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Research Article

Immobilization of *Acidithiobacillus ferrooxidans*-1333 on the Waste Ore Particles for the Continuous Oxidation of Ferrous Iron

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Background: The biooxidation of ferrous iron has a great potential for the regeneration of ferric iron, in operations such as bioleaching, bioremediation. Many natural inorganic materials were investigated for use as supports immobilizing *Acidithiobacillus ferrooxidans*. The waste chalcopyrite is another natural inorganic material of which particles are easy to prepare from the leached out ore heaps and the source is abundant.
Objectives: The aim of this work is to investigate several characteristics of the particles of waste ore that determines possibility of use as supports for immobilization of *Acidithiobacillus ferrooxidans* in the packed-bed bioreactor.
Materials and Methods: *Acidithiobacillus ferrooxidans*-1333 stored in Korean Centre for Culture Collection was used. The supports were prepared by sieving the particles of 5~30 mm in size out from the waste chalcopyrite ore heap. The cells were immobilized by the successive batch culture method and oxidation rate of the bioreactor was investigated in the continuous flow mode.
Results: The cell density of *Acidithiobacillus ferrooxidans*-1333 immobilized on the particles of waste chalcopyrite was 2.71×10^8 cells g^{-1} and the highest oxidation rate of the packed-bed bioreactor was $3.65 g.L^{-1}.h^{-1}$. Oxidation rate of the bioreactor was less influenced by the concentration of ferrous and ferric iron in the input solution as well as by the aeration rate and dilution rate than other materials mentioned in other previous works.
Conclusion: The waste chalcopyrite particle is efficient support material for immobilization of *Acidithiobacillus ferrooxidans* with comparable or superior characteristics to natural inorganic support materials reported before.
Keywords: Acidithiobacillus ferrooxidans, Biooxidation, Bioreactor, Chalcopyrite, Immobilization, Jarosite

1. Background

The iron oxidation ability of microbes such as *Acidithiobacillus ferrooxidans* has been applied to the bioleaching of copper, gold, uranium cobalt, nickel from several sulphide minerals for decades(1). In addition to the leaching of metals, bioreactors with acidophilic iron oxidizing microbes can also be adopted to the removal of excess iron, and other contaminants from hydrometallurgical process waters, to the removal of hydrogen sulphide from gaseous effluents, and desulfurization from coal and oil by regeneration of biological reagents for use in leaching processes (2-5).
The natural tendency of *A. ferrooxidans* to grow on surfaces makes it an ideal organism for cell immobilization. Generally attachment of these microbes on minerals often changes with pH (6), functional groups of mineral surface, and crystalline degree (7). Electrostatic force is considered the main factor that influences aggregation and attachment of bacterial cells on the surface of the sulfide mineral (8). While mineral surface is having a net negative charge in acidic, sulfate-rich environments and culture media, the extracellular polymeric substances(EPS) formed outside of bacterial cells in which ferric iron forms complexes with the uronic acid substituents is having net positive charge. Thus, ferric iron and EPS mediate the attachment of these bacterial cells to a sulfide surface (9, 10). The importance of EPS which mediate the bacterial adhesion to minerals was emphasised by Qian Li, Qianfen Wang, et al again (11). The environment, such as culturing conditions, affects the cell surface properties, so cells exhibit varied adhesion forces to the same substratum. YU Run-lan, et al (12) suggested that the extent of cell adhesion to chalcopyrite increased when EPS and ferric iron were added, and became weak when ferrous iron were added. This implies that the electrostatic interaction plays a main role in initial adhesion between bacteria and minerals.
However there must be other factors including biological characters. Bowen Tu, et al., (13) indicates

that P30, adhesin and Pilq proteins of *Acidithiobacillus ferrooxidans* are related to some aspects of cell attachment, such as recruiting the planktonic cells to attachment sites. When the supports was replaced with glass, expression of these genes became lower than that in pyrite. This suggests that A. ferrooxidans tend to attach more to pyrite than to glass.

Several kinds of organic material such as photo-cross-linkable resin, agar, calcium and sodium alginate, *k*-carrageenan gel, gelrite gel, polyurethane, PVA and there combinations were suggested in previous studies (14-17).

Several kinds of natural material such as activated carbon, clay tiles, ceramic beads, glass beads, nickel alloy fiber, siliceous stone were studied for immobilization of *A. ferrooixdans*(2, 18-21). It is important that sources of natural materials are abundant and they are also cheap to prepare than synthesized organic materials. However investigations for the use of waste ore particles as supports of *A.ferrooxidans* has been rarely reported.

The waste ore particles easily obtainable by sieving out from the leached out chalcopyrite ore dump have porous surface and are mechanically stable against intensive liquid and gas flow. It is the first for the waste ore particles to be used as supports immobilizing *A. ferrooxidans* in continuous iron oxidation bioreactor. As these waste ore particles were never investigated before and have good aspects as immobilizing supports, their characteristics such as immobilized cells density and effect of pH on the immobilization, effects of some key factors(ferrous and ferric concentration, aeration rate) on the continuous ferrous iron oxidation rate and bacteria producing rate in the reactor model were studied in our research.

2. Objectives

The aim of this work is to investigate several characteristics of the waste ore particles that determines using possibility of them as supports for immobilization of *Acidithiobacillus ferrooxidans* in the packed-bed bioreactor.

3. Materials and Methods

3.1. Bacterial Strain

Acidithiobacillus ferrooxidans-1333 stored in a Korean Centre for Culture Collection(Pyongyang, DPR Korea) was used.

3.2. Culture Media

Culture media used for immobilization and continuous oxidation experiment is 9K media(22).

3.3. Supports

Waste ore particles used as supports in this study were prepared by sieving the particles of 5~30 mm in size out from the waste chalcopyrite ore dump of Hwangbuk mine enterprise(North Hwanghae Province, DPR Korea).

3.4. Packed-bed Bioreactor

A PVC column with 90 mm in diameter and 25 cm in height was used as bioreactor. The working volume was 350 mL and the height of the support bed was 16 cm. (**Fig. 1**)

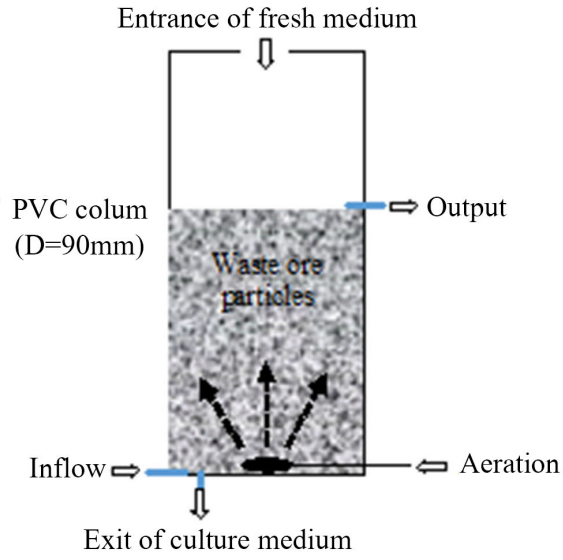


Figure 1. The schematic diagram of the bioreactor packed with waste ore particles as supports

3.5. Support Preparation

The particles were washed with water to remove slimes and then washed again with 3M sulfuric acid solution to remove acid soluble compounds. They were neutralized with water and dried before packing into the reactor.

3.6. Immobilization of Bacteria

The immobilization of cells was done by modified method reported before(2, 15, 23). The first culture, which starts with 10% inoculation, is completed after 95% of ferrous iron in the medium is oxidized, and is followed by the replacement of old medium with fresh medium without any inoculation and the second culture starts. All the other following cultures after the first are carried out without inoculation. These cultures are also completed when 95% oxidation of ferrous iron in the medium is achieved and starts with fresh medium. Such procedure is repeated until the required period for 95% oxidation of ferrous iron in the media is not shortened any more. This period will be inferred to the oxidation time in the next section of this paper.

3.7. Continuous Flow experiment

The bioreactor packed with immobilized biomass was continuously fed with diluted 9K media solutions with different $C_{Fe(II)}$ to estimate the effect of several factors on the R_{ox}. The stead state of the reactor was considered to be established if $C_{Fe(II)}$ in the effluent solution solution varied less than 5% in a period equal to 10 times of the mean residence time.

3.8. Analytical Methods

$C_{Fe(II)}$ was determined by titration with 0.001 M $KMnO_4$, $C_{Fe(III)}$ by titration with 0.01 M EDTA. Bacterial cell number was counted using a Petroff–Haüsser cell counter with the computer linked microscope which used camera instead of eye lens. *3.5 Support preparation* The particles were washed with water to remove slimes and then washed again with 3M sulfuric acid solution to remove acid soluble compounds. They were neutralized with water and dried before packing into the reactor.

4. Results

4.1. Immobilized Cell Density

The cell concentration in free cell culture of *Acidithiobacillus ferrooxidans*-1333 using 9K medium under the condition of temperature; 30°C, aeration rate; 0.5vvm, medium pH; 1.8 is 10^8cells.mL^{-1}. During the successive batch cultures of immobilization C_{cell} at the end of every culture was smaller than 10^8 cells.mL^{-1}. Difference between the cell concentrations in free cell culture and in the immobilizing cultures is reasonable for the cells immobilized to the supports. We calculated the total number of immobilized cells(N_c) using the following equation.

$$N_c = \sum_{i=1}^{n} (C_{max} - C_i)V$$

(C_{max}: the C_{cell} in free cell culture(cells.mL^{-1}), C_i: the C_{cell} of culture *i* (cells.mL^{-1}), *n*: the number of culture, *V*: the medium volume for one culture(mL))

The density of immobilized cells(D_c) was calculated by dividing the total number(N_c) by weight of support particles(W).

$$D_c = N_c / W$$

Density of immobilized cells on the waste ore particles after 8 successive batch cultures was 2.71×10^8cells. support g^{-1} and the oxidation time was 5.5h.

4.2. The Effect of pH to Immobilization

Density of immobilized cells on the particles and oxidation time of the 8th culture at various pH was investigated. Although the amount of immobilized biomass at pH 2.0 was the largest(3.27×10^8cells.support g^{-1}), oxidation time of the 8th culture at pH 2.0 was the longest(8.0h).

In order to investigate density of immobilized cells according to initial pH of medium and oxidation time which is related to activity of the immobilized cells, variation in iron concentration during immobilization cultures were monitored.

Decrease of iron concentration (the sum of $C_{Fe(II)}$ and $C_{Fe(III)}$) at the end of every culture accounts for the amount of iron precipitation formed on the surface of particles and inner surface of the reactor. The average value of these decreases at same pH was increased according to increase of input solution pH.

The average value of decreases of iron concentration because of precipitation on the surface of the supports and the reactor, and calculated amount of ferric iron precipitated on the surface of 1kg support were shown in **Table 1**.

Table 1. Decrease of iron concentration (mg. L^{-1}) caused by precipitation of ferric iron on the surface of supports and reactors at various pH

pH	a)	b)	c)	d)
1.5	438.1	210.4	227.7	637.5
1.8	1101.7	432.5	669.2	1873.7
2.0	2085.7	652.7	1433.0	4012.3

a): Average decrease of iron concentration(mg.L^{-1})
b): Decrease of iron concentration caused by the precipitation on the inner surface of the reactor(mg.L^{-1})
c): Decrease of iron concentration caused by the precipitation on the surface of the supports (mg. L^{-1})
d): Amount of ferric iron precipitated on the surface of 1kg support (mg. support kg^{-1})

Though jarosite is essential for formation of *A. ferrooxidans* biofilm, exceeding amount of it formed on the surface of the supports would cause the embedment of attached bacteria subsequently decrease of oxidation activity of the bioreactor. (2, 12, 24) The fact that activity of the biomass immobilized at pH 1.8 was higher than that at pH 1.5 or pH 2.0 indicates that amount of precipitates at pH 1.8, 1873.7 mg. support kg^{-}

[1], is appropriate for immobilization of *Acidithiobacillus ferrooxidans*-1333.

4.3. Effects of Several Factors on Oxidation Activity of the Bioreactor

Effect of concentration of Fe(II) to activity of the biomass in bioreactor was investigated.(**Fig. 2**)

If dilution rate is low, the residence time is long enough to oxidize almost all ferrous iron fed into the reactor, so the R_{ox} can be increased as the dilution rate increases. If dilution rate exceed above certain value the immobilized biomass are forced to be detached by the mechanical effect of liquid flow at high speed. In addition the residence time becomes short and the washing effect becomes significant so that free cell oxidation would be negligible and as a result the R_{ox} of the bioreactor decreases.

When $C_{Fe(II)}$ in the input solution was 12g.L^{-1}, R_{ox} was significantly lower than those when $C_{Fe(II)}$ was in 1~9g.L^{-1} because immobilized biomass of *A. ferrooxidans* is apparently affected by high substrate (ferrous iron) concentration.

As mentioned in many previous works, the biomass were not stable to liquid flow rate. But biomass immobilized on the waste ore particles was stable to dilution rate as high as 3h^{-1}.

C_{cell} in the effluent solution corresponding to various $C_{Fe(II)}$ in the input solution was shown in **Figure 3**.

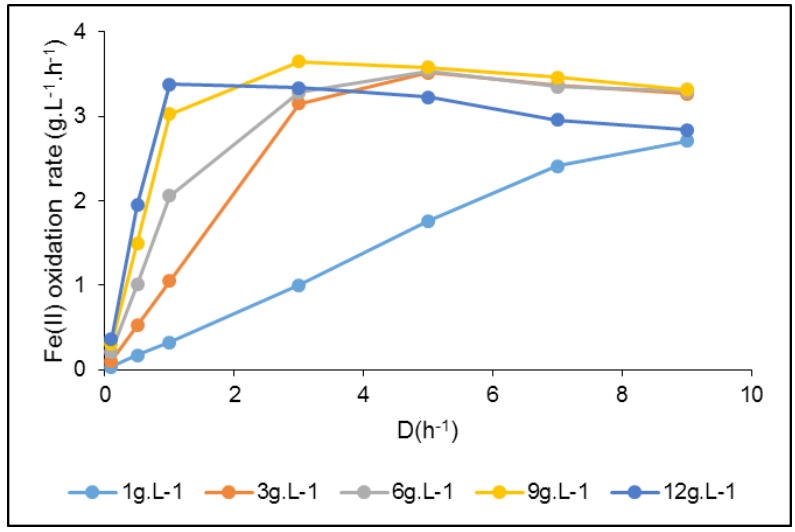


Figure 2. R_{ox} of the bioreactor corresponding to various $C_{Fe(II)}$ in the input solution (30°C, 1.0vvm, pH: 1.8)

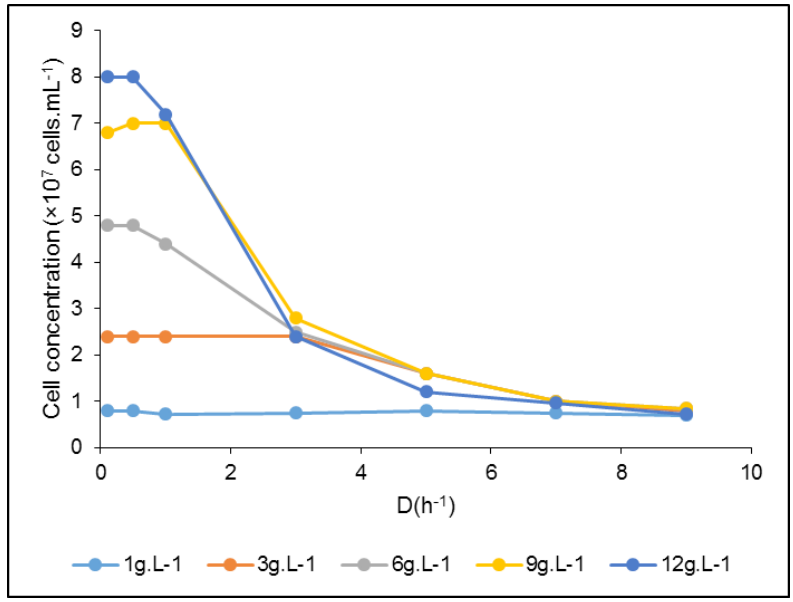


Figure 3. C_{cell} in the effluent solution corresponding to various $C_{Fe(II)}$ in the input solution (30°C, 1.0vvm, pH: 1.8)

As the number of immobilized cells remains constant, the number of grown bacterial cells in the bioreactor equals to the number of cells in effluence. The yield was calculated based on R_{ox} and C_{cell} in effluence as 8.0×10^9 cells.iron g^{-1}. R_{ox} corresponding to each $C_{Fe(III)}$ below $4g.L^{-1}$ were high and had no considerable difference between each other, but it was decreased when $C_{Fe(III)}$ was exceeded above $4g.L^{-1}$, the rate when $C_{Fe(III)}$ was $10g.L^{-1}$ was 37.2% of the rate when there was no Fe^{3+} in the input solution. If $C_{Fe(III)}$ in the input solution was $10g.L^{-1}$, no bacterial cell was detected in the effluent solution (**Fig. 4**).

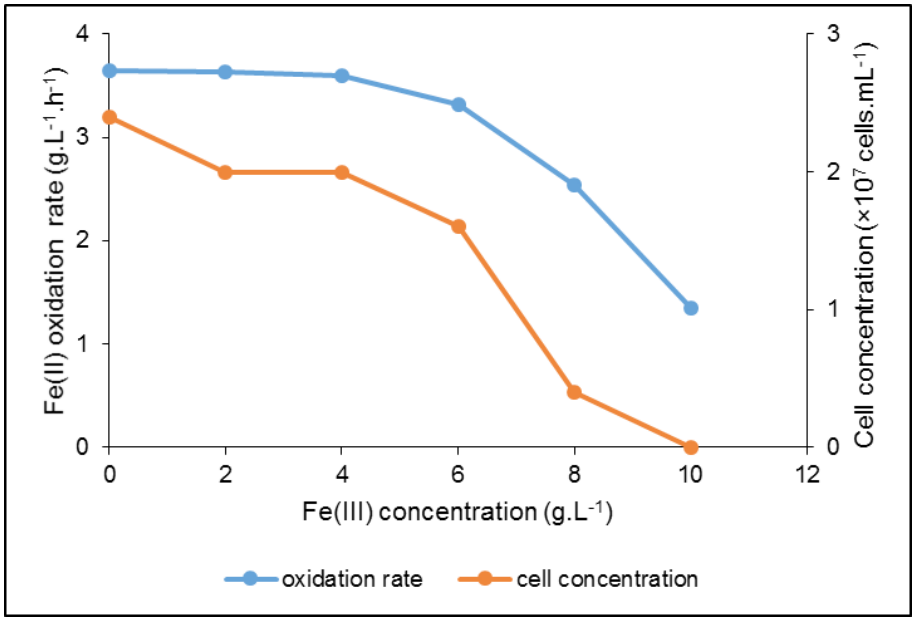
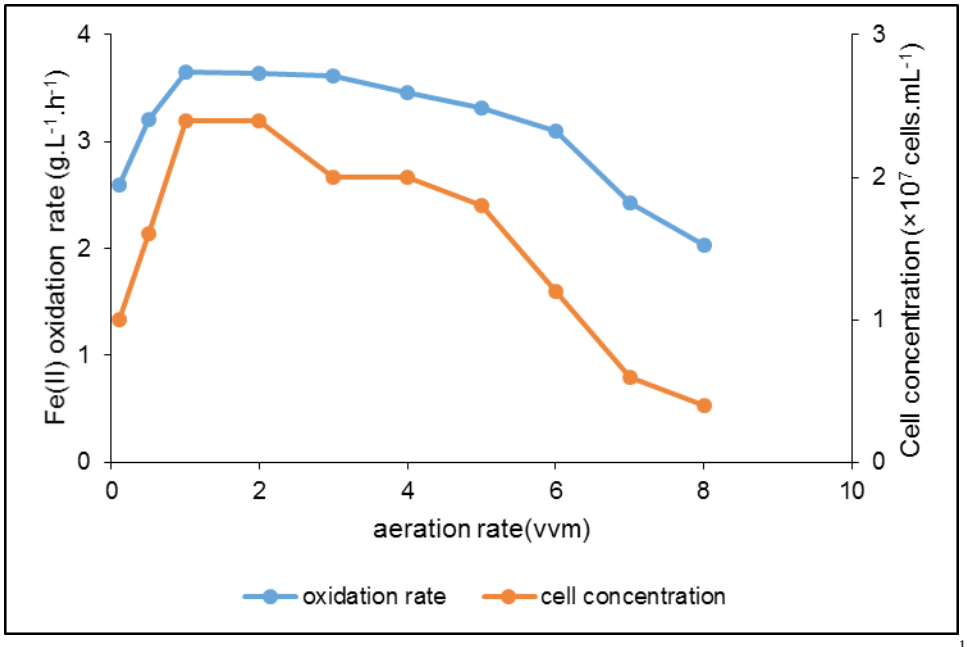


Figure 4. R_{ox} and C_{cell} in the effluent solution at various $C_{Fe(III)}$ in the input solution (30°C, 1.0vvm, D: $3.0h^{-1}$, pH: 1.8)

As shows in **Figure 5** the activity was low when R_{aer} was below than 1.0 vvm because of insufficient supply of oxygen necessary for the biomass to oxidize ferrous iron. And activity was low as well when R_{aer} was increased above 3.0 vvm because strong aeration would cause the detachment of immobilized cells.



Figigure 5. R_{ox} and C_{cell} in the effluent solution corresponding to various R_{aer} (pH: 1.8, 30°C, D: $3h^{-1}$)

5. Discussion

From the results we can conclude that the particles of waste chalcopyrite are able to be adopted for immobilization of *A. ferrooxidans* for several purpose.

Firstly, the immobilized cell density is rather high to be compared with other inorganic support materials such as glass beads. The cell density of fully immobilized waste ore particles within 8 times of successive batch culture was 2.71×10^8 cells.support g while the cell density of fully immobilized glass beads was 1.2×10^8 cells.support g which was metioned in reference 2, although it was smaller than that of organic polymers such as gelrite gel bead(2.1×10^8 cells/bead (6.1×10^9 cells/ml of gel), reference 16) and polyurethane(3.4×10^9 cells.support mL, reference 15),

The iron oxidation rate, which are the main characteristic of the bioreactor of immobilized *A.ferrooxidans* , is also comparable than other support materials. While the highest iron oxidation rate of immobilized waste ore particles was $3.65gL^{-1}h^{-1}$, oxidation rate of Caolin clay tiles(18) was $0.89gL^{-1}h^{-1}$, siliceous stone particles(21) was $5.8gL^{-1}h^{-1}$, ceramic beads(2) was 6.7 $gL^{-1}h^{-1}$. Cobblestone and active carbon was also investigated by ZHOU Hong-bo(25). In continuous operation mode, the highest ferric iron productivity in reactor with cobblestone as supports was 1.54 $gL^{-1}h^{-1}$. The highest ferric iron productivity in reactor with active carbon as supports is 1.89 $gL^{-1}h^{-1}$. Mousavi SM, et al showed that using low density polyethylene(LDPE) the highest oxidation rate of 2.9 $gL^{-1}h^{-1}$ was obtained at the dilution rate of 0.4 h^{-1} (26). Among several support materials, the waste ore particles can be appraised to have competitive characteristic in aspect of oxidation rate.

In addition to high oxidation rate, the immobilized biomass on these particles are shown to be stable as well as the immobilized biomass on the other support materials to the high concentration of ferrous and ferric iron in input solution. Mousavi SM, et al indicated that inhibitory effect of the ferrous and ferric concentrations was observed at the input iron concentrations of 25 and 1 gL^{-1} respectively (26) while the inhibitory ferrous and ferric iron concentration of the biomass immobilized on waste ore particles was above 9 and $4gL^{-1}$. Cells immobilized on ceramic beads were affected by the high concentration of ferrous iron above $8.34gL^{-1}$ (2) and those on polyurethane form were affected by the high concentration of ferrous and ferric iron above 9 and $9g.L^{-1}$ respectively(15).

Another remarkable character of the immobilized cells on waste ore particles was high physical stability such as anti-detachment against high liquid flow or air rate. In our study, the immobilized biomass was stable to high dilution rate up to $9.0h^{-1}$, and to high aeration rate up to 3.0vvm, however stability of the biomass in such conditions were rarely reported before. This shows that waste ore particle-bacterial cell interaction is very strong than any other case reported before.

In conclusion, we might estimate that good characteristics as immobilizing supports, abundance and ease to prepare make these particles of waste chalcopyrite promising support material for immobilizing *A.ferrooxidans* in the fields of biomining, bioremediation and biodesulfurization.

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