ENHANCED CELL DENSITY CULTURE OF *THIOBACILLUS FERROOXIDANS* IN MEMBRANE-TYPE BIOREACTOR WITH ELECTROLYTIC REDUCTION UNIT FOR FERRIC ION

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Enhanced cell density culture of *Thiobacillus ferrooxidans* was achieved by using a membrane-type bioreactor with cell filtration and iron recycling units. Using an electrolytic bath with an anion exchange membrane, microbially produced Fe^{3+} could be reduced to Fe^{2+} without loss of main nutrients in the medium. It was confirmed that *T. ferrooxidans* exhibited active growth in the electrochemically regenerated medium. In the repeated-batch culture with cell filtration and feeding of the regenerated medium, the cell concentration obtained was at least 4-11 times higher than that in a conventional batch culture. In this culture system, the measurement of medium redox potential made it possible to estimate the concentrations of cells and iron, and to determine the time for culture filtration and medium-feeding operations.

Introduction

Microbial processes for coal desulfurization and ore leaching have become of interest in connection with the effective utilization of fossil and mineral resources.^{3,6,12)} *Thiobacillus ferrooxidans* is a primary microorganism applied to these processes because of its ability to oxidize iron and sulfur compounds. As *T. ferrooxidans* is a chemoautotrophic aerobe and thus its cell yield is considerably low on the basis of energy source utilized, it is important to achieve high cell density of *T. ferrooxidans* for the enhancement of bioleaching rates.⁵⁾

Recently, the membrane-type bioreactor has been proposed to achieve high-cell density cultures of various microorganisms by cell holding in a fermentor with a microfiltration unit.^{9,11,14)} However, a major disadvantage of this bioreactor system is that it requires an extremely large amount of medium (water and main nutrients) in proportion to the cell mass obtained.

In the present study we examine a membrane-type bioreactor system to obtain a concentrated cell suspension of *T. ferrooxidans* for use in bioleaching processes. Moreover, we incorporate a recycling unit for ferrous ion (the energy source for the bacterium) into the bioreactor system to minimize the medium

volume used. In this unit, ferric ion produced by *T. ferrooxidans* is electrochemically reduced to ferrous ion on a cathode in an electrolytic bath. The ferrous ion is then reused to cultivate the iron-oxidizing bacterium.

This paper describes the performance of the enhanced cell density culture of *T. ferrooxidans* and the operational conditions for a membrane-type bioreactor system with cell filtration and iron recycling units.

1. Experimental

1.1 Microorganism

Throughout these experiments, *Thiobacillus ferrooxidans* Y5-9 (obtained from Dowa Mining Industry Co.) was used. This strain was maintained using 9K medium¹⁰⁾ and was stored at 4°C.

1.2 Medium and culture conditions

The basal composition of medium (in $\text{kg} \cdot \text{m}^{-3}$) was as follows; $(\text{NH}_4)_2\text{SO}_4$: 3.0; K_2HPO_4 : 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 0.5; KCl : 0.1; $\text{Ca}(\text{NO}_3)_2$: 0.01; and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: 40. As the bacterium can grow chemoautotrophically by the utilization of CO_2 in air, no carbon source was added to the medium. The medium was autoclaved at 121°C for 0.3 h and total iron concentration was 125-143 $\text{mol} \cdot \text{m}^{-3}$ after autoclaving owing to the precipitation of iron hydroxides. The pH of the medium was adjusted to 2.0 with H_2SO_4 to minimize iron loss by this precipitation. In the

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experiments for medium reuse, the following minor components were added to the medium after electrolytic reduction of Fe^{3+} (in $\text{g} \cdot \text{m}^{-3}$): K_2HPO_4 : 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$: 10; KCl : 2.0; and $\text{Ca}(\text{NO}_3)_2$: 0.2.

All cultivations were carried out at 27°C using an L-tube (10 cm^3 of medium), or a jar fermentor (see below). Inoculum size was 10 vol%.

1.3 Membrane-type bioreactor system

The experimental apparatus is shown in Fig. 1. *T. ferrooxidans* was cultivated in a jar fermentor (Model TBR-2, Sakura Seiki Co., 1 dm^3 of working volume). The culture was agitated with an impeller at 500 rpm and sparged with air at a flow rate of $90\text{ dm}^3 \cdot \text{h}^{-1}$. The filtration of culture broth was done with a hollow-fiber module (Plasmacure M, Kuraray Co., $0.2\text{ }\mu\text{m}$ nominal pore size, 0.3 m^2 available surface area) and a diaphragm pump (Type MD 281-32, Metronix Co.). The module was used after sterilization with $50\text{ mol} \cdot \text{m}^{-3}$ H_2SO_4 and washing with sterile water. The filtrate volume was 0.8 dm^3 and the same volume of electrolytically regenerated medium (or fresh medium) was fed to keep the working volume constant.

The filtration was carried out by the following operations (see Fig. 1). 1) Valves 3-1, 3-2 and 3-3 were opened, and valve 3-4 was closed. When valve 3-2 was turned to half-closed position, cross-flow filtration started and filtrate was collected for subjection to Fe^{3+} reduction. 2) Valves 3-1 and 3-3 were closed and valves 3-2 and 3-4 were fully opened. Electrolytically regenerated medium (or fresh medium), which had been stored in a reservoir after Fe^{3+} reduction of filtrate obtained by preceding filtration, was introduced into the fermentor through the hollow-fiber module in back-flashing mode. 3) Valve 3-4 was closed and valve 3-1 was opened. To peel off cell cake on the inner surface of fibers, the medium was sufficiently circulated in the module. 4) All valves were closed and cultivation was resumed.

The details of the electrolytic bath are shown in Fig. 2. The bath was partitioned into two chambers by an anion exchange membrane, HKA-3 (Asahi Chemical Industry Co., 64 cm^2 of available surface area) to prevent ferrous ion from being oxidized on the anode. A platinum electrode plate was set in each chamber as cathode and anode respectively (22 cm^2 of surface area, 15 cm apart). In the cathode chamber 0.8 dm^3 of culture filtrate was introduced, and one volume of $50\text{ mol} \cdot \text{m}^{-3}$ H_2SO_4 was poured into the anode chamber. Unless otherwise noted, this electrolytic bath was used. The electrodes were connected to appropriate terminals on a direct current (DC) power supply (Model GPT 322, Takasago Seisakusho Co.), which was operated in constant-current mode. Both chambers were vigorously agitated with magnetic stirrers. Depending on the experimental objective, a

Fig. 1. Experimental apparatus, showing membrane-type bioreactor

Fig. 2. Schematic diagram of Fe^{3+} reduction in electrolytic system with anion exchange membrane

small-scale electrolytic bath was also used. Its configuration was as follows; cathode or anode chamber volume: 0.2 dm^3 ; available surface area of anion exchange membrane: 16 cm^2 ; electrode surface area: 15 cm^2 ; and electrodes separation: 15 cm .

1.4 Analyses

Cell concentration was determined by microscopic counting with a hemacytometer. The concentrations of Fe^{2+} and Fe^{3+} were measured colorimetrically using thiocyanate and thioglycolic acid.⁴⁾ The con-

centration of SO_4^{2-} was determined by a turbidimetric method with BaCl_2 .⁴⁾ The concentrations of NH_4^+ and PO_4^{3-} were determined using phenol¹⁵⁾ and molybdate¹³⁾ respectively. The amounts of K^+ and Mg^{2+} were measured by atomic absorption spectrometry (Model AA-880 Mark II, Japan Jarrell-Ash Co.).

Redox potential was measured at 27°C using a Model F-7 pH/millivolt meter with a combined redox electrode (No. 6810, Horiba Seisakusho Co.).

2. Results

In a typical batch culture of *T. ferrooxidans*, the ultimate cell concentration was at most 20×10^{13} cells $\cdot \text{m}^{-3}$ with the consumption of about $140 \text{ mol} \cdot \text{m}^{-3}$ of Fe^{2+} (initial cell concentration: $1.5\text{--}2.0 \times 10^{13}$ cells $\cdot \text{m}^{-3}$). Thus the autotrophic growth of *T. ferrooxidans* required the oxidation of an extremely large amount of Fe^{2+} . Therefore, it is desired to reuse bacterially produced Fe^{3+} for the effective utilization of energy source in the enhanced cell density culture of *T. ferrooxidans*.

2.1 Electrolytic reduction of Fe^{3+}

The electrochemical reduction of Fe^{3+} to Fe^{2+} was investigated for iron recycling. In this case, it is necessary to separate the anode from the $\text{Fe}^{2+}/\text{Fe}^{3+}$ mixture to avoid the re-oxidation of Fe^{2+} . In the present study, we constructed an electrolytic unit with an anion exchange membrane as partition (Figs. 1 and 2).

Figure 3 shows the reduction profiles of Fe^{3+} in the small-scale electrolytic bath, using $95 \text{ mol} \cdot \text{m}^{-3}$ $\text{Fe}_2(\text{SO}_4)_3$ (pH = 2.0). At low current values ($I = 0.25$ and 0.50 A), total iron concentration ($[\text{Fe}^{3+} + \text{Fe}^{2+}]$) hardly varied with reaction time. At high current values ($I = 0.68$ and 0.90 A), on the other hand, a decrease in total iron concentration was observed after 1–2 h owing to the substantial precipitation of metallic iron. This phenomenon resulted from over-reduction of Fe^{2+} to Fe (elemental iron) on the cathode, which was accompanied by Fe^{2+} accumulation in the reaction mixture with Fe^{3+} reduction. About $190 \text{ mol} \cdot \text{m}^{-3}$ of Fe^{3+} was almost reduced for 3–5 h at the current values tested. The reduction rate of Fe^{3+} fell with reaction progress except for the experiment at $I = 0.25$ A. Figure 4 shows the relationship between the initial reduction rate of Fe^{3+} and the current value applied in the small-scale electrolytic bath. Up to about 0.6 A the reduction rate increased linearly with increasing current value and agreed closely with the result calculated by Eq. (1) (the solid line in Fig. 4);

$$-r = I/n \cdot F \quad (1)$$

where $F = 9.65 \times 10^4 \text{ C} \cdot \text{mol}^{-1}$ and $n = 1$. In this current range, all electrons thus seemed to act for the

Fig. 3. Profiles of electrolytic Fe^{3+} reduction at various current values

Fig. 4. Relationship between reduction rate of Fe^{3+} and current value applied

reduction of Fe^{3+} to Fe^{2+} . It was beyond a current of about 0.6 A that the rate reached a plateau and metallic iron began to precipitate. The voltage values between electrodes were 16–19 V at the critical current values of $0.6\text{--}0.68$ A against over-reduction of Fe^{2+} . Therefore, current and voltage less than these values were employed in subsequent experiments to prevent the loss of iron in culture broth.

2.2 Reuse of medium with electrolytically regenerated Fe^{2+}

During the electrolytic reduction of Fe^{3+} , the ionic (especially anionic) nutrients may permeate through the membrane into the anode chamber, which leads to the loss of these nutrients from the medium. As shown in Table 1, the concentrations of main nutrients in the cathode chamber were compared before and

Table 1. Change of main medium components after electrolytic reduction of Fe^{3+}

Component	Concentration [$\text{mol} \cdot \text{m}^{-3}$]	
	Before reduction	After reduction
NH_4^+	39	39
K^+	5.9	6.1
Mg^{2+}	1.2	1.3
Fe^{2+}	ND*	125
Fe^{3+}	136	16
SO_4^{2-}	240	200
PO_4^{3-}	2.9	2.8

* ND: Not detectable.

after the electrolysis of culture filtrate. The electrolysis was carried out for 10 h at $I=0.5$ A and $v=8$ –10 V, and it was found that most components were retained in the cathode chamber. The dominant permeate through the membrane was SO_4^{2-} , and its concentration dropped to 83% after almost complete reduction of Fe^{3+} .

Figure 5 shows the repeated use of medium. In each batch culture of *T. ferrooxidans* except for the first run, the filtrate obtained from the preceding culture was used as medium after the electrolytic reduction of Fe^{3+} for 6 h at $I=0.5$ A and $v=12$ –14 V using the small-scale electrolytic bath. The cultures were carried out using L-tubes containing regenerated medium sterilized by filtration. No significant differences were observed with respect to cell growth or Fe^{2+} oxidation during the repeated cultures. Therefore, it was found that electrochemically regenerated medium could be repeatedly used without substantial deterioration.

2.3 Monitoring of culture by redox potential

For consecutive culture with the membrane-type bioreactor, it is important that the concentrations of cells and substrate be estimated to know the operation time for cell filtration and Fe^{3+} reduction. The redox potential of culture broth was considered as an indicator for this purpose. On the basis of the Nernst equation, the redox potential was correlated with the logarithmic ratio of $[\text{Fe}^{2+}]$ to $[\text{Fe}^{3+}]$ as follows.

$$Eh = -\alpha \cdot \log([\text{Fe}^{2+}]/[\text{Fe}^{3+}]) + \beta \quad (2)$$

In the case of *T. ferrooxidans* culture, the medium pH was very low ($\text{pH}=2.0$) and various ions were included in the medium. Therefore, it was preferable to evaluate the values of α and β in Eq. (2) by experimental analysis. As shown in **Fig. 6A**, the redox potential was measured for solutions containing various proportions of Fe^{2+} and Fe^{3+} . The data were obtained using culture broth of *T. ferrooxidans*, or FeSO_4 and $\text{Fe}_2(\text{SO}_4)_3$ mixture (total iron concentration = $190 \text{ mol} \cdot \text{m}^{-3}$, $\text{pH}=2.0$). From this result, the α value was 59.4 mV in agreement with the

Fig. 5. Repeated use of electrolytically regenerated medium for *T. ferrooxidans* culture

Fig. 6. Relationships between redox potential and $\log([\text{Fe}^{2+}]/[\text{Fe}^{3+}])$ (A), and cells grown and Fe^{2+} utilized (B)

coefficient ($=2.30RT/nF$) in the Nernst equation, and the β value was determined as 425 mV. Here, the loss of iron was assumed to be negligible in the culture system.

$$[\text{Fe}^{2+}] + [\text{Fe}^{3+}] = \text{constant (total iron concentration)} \quad (3)$$

For the calculation of cell concentration, we estimated cell yield on the basis of Fe^{2+} utilized.

$$Y_{\text{Fe}} = -\Delta x / \Delta[\text{Fe}^{2+}] \quad (4)$$

Assuming that the cell yield is constant during the culture, this value was experimentally determined as $Y_{\text{Fe}} = 1.4 \times 10^{12} \text{ cells} \cdot \text{mol}^{-1}$ (Fig. 6B).

Thus it was possible to acquire information on Fe^{2+} and cell concentrations and to determine the time for the filtration and reduction of culture broth by the measurement of redox potential.

2.4 Enhanced cell density culture of *T. ferrooxidans*

T. ferrooxidans was cultivated in the bioreactor associated with cell filtration and Fe^{3+} reduction, as shown in Fig. 7. In this case, we intended to perform the filtration at a culture time when the redox potential value reached about 500 mV and the ratio of Fe^{2+} to total iron in culture broth was less than about 10% (see the arrows in Fig. 7). The redox potential values at filtration times are shown in Fig. 7 together with the arrows. Subsequently to the filtration operations, culture filtrate containing electrochemically regenerated Fe^{2+} was fed to the fermentor (fresh medium feeding only at the first operation time). In this experiment, the culture filtrate was subjected to electrolysis at $I=0.5 \text{ A}$ and $v=8\text{--}10 \text{ V}$ until the operation time for medium feeding. During the 7 times-repeated batch cultures, cell concentration reached $84 \times 10^{13} \text{ cells} \cdot \text{m}^{-3}$. The solid lines in Fig. 7 show the calculated results for cell and Fe^{2+} concentrations, based on Eqs. (2)–(4) using initial cell concentration $= 2.2 \times 10^{13} \text{ cells} \cdot \text{m}^{-3}$ and total iron concentration $= 134 \text{ mol} \cdot \text{m}^{-3}$. The calculated values agreed well with the experimental ones. Therefore, it was found that the redox potential was a satisfactory indicator for monitoring the *T. ferrooxidans* culture.

Table 2 summarizes the various culture results for *T. ferrooxidans*. In the conventional batch culture (Run 1), the final cell concentration was $20 \times 10^{13} \text{ cells} \cdot \text{m}^{-3}$. In the cultures with filtration and Fe^{3+} reduction (Runs 3 and 4), on the other hand, the cell concentration could be increased by 4–11 times, depending on times of cell filtration and medium feeding. In these cultures, moreover, higher ratios of cells grown to total amount of iron in the culture systems were obtained.

3. Discussion

Recent studies^{5,8)} of microbial desulfurization of coal reported that the desulfurization rate depended on the adsorption of *T. ferrooxidans* to pyritic moiety of coal and that larger inoculum size increased the removal rate of coal pyrite sulfur. Thus the cell concentration of iron-oxidizing bacterium is an important factor in effective bioleaching performance. Some workers^{1,2)} reported that a ratio of at least

Fig. 7. Cultivation of *T. ferrooxidans* in membrane-type bioreactor

Table 2. Summary of various cultures of *T. ferrooxidans*

Culture mode	Medium used [dm ³]	Final cell concentration [$\times 10^{13} \text{ cells} \cdot \text{m}^{-3}$]	Ratio of cells grown to total iron in medium [$\times 10^{12} \text{ cells} \cdot \text{mol}^{-1}$]
Batch (Run 1)	1.0	20	1.4 (1.5*)
Filtration without Fe^{3+} reduction, 6 times repeated (Run 2)	4.5	86	1.2 (1.3*)
Filtration with Fe^{3+} reduction, 7 times repeated (Run 3)	1.8	84	3.5
Filtration with Fe^{3+} reduction, 25 times repeated (Run 4)	1.8	230	9.1

* Cell yield value calculated from (cells grown)/(Fe^{2+} utilized). Culture time [h]; Run 1: 42; Run 2: 122; Run 3: 100; and Run 4: 285.

$10^{14}\text{--}10^{15} \text{ cells} \cdot (\text{kg-pyrite})^{-1}$ was required, otherwise cell concentration was limiting in bioleaching process. For example, when bacterial treatment of pulverized coal with 1–2 wt% pyrite is carried out at a concentration of 50 wt% coal slurry, the cell suspension on the order of $10^{15}\text{--}10^{16} \text{ cells} \cdot \text{m}^{-3}$ must be prepared.

In the present study, we proposed a membrane-type bioreactor system to achieve an enhanced cell density culture of *T. ferrooxidans* to be employed for coal desulfurization or ore refining. A novel bioreactor design in which a microfiltration unit was incorporated has been successfully applied to various culture processes.^{9,11,14)} In many cases, however, a huge amount of medium was consumed to retain microorganisms at high cell density. For the recycling of energy (iron) source for *T. ferrooxidans* culture, in the present study the spent medium was regenerated by

the reduction of microbially produced Fe^{3+} in an electrolytic bath with an anion exchange membrane. It was found that the regenerated medium retained the main nutritional components, including Fe^{2+} , and sustained sufficient growth of *T. ferrooxidans*.

Kinsel and Umbreit,⁷⁾ and Yunker and Radovich¹⁶⁾ investigated cultures of iron-oxidizing bacteria accompanying the electrolytic reduction of Fe^{3+} . In their system, electrodes were directly inserted into fermentors and direct current was applied to culture broth containing bacterial cells. Yunker and Radovich¹⁶⁾ used a strain of *T. ferrooxidans* adapted to the current-applied condition because of the inhibitory effect of current on bacterial growth. We examined the repeated-batch culture of *T. ferrooxidans* with the reuse of regenerated medium using a system with cell microfiltration unit and electrolytic bath separated from the fermentor. In this culture system the bacterial cells were protected from exposure to applied current, and the cell concentration of *T. ferrooxidans* could be increased without loss of iron source, unlike the conventional batch culture system. The cell concentration is possibly enhanced by greater repetition of culture filtration and regenerated medium feeding.

Conclusion

(1) A membrane-type bioreactor was used in order to achieve the enhanced cell density culture of *T. ferrooxidans* to be employed for coal desulfurization or ore leaching.

(2) Using an electrolytic bath with an anion exchange membrane, Fe^{3+} could be reduced to Fe^{2+} without loss of total iron in operations below 0.6–0.68 A direct current and voltages of 16–19 V.

(3) The main nutrients such as iron were substantially retained in the electrolytically regenerated medium, which supported the growth of *T. ferrooxidans* successfully.

(4) In repeated-batch culture with cell filtration and recycling of regenerated medium, the enhanced cell density culture of *T. ferrooxidans* was demonstrated. The time for cell filtration and medium feeding could be determined on the basis of redox potential measurement.

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Nomenclature

Eh	= redox potential	[mV]
F	= Faraday constant	[C·mol ⁻¹]
I	= current	[A]
n	= number of electrons involved in redox reaction	[—]
r	= initial reduction rate of Fe^{3+}	[mol·s ⁻¹]
R	= gas constant	[J·K ⁻¹ ·mol ⁻¹]
T	= temperature	[K]
v	= voltage between electrodes	[V]
x	= cell concentration	[cells·m ⁻³]
Y_{Fe}	= cell yield base on Fe^{2+} utilized	[cells·mol ⁻¹]
α, β	= empirical coefficients in Eq. (2)	[mV]
[]	= concentration of species in brackets	[mol·m ⁻³]

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