence of magnetite and ferrihydrite while the reduction peaks had no change, which indicated that the oxidizing reaction readily occurred under a low potential in the presence with magnetite and ferrihydrite [34]. Other redox peaks occurred in the presence of magnetite, which indicated that magnetite addition may induce the release of other kinds of redox mediators and thus resulted in a higher total extracellular electron output efficiency. Furthermore, magnetite addition significantly increased the reduction current, which indicated that a higher electrochemical activity for magnetite reduction was revealed (Figure 5(a)).

### Table 1 The total extracellular electron output efficiency of \(C. bifermentans\) in the presence of magnetite and ferrihydrite

|                        | Control  | 50 (mmol/L) | 100 (mmol/L) | 200 (mmol/L) | 50 (mmol/L) | 100 (mmol/L) | 200 (mmol/L) |
|------------------------|----------|-------------|--------------|--------------|-------------|--------------|--------------|
| \(H_2\) (μmol)         | 297.4    | 251.0       | 224.7        | 153.2        | 250.3       | 235.3        | 149.1        |
| Fe(II) (μmol)          | 0.0      | 188.7       | 337.6        | 524.7        | 391.4       | 442.3        | 456.8        |
| AEE (μmol)             | 594.8    | 690.7       | 787.0        | 831.1        | 892.0       | 912.9        | 755.0        |
| EOE (%)                | 100.0    | 116.1       | 132.3        | 139.7        | 150.0       | 153.5        | 126.9        |

Note: AEE=2H₂+Fe(II), the amount of exporting electrons; EOE=(AEEₓ-AEEₓ₋₁)/AEEₓ₋₁+1, the total extracellular electron output efficiency.
However, the addition of ferrihydrite had no obvious influence on the reduction current and only one redox mediator was released by *C. bifermentans* (Figure 5(b)). These results reveal that the influence of magnetite on *C. bifermentans* electrochemical activity was different from that of ferrihydrite. Most intuitively, magnetite is conductive while ferrihydrite is insulative [6]. Therefore, the difference in electron extraction between magnetite and ferrihydrite may partly contribute to electrochemical properties.

4 Conclusions

In this study, the experimental results showed that magnetite and ferrihydrite could shunt electrons in a mixed system with bacteria and minerals, which results in lowering hydrogen production. With stimulation of the acetate production pathway in the presence of magnetite and ferrihydrite, a higher total extracellular electron output efficiency emerged. This work broadens the understanding of the interaction between iron oxide and Fe(III) reduction microorganisms during the hydrogen evolution process.

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