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Immobilization of *Acidithiobacillus ferrooxidans* and ferric iron production

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Abstract: Cobblestone, glass beads and active carbon were selected as bacterial supports to study immobilization of *Acidithiobacillus ferrooxidans* in packed bed reactors. The production of ferric iron was then investigated in these immobilized reactors in batch and continuous operation modes. The results show that stable biofilm forms in cobblestone and active carbon supports, thus these two kinds of supports are suitable for immobilization of *A. ferrooxidans*. In batch culture, ferric iron productivity in reactor with cobblestone as supports is 0.61 g/(L·h), which is 1.49 times higher than that in suspended culture reactor. In continuous operation mode, the maximum ferric iron productivity in reactor with cobblestone as supports is 1.54 g/(L·h), which is 3.76 times higher than that in suspended culture reactor. The maximum ferric iron productivity in reactor with active carbon as supports is 1.89 g/(L·h), which is 4.61 times higher than that in suspended culture reactor. In addition to bacteria, the results of X-ray diffraction and scanning electronic microscope analysis show that there is a lot of exopolysaccharide, jarosite and ammoniojarosite in biofilm, which plays important role in the formation of biofilm.

Key words: *Acidithiobacillus ferrooxidans;* immobilization; biofilm; ferric iron productivity

1 Introduction

Acidithiobacillus ferrooxidans is a kind of Gram negative, aerobic, acidophilic and chemolitho-autotrophic bacteria which is mainly applied in bioleaching of sulphide ores for metal recovery[1]. Compared with the traditional metallurgy techniques, biohydrometallergy technology has the advantages of short process, less energy consumption, environment friendly and lower cost. Thus, biohydrometallurgy is one of the most important aspects in the process of sulphide ores. *A. ferrooxidans* can also be applied in the removal of H_2S in gases[2], desulphurization of coal and treatment of mine wastewater containing Fe^{2+}[3]. *A. ferrooxidans* obtains energy from the oxidation of ferrous iron(Ⅱ) to ferric iron(III). Ferric iron is a kind of strong oxidizing agent, which plays an important role in bioleaching of sulphide ores, desulphurization of coal and the removal of H_2S in acid gases[4−6], so it is important to produce ferric irons quickly and in large quantities.

The cell density is low in suspended culture since *A. ferrooxidans* is chemolitho-autotrophic and grows slowly, so it is difficult to produce ferric irons quickly. Supports for immobilization can provide large surface area for bacteria adsorption, and allow the formation of biofilm[7]. Biofilm increases the biomass in the reactor and enhances the oxidation rate of ferrous iron. So far, the studies on the immobilization of *A. ferrooxidans* in domestic or overseas literature are mainly focused on the kinds of carriers, reactor types and the influence of operating parameters[8−11]. However, few studies have investigated the difference of ferric productivity between immobilized culture reactor and suspended culture reactor, and the research on the biofilm formation during the immobilization of *A. ferrooxidans* have not attracted much attention.

This paper studies the techniques for biofilm formation in order to enhance the ferric irons productivity. The biofilm formed in packaged bed reactors by using cobblestone, glass beads and active

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carbon as supports is investigated. Then the influence of immobilized culture and suspended culture on ferric iron productivity is compared in reactors operating in batch and continuous modes. The chemical composition of biofilm was also analyzed by X-ray diffraction(XRD), the morphological observation of biofilm is done by scanning electronic microscope(SEM) and the role of exopolysaccharide, jarosite and ammoniojarosite in the formation of biofilm is discussed.

2 Experimental

2.1 Inoculum

A. ferrooxidans isolated by the Key Laboratory of Biometallurgy of Ministry of Education of China was used as inoculum.

2.2 Medium

The compositions of 9 K nutrient medium are as follows: $(NH_4)_2SO_4$ 3 g/L; KCl 0.1 g/L; K_2HPO_4 0.5 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $Ca(NO_3)_2$ 0.01 g/L; $FeSO_4 \cdot 7H_2O$ 44.7 g/L[12].

2.3 Support materials for immobilization of bacteria

Cobblestone(diameter 4−6 mm), active carbon(1 cm ×1.5 mm), glass beads(diameter 4−6 mm) were selected as inert supports. All the supports had been dipped into 1 mol/L sulfuric acid for 72 h before being packaged into the reactors to remove the soluble compounds.

2.4 Reactor and operating mode

Fig.1 shows the illustration of experimental device. The reactor is a cylinder with water jacket, the ratio of height/diameter is 3, the effective volume is 500 mL. Four fifths of the reactor volume was filled with inert supports, and the liquid volume in the reactor was 300 mL after the supports had been filled. Three operation modes are described as follows. 1) Batch culture: air was aerated in at the bottom of the reactor with a flow rate of 150 mL/min, culture medium was filled from the influent reservoir and not recycled. 2) Batch cycled culture: air was aerated in at the bottom of the reactor with a flow rate of 150 mL/min; culture medium was filled from the influent reservoir and then recycled from the effluent reservoir by a constant peristaltic pump. 3) Continuous culture: fresh medium was fed from the influent reservoir by a constant peristaltic pump continuously; air was aerated in at the bottom of the reactor with a flow rate of 150 mL/min.

2.5 Analytical methods

Ferrous iron (Ⅱ) was titrated by potassium dichromate [4]. The chemical composition of biofilm was analyzed by XRD[13]. The morphological observation of biofilm was done by SEM.

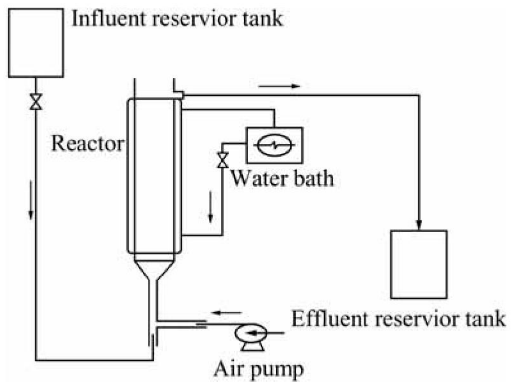

Fig.1 Experimental device

The preparation of sample for SEM analysis was as follows: 30 mL of carrier supports were taken from the reactor and transferred into a 150 mL Erlenmeyer flask when biofilm steadily formed on the surface of the carrier. 50 mL distilled water was put into the flask and the flask was shaken at a speed of 200 r/min for 30 min. The carrier was discarded, the liquid was centrifuged at a speed of 10 000 r/min for 10 min, most of the supernatant was discarded and the sample was fixed with 3% glutaraldehyde, then kept in the refrigerator at 4 ℃ for 24 h. The precipitation and the solution were mixed and a little mixed liquid was spread onto the coverslip uniformly. After being dehydrated, dried and sprayed with gold ions on the surface of the glass patch, the sample was observed with SEM[14].

2.6 Determination of biomass on carrier

30 mL inert supports were taken from the reactor and transferred into a 150 mL Erlenmeyer flask together with 30 mL distilled water after the biofilm steadily formed. The flask was vibrated at a speed of 200 r/min for 1 h in order to peel off the biofilm from the surface of the carrier. Then the carrier was discarded, some glass bead was added into the flask and the flask was shaken at a speed of 200 r/min for 60 min to break the biofilm into free swimming bacteria. Cell numbers were determined by a counting chamber under an optical microscope at 400 magnifications. The cell densities were then calculated as an average of more than 5 determinations of each culture sample.

3 Results and discussion

A. ferrooxidans can grow in 9 K medium, the only energy is ferrous sulfate. The bacteria obtain energy for growth from the oxidation of ferrous irons (reaction 1):

$$4Fe^{2+} + 4H^{+} + O_2 \xrightarrow{A.\ ferrooxidans} 4Fe^{3+} + 2H_2O \qquad (1)$$

Since the behavior of ferrous irons in the medium is correlated closely to the growth of *A. ferrooxidans*, the ferrous iron oxidation rate and ferric iron productivity are applied to illustrate the growth of bacteria and the ability of the reactors to produce ferric irons.

Ferric iron productivity=the amount of ferrous iron oxidized in unit volume × the volume of effluent/(effective reactor volume×reaction time).

Where the reaction time refers to culture time in batch culture mode and in batch recycled culture mode, it refers to hydraulic retention time(HRT) in continuous culture mode.

3.1 Formation of biofilm

Cobblestone, glass beads and active carbon particles were selected as supports to study immobilization of *A. ferrooxidans* in packed bed reactors. The reactors were inoculated with seed culture (cell density is 1×10^{8} mL^{-1} after inoculation) and run in batch recycled mode. The culture conditions were: 30 ℃, initial pH 1.5, initial ferrous iron (Fe^{2+}) concentration 9 g/L, upflow velocity 1.06 m/h. The ferrous iron concentration was determined during the process of biofilm formation. When the ferrous iron oxidation rate increased up to 95%, the used liquid was replaced with fresh medium (inoculated by 10%). This process was repeated several times till the brown biofilm formed in the surface of supports after 15 d. Fig.2 shows the photos of supports before and after the biofilm formation(the photos in the left are supports before biofilm formation, in the right are supports after biofilm formation).

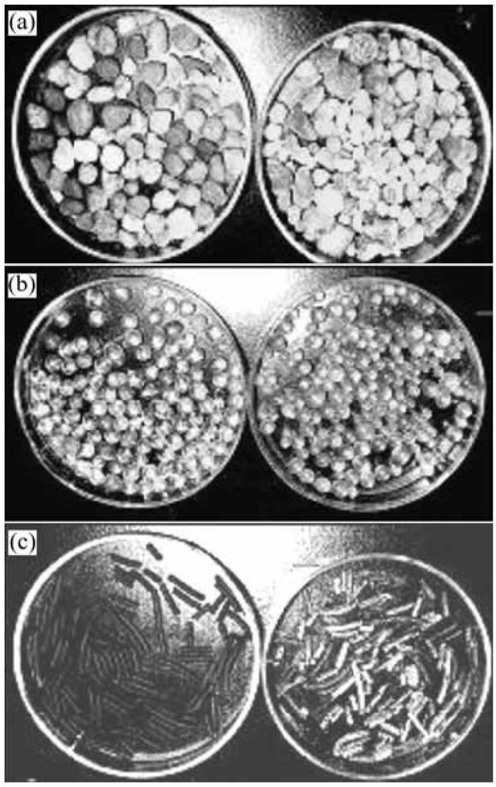

Fig.2 Photos of supports without and with biofilm: (a) Cobblestone; (b) Glass beads; (c) Active carbon

3.2 Ferric iron production in immobilized reactors operating in batch mode

After the biofilm formed, the reactors were pumped empty, and filled with fresh medium to operate in batch recycled mode. The ferrous iron oxidation ability in the reactor with cobblestone as supports was compared with the suspended culture reactor, both reactors were inoculated with seed culture (cell density was 1×10^{8}/mL after inoculation). The culture conditions were: 30 ℃, initial pH 1.5, initial Fe^{2+} 9 g/L.

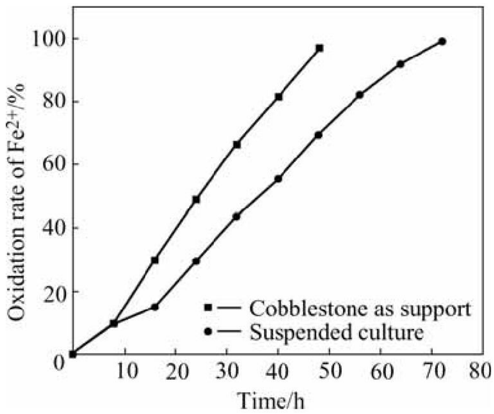

Fig.3 Comparison of ferrous iron oxidation ability of immobilized and suspended *A. ferrooxidans*

Fig.3 shows that it takes 48 h to completely oxidize the ferrous irons in immobilized culture reactor operating in batch mode, the ferric iron productivity is 0.61 g/(L·h). While in the suspended culture reactor, the data are 72 h and 0.41 g/(L·h), respectively. The ferrous iron oxidation rate in the immobilized culture reactor is 1.49 times higher than that in the suspended culture reactor.

In search of the better support for immobilization of *A. ferrooxidans*, the ferrous iron oxidation rates in immobilized culture reactors with three types of supports were compared. The culture conditions were: 30 ℃, initial pH 1.5, initial Fe^{2+} 40 g/L.

Fig.4 shows that the ferrous iron oxidation rate of immobilized *A. ferrooxidans* using active carbon as support is the fastest in batch culture. The ferric productivities are 0.69, 0.64, 0.51 g/(L·h) for active carbon, cobblestone and glass beads supports respectively. It is suggested that it is difficult for *A. ferrooxidans* to adsorb on glass beads since the surface is very smooth, thus the biofilm formation is slow and unstable, especially under the scouring of aeration and water stream. Therefore, reactors using active carbon, cobblestone particles as supports for immobilization were selected for further continuous culture.

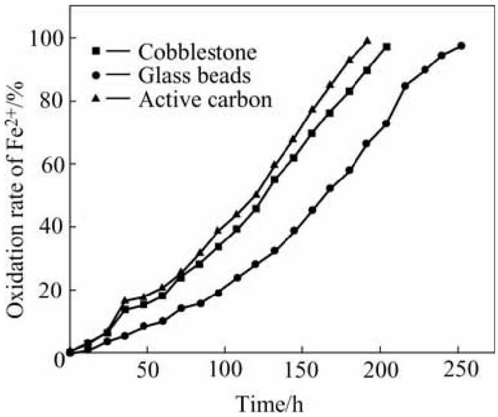

Fig.4 Effects of immobilization of *A. ferrooxidans* by different supports on ferrous iron oxidation rate

3.3 Ferric iron production in immobilized reactor operating in continuous mode

It is suitable to operate the reactor in continuous mode to obtain high ferric iron productivity since large amount of biomass can retain in the immobilized culture reactor. Therefore, the ferrous iron oxidation in immobilized reactor operating in continuous mode was investigated.

3.3.1 Tests for biofilm stability

Under the conditions of 30 ℃, initial pH 1.50, initial Fe^{2+} 4.2 g/L, the flow rate was adjusted to 100, 200, 300 mL/h to operate the immobilized culture reactor in continuous mode. After the reactors had been operated for 10 HRT to reach steady-state, the ferrous iron oxidation rates were determined from time to time.

From the results shown in Fig.5, it can be seen that at different flow rates, in both immobilized reactors with cobblestone and active carbon as supports, there is no big fluctuation in ferrous iron oxidation rates, which means that the stable biofilm has already formed in supports.

3.3.2 Ferric iron production of immobilized reactors running in continuous culture mode at different flow rates

Under the conditions of 30 ℃, initial pH 1.50, initial Fe^{2+} 4.5 g/L, the flow rate was adjusted to run the immobilized reactor in continuous operation mode at different dilution rates. After the reactors had run for 10 HRT to reach steady-state, the ferrous iron oxidation rates were determined and the ferric iron productivities were calculated.

Fig.6 shows the ferric iron productivities in immobilized culture reactors operating in continuous mode at different flow rates. In reactor with cobblestone as supports, when dilution rates range from 0.33 to 0.67 h^{-1}, the ferrous iron oxidation rates and ferric productivities are relatively high. At dilution rate of 0.67 h^{-1}, the ferrous iron oxidation rate is 51.35%, while the ferric iron productivity reaches the maximum value of 1.54 g/(L·h), which is 3.76 times higher than that of the suspended batch culture. The ferrous iron oxidation rate and ferric productivity decrease when the dilution rate increases further. In reactor with active carbon as supports, the ferrous iron oxidation rate and ferric productivity are relatively high at the dilution rate ranging between 0.33 and 0.72 h^{-1}. At the dilution rate of 0.72 h^{-1}, the ferrous iron oxidation rate is 58.66%, while the ferric iron rate reaches the maximum value of 1.89 g/(L·h), which is 4.61 times higher than that of the suspended batch culture. The ferrous iron oxidation rate and ferric productivity decrease when the dilution rate increases further.

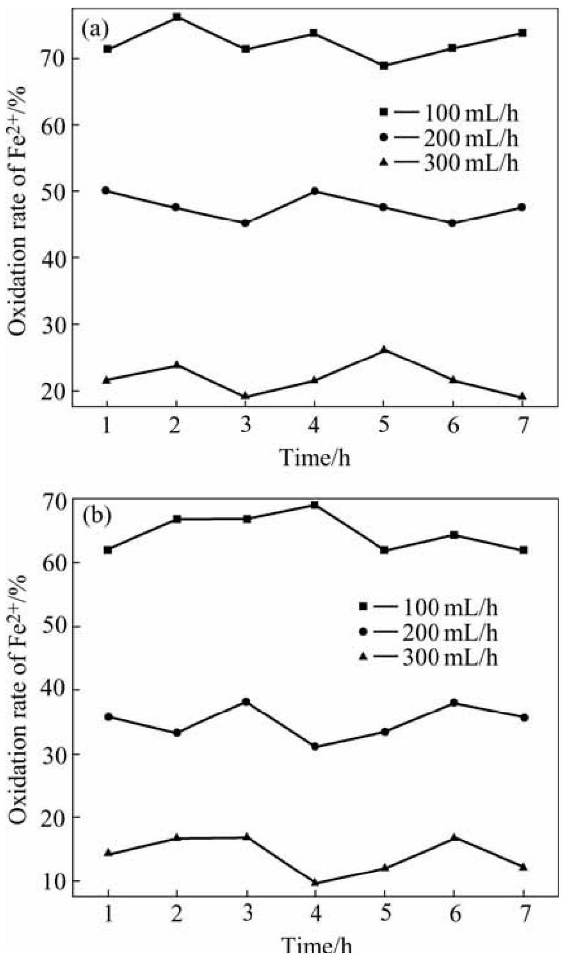

Fig.5 Ferrous iron oxidation rates in immobilized reactors under different flow rates: (a) Active carbon supports; (b) Cobblestone supports

3.4 Analysis of biofilm

The cell densities on the cobblestone and on the surface of activated carbon are 4.2×10^8 cells/cm^3 and 5.7×10^8 cell/cm^3 respectively. The numbers are obviously higher than that in the suspended continuous culture, which is usually around $(1-9)\times10^7$ cells/mL (data from our lab). This means the immobilization of *A.*

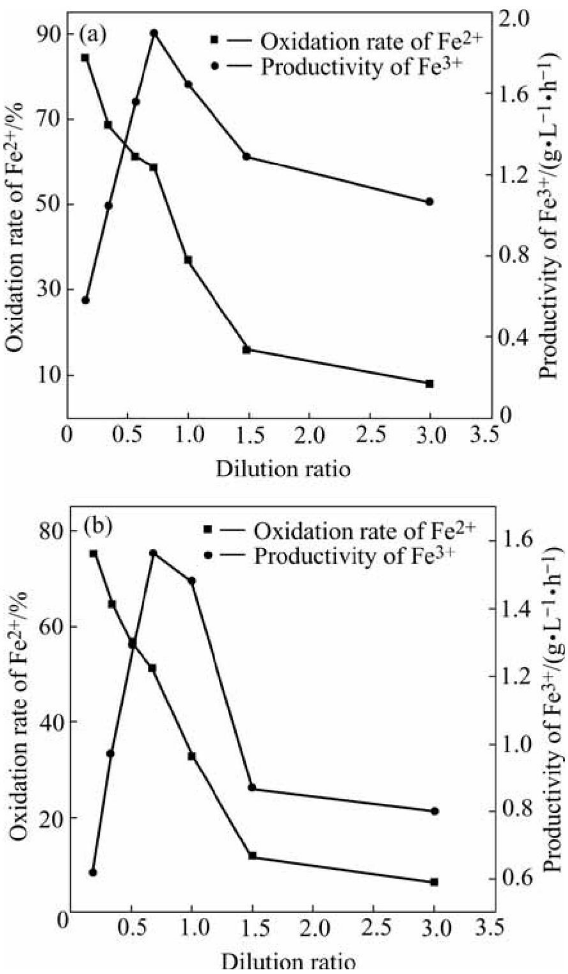

Fig.6 Ferrous iron oxidation rates and ferric iron productivities in immobilized culture reactors under different flow rates: (a) Active carbon supports; (b) Cobblestone supports

ferrooxidans can increase the biomass in the reactor for a certainty.

The precipitates formed during the biooxidation process were analyzed by SEM (Fig.7). At the end of experiment, biofilm in reactor with cobblestone particles as bacterial support was peeled by mechanical method to do SEM and XRD analysis (Figs.8 and 9).

Fig.7 shows that the surface of the precipitates formed in suspended batch culture is rough and without the cream-like substance, the number of adsorbed bacteria is small. However, it can be seen from Fig.8 that the biofilm consists of small particles, bacteria and the cream-like substance. The rod-shaped bacteria either adsorbed or embedded in the particles and in the cream-like substances (as shown by the arrows in Fig.8). As reported by Ref.[15], the *A. ferrooxidans* produced exopolysaccharide(EPS) when adsorbed to the solid surface, however, there was no EPS produced in suspended culture[16]. According to the results in Ref.[17], and considering the results we obtained, the

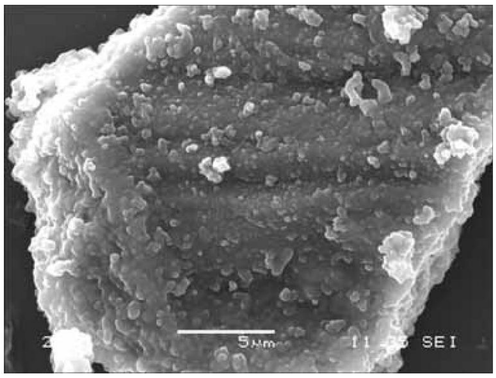

Fig.7 SEM image of precipitates produced in suspended culture reactor

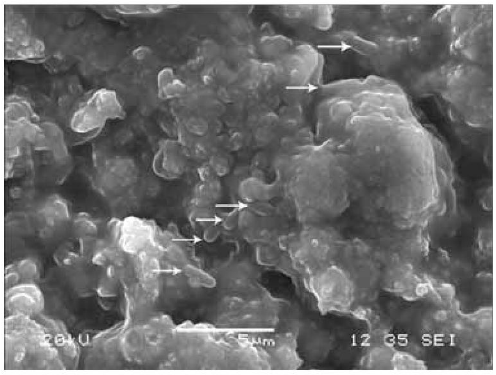

Fig.8 SEM image of biofilm

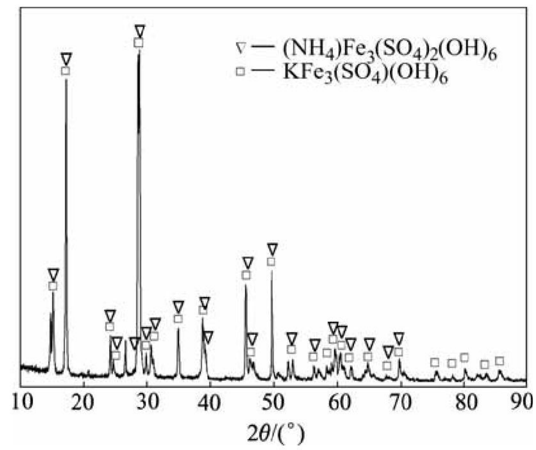

Fig.9 X-ray diffraction pattern of biofilm

authors suggest that the cream-like substance is EPS.

The results of XRD analysis (Fig.9) shows that there is a large amount of jarosite and ammoniojarosite precipitates in the biofilm. The small particles observed by SEM are suggested as mixture of jarosite and ammoniojarosite precipitates. It is suggested that with the reaction taking place, the hydrolysates of ferric irons such as jarosite (reactions (1) and (2)) and ammoniojarosite (reactions (1) and(3)) precipitate in the surface of the bacterial supports and become part of the biofilm.

$$3Fe^{+3} + K^{+} + 6H_2O + 2SO_4^{2-} \rightarrow KFe_3(SO_4)_2(OH)_6 + 6H^{+} \quad (2)$$

$$3Fe^{3+} + NH_4^{+} + 6H_2O + 2SO_4^{2-} \rightarrow NH_4Fe_3(SO_4)_2(OH)_6 + 6H^{+} \quad (3)$$

The jarosite and ammoniojarosite precipitates are loose and porous materials, which are very nice supports for bacterial adsorption[18]. It was reported that in the reactor with immobilized *A. ferrooxidans*, the biomass in the biofilm accounted for 90% of the total biomass[19]. Considering the results we acquired and reported in literature, the process of biofilm formation can be suggested as follows. On one hand, some free swimming bacteria adsorb to support materials and excrete EPS which entrap more free swimming bacteria. On the other hand, the jarosite and ammoniojarosite precipitates formed in the process deposit on the surface of bacterial supports and also adsorb free swimming bacteria. The adsorbed or the embedded bacteria reproduce more bacteria. Thus, from time to time, the bacteria, EPS, jarosite and ammoniojarosite mix and overlay each other and form biofilm with certain thickness. Once the biofilm has been formed, a number of immobilized bacteria and free swimming bacteria are retained in the reactor, therefore the biomass increases and the ferrous iron oxidation rate is enhanced.

4 Conclusions

1) *A. ferrooxidans* form biofilms in the bacterial supports such as cobblestone, glass beads and active carbon particles in packed bed reactors. The biofilms formed in cobblestone and active carbon particles show strong ferrous iron oxidation ability. In batch operation mode, the ferric iron productivity in reactor using cobblestone as bacterial supports is up to 0.61 g/(L·h), which is 1.49 times higher than that of the suspended culture.

2) The biofilm formed in the packed bed reactors consists of a large number of bacteria, EPS and jarosite and ammoniojarosite precipitates. The formation of biofilm increases the biomass in the reactor.

3) In continuously operated packed bed reactors, the ferric iron productivity in reactor using cobblestone particles as bacterial supports is up to 1.54 g/(L·h), which is 3.76 times higher than that of the suspended culture; the ferric iron productivity in reactor using active carbon particles as bacterial supports is up to 1.89 g/(L·h), which is 4.61 times of that of the suspended culture. The immobilization of *A. ferrooxidans* enhances the ferric iron productivity by a large extent.

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