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Effects of *Acidiphilium cryptum* on biosolubilization of rock phosphate in the presence of *Acidithiobacillus ferrooxidans*

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Abstract: The bioleaching of pyrite and biosolubilization of rock phosphate (RP) in 9K basal salts medium were compared by the following strains of an autotrophic acidophilic bacterium, *Acidithiobacillus ferrooxidans*, a heterotrophic acidophilic bacterium, *Acidiphilium cryptum*, and mixed culture of *At. ferrooxidans* and *A. cryptum*. The results show that *A. cryptum* is effective in enhancing the bioleaching of pyrite and biosolubilization of RP in the presence of *At. ferrooxidans*, although it could not oxidize pyrite and solubilize RP by itself. This effect is demonstrated experimentally that *A. cryptum* enhances a decrease in pH and an increase in redox potential, concentration of total soluble iron and planktonic part bacterial number in the broth during pyrite bioleaching processes by *At. ferrooxidans*. The mixed culture of *At. ferrooxidans* and *A. cryptum* leads to the most extensive soluble phosphate released at 30 °C. Pulp density exceeding 3% is shown to adversely influence the release of soluble phosphate by the consortium of *At. ferrooxidans* and *A. cryptum*. It is essential to add pyrite to the 9K basal salts medium for the biosolubilization of RP by the mixed culture of *At. ferrooxidans* and *A. cryptum*, and the percentage of soluble phosphate released is the greatest when the mass ratio of RP to pyrite is 1:2 or 1:3.

Key words: *Acidiphilium cryptum*; bioleaching; pyrite; rock phosphate; biosolubilization; *Acidithiobacillus ferrooxidans*

1 Introduction

Phosphorus is one of the most important essential elements for crop production. Despite phosphorus being widely and abundantly distributed in the soils in both its inorganic and organic forms, many soils throughout the world are deficient in phosphorus. Hence, large amounts of soluble phosphate are applied to soils as fertilizer for sustaining profitable agricultural production.

Natural rock phosphate (RP) is a complex raw material and is mainly used in the manufacture of phosphate fertilizer [1]. Almost 80% of RP all over the world is low-grade and not suitable for direct application to soils as a phosphate fertilizer because of its low phosphorus content and poor solubility [2]. Conventionally, RP is chemically processed with sulfuric acid or phosphoric acid into phosphate fertilizer. This process makes the fertilizer more expensive and contributes to environmental pollution [3]. Consequently, a more efficient process to release soluble phosphate from RP is being sought.

As an alternative to the direct use of sulfuric or phosphoric acid, microorganisms may be considered a source of solubilizing agents for insoluble mineral phosphates [4]. Some microorganisms, including bacteria and fungi, are known to be involved in the solubilization of RP [5−7]. These phosphate-solubilizing micro-organisms used for industrial production of phosphate fertilizer lower the production cost. Their activity may also be exploited when insoluble mineral phosphate is applied directly to soils [8,9].

Autotrophic acidophilic bacteria have special interest recently as phosphate-solubilizing micro-organisms because these bacteria, including iron- and sulfur-oxidizing microorganisms, are commonly used in bioleaching and play an important role in hydrometallurgy because of the ease of handling them,

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the low cost of their application, and the ability to control the impact they have on the environment [10]. Owing to the numerous applications of autotrophic acidophilic bacteria in the bioleaching of sulfide minerals, several studies have been done with autotrophic acidophilic bacteria, such as *Acidithiobacillus ferrooxidans* and *Acidithiobacillus thiooxidans*, to solubilize RP [11−13]. However, most of the earlier experiments with autotrophic acidophilic bacteria were performed on a small scale. Although RP can be solubilized successfully by these bacteria, the low release of soluble phosphate was a major challenge. Thus, enhancement of the release of soluble phosphate from RP would make the use of RP in agriculture more effective and economic.

At. ferrooxidans is most studied in the bioleaching acidophiles and was thought to be the most important contributor to enhance bioleaching, largely because it flourished in laboratory cultures. However, its ability to oxidize ferrous ion and/or sulfur is repressed to different degrees by the addition of naturally-occurring organic compounds in growth media [14]. Several heterotrophic microorganisms have been reported to remove this inhibition by metabolizing the organic materials, consequently enhance the growth of the autotrophs and as a result improve the leaching rate of metal sulfides [15]. In this study, a heterotrophic acidophilic bacterium *Acidiphilium cryptum* was introduced into an autotrophic acidophilic bacterium *At. ferrooxidans* for the bioleaching of pyrite and biosolubilization of RP in the presence of pyrite. The effects of temperature, pulp density and mass ratio of RP to pyrite on the biosolubilization of RP by the consortium of *At. ferrooxidans* and *A. cryptum* were also investigated.

2 Experimental

2.1 Bacteria and culturing techniques

The bacteria used in this experiment were autotrophic acidophilic bacterium *At. ferrooxidans* and heterotrophic acidophilic bacterium *A. cryptum*, which were kindly supplied by Central South University (Changsha, China). *At. ferrooxidans* was routinely cultured in 250 mL flasks containing 100 mL 9K basal salts medium with 44.2 g/L $FeSO_4·7H_2O$ as an energy source [16], and previously adjusted to pH of 2.5 using 20% sulfuric acid. *A. cryptum* was grown in 250 mL flasks containing 100 mL 9K basal salts medium with 1 g/L glucose and 0.3 g/L yeast extract as an energy source, and adjusted to initial pH of 3.5 using 20% sulfuric acid. Flasks were incubated at 30 °C on a rotary shaker at 160 r/min. The cultures that were used had been subcultured through five transfers in pyrite-containing medium in order to adapt the bacteria under the experimental conditions.

2.2 Assays of bioleaching of pyrite

The pyrite sample was obtained from Daye iron mines (Hubei, China), which mainly contained 90% FeS_2 and some quartz. The pyrite was crushed, ground and dry-sieved to a particle size of 75−147 μm. Prior to use, the pyrite was washed with 2 mol/L hydrochloric acid to remove any surface oxidized deposits, rinsed repeatedly with distilled water, and then dried at 50 °C for 24 h and sterilized by UV for 24 h. Bioleaching of pyrite was carried out in 250 mL flasks with 100 mL 9K basal salts medium containing 2 g pyrite. The pH of the medium was initially adjusted to 2.5 except for that of the pure culture of *A. cryptum*, which was adjusted to pH 3.5. The cell density of each culture was adjusted previously at 1×10^7 cell/mL and inoculated at 10% (v/v). Cultures of *At. ferrooxidans* and *A. cryptum* were mixed at 1:1 (v/v) for the assays of the consortium. All the assays were performed at 30 °C on a rotary shaker at 160 r/min for 14 d, and samples were taken out every day to determine pH, redox potential, total soluble iron concentration and planktonic part bacterial number. Autoclaved, uninoculated medium containing 20 g/L pyrite was served as abiotic control. All experiments were performed in triplicate.

2.3 Assays of biosolubilization of RP

The RP sample was obtained from Yichang phosphate mines (Hubei, China), and was crushed, ground and dry-sieved to a particle size of 75−147 μm. X-ray diffraction analysis showed that the sample was mainly composed of hydroxyapatite and a small quantity of quartz and montmorillonite. Biosolubilization of RP was tested in 250 mL flasks with 100 mL 9K basal salts medium containing 2 g pretreated pyrite and 1 g RP (mass ratio of RP to pyrite: 1:2, pulp density: 3% (w/v), the same below if not mentioned). The pH of the medium was initially adjusted to 2.5 except for that of the pure culture of *A. cryptum*, which was adjusted to 3.5. The cell density of each culture was adjusted previously at 1×10^7 cell/mL and inoculated at 10% (v/v). Cultures of *At. ferrooxidans* and *A. cryptum* were mixed at 1:1 (v/v) for the assays of the consortium. All the assays were performed at 30 °C on a rotary shaker at 160 r/min for 14 d, and samples were taken every day and filtered through a 0.45 μm pore-size filter paper. Then the filtrate was centrifuged at 11000 ×*g* for 20 min, and the supernatant was assayed for the content of soluble phosphate. Autoclaved, uninoculated medium containing 20 g/L pyrite and 10 g/L RP was served as abiotic control. To investigate the optimal temperature for the solubilization

of RP by the consortium of *At. ferrooxidans* and *A. cryptum*, the flasks were shaken at different temperatures (20, 25, 30, 35, and 40 °C, respectively). Subsequently, the effect of pulp density (w/v) was studied at 1%, 2%, 3%, 4%, and 5% (w/v), respectively. Finally, the effect of mass ratio of RP to pyrite (3:1, 2:1, 1:1, 1:2, and 1:3, respectively) in the broth on RP biosolubilization was studied. All experiments were performed in triplicate.

2.4 Analytical methods

The pH value was recorded with a pH meter equipped with glass electrode. The redox potential was measured using a platinum electrode combined with Ag/AgCl reference electrode and converted to E_h values (i.e. relative to a hydrogen reference electrode) by adding 234 mV to the measured values [17]. Total soluble iron was determined by atomic absorption spectrometry. The planktonic part of the bacterial number was counted by means of a haemocytometer under microscope. Content of soluble phosphate was determined by the vanadium−ammonium molybdate colorimetric method with a UV-vis 8500 spectrophotometer at 490 nm [18]. Values were given as mean ± standard deviation for triplicate samples.

3 Results and discussion

3.1 Bioleaching of pyrite by *At. ferrooxidans* and *A. cryptum*

The bioleaching of pyrite has been studied extensively, and recent work has provided strong evidence that the pyrite bioleaching by autotrophic acidophilic bacteria occurs via a multiple subprocess mechanism [19−22].

In this study, the dynamics of the bioleaching of pyrite was followed by determining the pH, redox potential, concentration of total soluble iron and planktonic part bacterial number in the broth. The results obtained in 14 d experiments are shown in Fig. 1. The pH in the broth decreased gradually after being inoculated with *At. ferrooxidans*, and at the end of the experiment, the pH decreased to 1.44 from an initial pH of 2.5 (Fig. 1(a)). An obvious increase of redox potential in the biotic test was consistent with the oxidizing activity of *At. ferrooxidans*. This trend was confirmed by the measured E_h, which increased from 651 to 825.7 mV in the *At. ferrooxidans* culture (Fig. 1(b)). The activity was also manifested through the increase of the

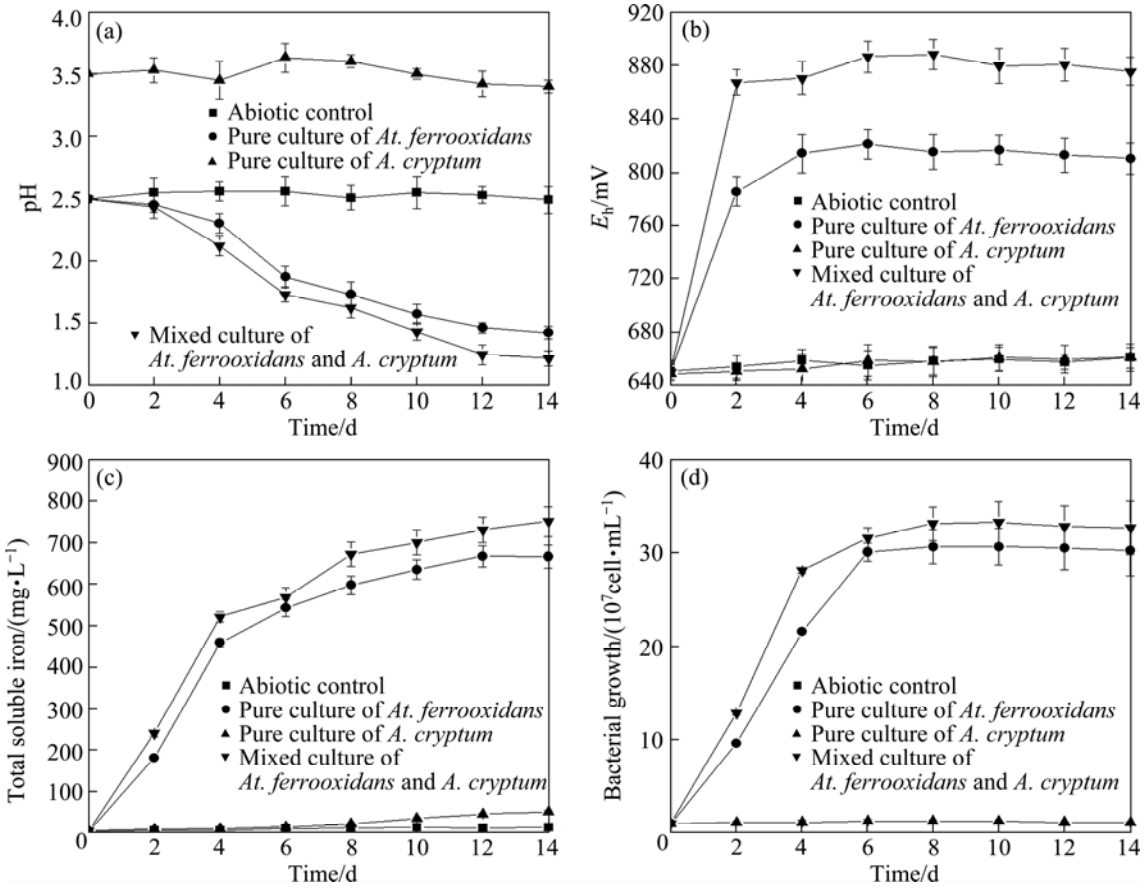

Fig. 1 Changes in pH (a), redox potential (b), concentration of total soluble iron (c), and planktonic part bacterial number (d) in broth during pyrite bioleaching processes by *At. ferrooxidans* and *A. cryptum*

concentration of total soluble iron in the broth, in which the highest concentration of 680.3 mg/L was obtained (Fig. 1(c)). Furthermore, *At. ferrooxidans* grew well in the presence of pyrite, and the bacterial abundance increased after inoculation and reached a maximum of about 3.1 $\times 10^8$ cell/mL after 14 d (Fig. 1(d)). However, the changes of pH, redox potential and concentration of total soluble iron in the abiotic control were negligible.

Autotrophic acidophilic bacteria can obtain energy for growth by oxidizing Fe (II) and/or reduced forms of sulfur [23,24]. However, their oxidizing activity is always repressed to different degrees by the production of organic compounds in the broth [25]. It is interesting to note that some heterotrophic acidophilic bacteria, which are able to metabolize organic compounds as a source of energy and detoxify the growth environment for autotrophic acidophilic bacteria, were reported to form a mutualism with autotrophic acidophilic bacteria in mineral bioleaching process [26−29]. In this study, a heterotrophic acidophilic bacterium, *A. cryptum*, was added to the broth as it was hypothesized that it could cooperate with *At. ferrooxidans* by feeding on organic excretions from the latter. This heterotroph does not oxidize iron(II) or sulfur as sole energy substrate but able to reduce Fe^{3+} and metabolize a small amount of organic compounds, such as glucose and yeast extract, but a large amount of organic compounds inhibit its growth [14].

Figure 1(a) shows that the mixed culture of *At. ferrooxidans* and *A. cryptum* appeared to reduce the pH more effectively than the pure culture of *At. ferrooxidans*, which is decreased by 8.3% (compared with the pH at the end of the experiment). Results also show that *A. cryptum* was able to improve the bioleaching of pyrite by *At. ferrooxidans*, and the redox potential and concentration of total soluble iron were elevated by 7.1% and 10.7%, respectively, at the end of the experiment in the presence of *A. cryptum* (Figs. 1(b) and (c)). The planktonic part bacterial number in the broth inoculated with mixed culture of *At. ferrooxidans* and *A. cryptum* was higher than that with pure culture of *At. ferrooxidans* (Fig. 1(d)). The result indicated that although *A. cryptum* did not grow in the medium without any organic matter addition, it was able to enhance the cell growth of *At. ferrooxidans*. It also displayed a fast growth of the mixture of *At. ferrooxidans* and *A. cryptum*, which was 10.7% higher than the rate observed for the pure culture of *At. ferrooxidans*. However, as for the pure culture of *A. cryptum*, since it was not able to oxidize pyrite as its growth source, it did not grow well in the broth. These results suggest that the presence of a consortium of *At. ferrooxidans* and *A. cryptum* may determine a higher pyrite oxidizing efficiency due to a coupled and synergistic metabolism [30]. Heterotrophs were reported to assist bioleaching environments by metabolizing organic matter generated by autotrophs, and thus detoxifying the growth environment for autotrophs [31]. At the same time the growth of autotrophs and the release of labile organic material from their metabolism seem to be enough for the heterotrophic needs of the Fe-reducing bacteria.

3.2 Biosolubilization of RP by *At. ferrooxidans* and *A. cryptum*

Autotrophic acidophilic bacteria have a key role in the production of sulfuric acid from the oxidation of pyrite. The sulfuric acid creates an acid environment and thus benefits the solubilization of RP. The hydroxyapatite, which is the major mineral present in the RP sample, is solubilized according to the following chemical reaction:

$$5H_2SO_4+Ca_5(OH)(PO_4)_3 \longrightarrow 5CaSO_4+3H_3PO_4+H_2O$$

In this study, both the pure culture of *At. ferrooxidans* and the mixed culture of *At. ferrooxidans* and *A. cryptum* could effectively release soluble phosphate from RP compared with the abiotic control, and the addition of pyrite was very important to the biosolubilization of RP (Fig. 2(a)). At the end of the experiment, the pyrite-added system achieved the highest percentage of soluble phosphate released of 26.1% and 28.5%, respectively, for the pure culture of *At. ferrooxidans* and mixed culture of *At. ferrooxidans* and *A. cryptum*, compared with 4.3% and 4.4%, respectively, for the same system without the additional pyrite. The mixed culture of *At. ferrooxidans* and *A. cryptum* released more soluble phosphate (9.2% elevated at the end of the experiment) compared with the pure culture of *At. ferrooxidans*. This indicated that the application of the heterotrophic acidophilic bacterium, *A. cryptum*, was an effective method to promote the biosolubilization of RP by the autotrophic acidophilic bacterium *At. ferrooxidans*. It seems to be a feasible and attractive approach to enhance the soluble phosphate releasing efficiency from RP with coinoculation of *At. ferrooxidans* and *A. cryptum*.

Figure 2(b) shows that although there is obvious increase of pH as a result of the consumption of acid by the proton attack on RP for the pure culture of *At. ferrooxidans* and mixed culture of *At. ferrooxidans* and *A. cryptum*, respectively, at the 4th day, gradual decrease of pH was observed after inoculation for 4 d. However, no pH reduction but sharp increase of pH was observed in abiotic control or in the biotic test without pyrite addition during the experiment. It indicated that the pyrite addition had a significant influence on the bacterial performance. Results also exhibited that the pH of the broth inoculated with the mixed culture of *At. ferrooxidans* and *A. cryptum* was lower than that

inoculated with the pure culture of *At. ferrooxidans* during the solubilizing processes, and decreased by 9.6% in pH at the end of the experiment. The heterotrophic acidophilic bacterium *A. cryptum* could improve the pH-reducing ability of autotrophic acidophilic bacterium *At. ferrooxidans*, since it was not able to reduce pH by itself.

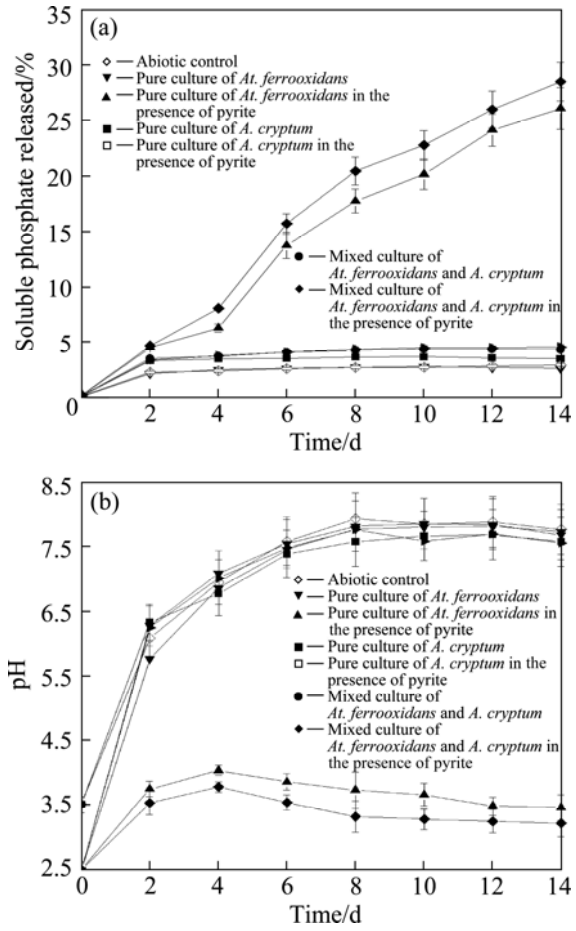

Fig. 2 Changes in percentage of soluble phosphate released (a) and pH (b) during RP solubilizing processes by *At. ferrooxidans* and *A. cryptum*

3.3 Optimization of temperature, pulp density, and mass ratio of RP to pyrite for biosolubilization of RP by consortium of *At. ferrooxidans* and *A. cryptum*

Biosolubilization of RP is a complex natural process affected by a number of factors including temperature, pulp density, pH, particle size, presence of toxic elements etc., controlling the activity of bacteria and the chemistry of the solubilizing process. Therefore, the effects of temperature, pulp density, and mass ratio of RP to pyrite on the biosolubilization of RP by the mixed culture of *At. ferrooxidans* and *A. cryptum* were investigated.

The effect of temperature on the soluble phosphate released during 14 d of RP solubilizing experiments with mixed culture of *At. ferrooxidans* and *A. cryptum* in the presence of pyrite is shown in Fig. 3. Results show that the optimal temperature for the biosolubilization of RP by the consortium of *At. ferrooxidans* and *A. cryptum* was 30 °C, and when the temperature was higher or lower than the optimal temperatures, the percentage of soluble phosphate released decreased.

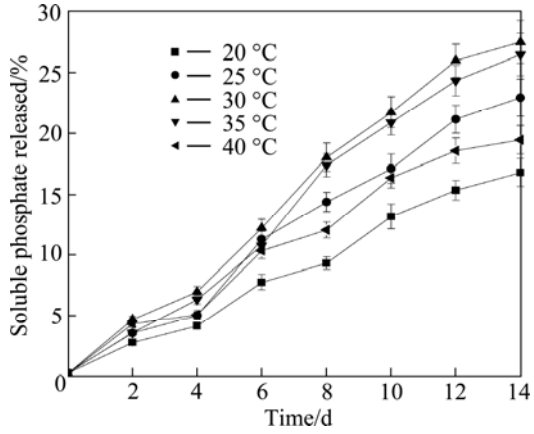

Fig. 3 Effect of temperature on soluble phosphate released during RP solubilizing processes by mixed culture of *At. ferrooxidans* and *A. cryptum* in the presence of pyrite

Figure 4 shows the greatest percentage of soluble phosphate released during 14 d of RP solubilizing experiments by mixed culture of *At. ferrooxidans* and *A. cryptum* in the presence of pyrite at a pulp density of 3% (w/v). Increasing pulp density exceeding by 3% adversely influenced the biosolubilization of RP. The adverse effect of increasing pulp density could be attributed to the inhibitory effect of increasing concentrations of ferric iron, the limited availability of nutrients and, O_2 and CO_2 with increasing pulp density and the mechanical damage to bacterial cells by solids [32−34].

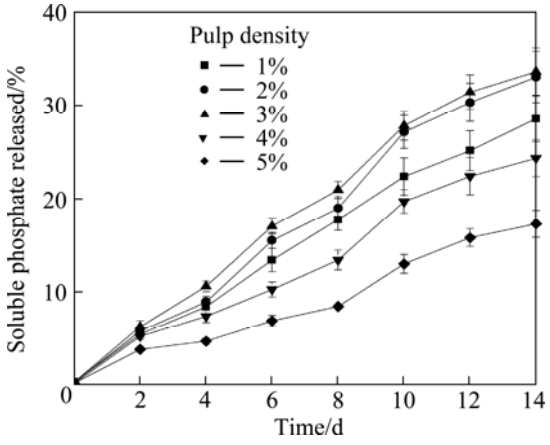

Fig. 4 Effect of pulp density on soluble phosphate released during RP solubilizing processes by mixed culture of *At. ferrooxidans* and *A. cryptum* in the presence of pyrite

High mass ratio of RP to pyrite had a negative influence on biosolubilization of RP as illustrated in Fig. 5. Percentage of soluble phosphate released was the greatest and nearly similar in extent when the mass ratio of RP to pyrite was 1:2 or 1:3. Pyrite was the energy source for the growth of *At. ferrooxidans* during the RP solubilizing process, therefore, relatively low mass ratio of RP to pyrite was more effective in the biosolubilization of RP by the consortium of *At. ferrooxidans* and *A. cryptum*.

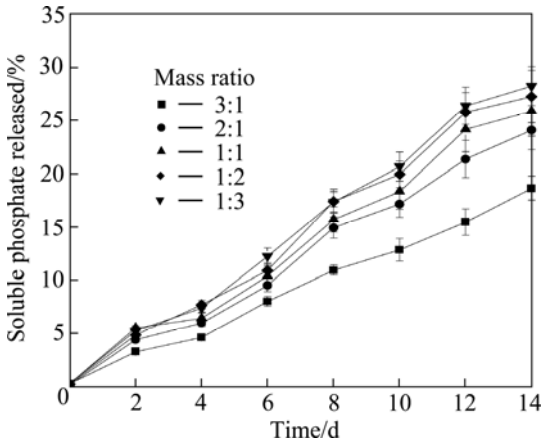

Fig. 5 Effect of mass ratio of RP to pyrite on soluble phosphate released during RP solubilizing processes by mixed culture of *At. ferrooxidans* and *A. cryptum* in the presence of pyrite

4 Conclusions

1) A heterotrophic acidophilic bacterium *A. cryptum* was found to enhance the bioleaching of pyrite by the autotrophic acidophilic bacterium *At. ferrooxidans*. A combined bioleaching experiment of pyrite (coupling *At. ferrooxidans* and *A. cryptum*) indicated that the lowering of pH and the total soluble iron released from pyrite were significantly improved. As a result the redox potential was elevated, which benefited bacterial growth.

2) The introduction of *A. cryptum* into the RP solubilizing system by *At. ferrooxidans* was manifested experimentally to improve the RP solubilizing efficiency of *At. ferrooxidans*.

3) Effects of temperature, pulp density and mass ratio of RP to pyrite on biosolubilization of RP in pyrite-containing 9K basal salts medium inoculated with the mixed culture of *At. ferrooxidans* and *A. cryptum* were investigated, and the maximum percentage of soluble phosphate released was recorded at temperature 30 °C, pulp density 3% and mass ratio of RP to pyrite 1:2 or 1:3, respectively.

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Acidiphilium cryptum 对 *Acidithiobacillus ferrooxidans* 生物溶解磷矿的作用

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摘 要：比较了嗜酸自养菌 *Acidithiobacillus ferrooxidans*、嗜酸异养菌 *Acidiphilium cryptum*、*At. ferrooxidans* 和 *A. cryptum* 的混合菌在 9K 基体盐培养基中对黄铁矿的生物浸出以及磷矿的生物溶解。结果表明，虽然 *A. cryptum* 自身不能氧化黄铁矿和溶解磷矿，但能有效促进 *At. ferrooxidans* 对黄铁矿的生物浸出以及磷矿的生物溶解。这种促进效应可通过 *A. cryptum* 促进 *At. ferrooxidans* 生物浸出黄铁矿体系中 pH 的降低以及氧化还原电位、总铁浓度和浮游细菌数目的升高的实验结果来证明。*At. ferrooxidans* 和 *A. cryptum* 的混合菌液在 30 °C 条件下溶解磷矿时可最大程度地释放其中的可溶性磷。矿浆浓度大于 3%时会给 *At. ferrooxidans* 和 *A. cryptum* 的混合菌液释放可溶性磷带来不利影响。在 9K 基体盐培养基中添加黄铁矿对 *At. ferrooxidans* 和 *A. cryptum* 的混合菌液溶解磷矿是很有必要的，且磷矿和黄铁矿的质量比为 1:2 或 1:3 时可溶性磷浸出率较高。

关键词：*Acidiphilium cryptum*；生物浸出；黄铁矿；磷矿；生物溶解； *Acidithiobacillus ferrooxidans*

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