Effect of Rotation Speed and Fulvic Acid Concentration on Biogenic Secondary High-Iron Mineral Synthesis

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Abstract: From an engineering standpoint, investigating the effects of rotation speed and fulvic acid concentration on the development of secondary high-iron minerals is crucial for treating acid mine drainage. The Fe^{2+} oxidation mechanism by Acidithiothrix (A.) ferrooxidans to synthesise secondary high-iron minerals was examined in this study using shaking flask tests under various conditions: fulvic acid concentrations of 0, 0.2, or 0.4 g/L and rotation speeds of 180 r/min or 100 r/min. The pH, Fe^{2+} oxidation rate, total iron precipitation rate, secondary high-iron mineral functional groups and ore equivalent indicators were also investigated. The results demonstrated that at a fulvic acid concentration of 0 g/L, the pH decreased from 2.5 to 2.17 at 180 r/min. At 0.2 g/L, it decreased from 2.5 to 2.05. Finally, at 0.4 g/L, it decreased from 2.5 to 2.07. Fe^{2+} was completely oxidised after 48 h, and the final total iron precipitation rate ranged from 26.2% to 33.4%. The synthesised secondary high-iron minerals were uniformly dispersed in the solution. When the rotation speed was 100 r/min, the pH reduced from 2.5 to 2.05. Finally, at 0.4 g/L, it decreased from 2.5 to 2.07. Fe^{2+} was completely oxidised after 48 h, and the final total iron precipitation rate ranged from 26.2% to 33.4%. The synthesised secondary high-iron minerals were blocky and adhered to the bottom of the shaking flask. In summary, at a rotation speed of 180 r/min or 100 r/min, the Fe^{2+} oxidation rate and total iron precipitation rate of the experimental group with a fulvic acid concentration of 0.2 g/L were higher than those of the control group and the experimental group with a fulvic acid concentration of 0.4 g/L. A fulvic acid concentration of 0.2 g/L enhanced the activity of Acidithiothrix (A.) ferrooxidans. The minerals obtained from these experiments were characterised and identified as schwertmannite and jarosite.

Keywords: fulvic acid; rotation speed; Acidithiothrix (A.) ferrooxidans; bio-oxidation; biological mineralisation

1. Introduction

In mining, metal sulphides, represented by pyrite, undergo biochemical reactions via the synergistic action of water, air and organisms, leading to acid mine drainage (AMD) [1–3]. AMD is characterised by a low pH and a large amount of iron ions, sulphate ions and heavy metals (Zn, Cu, As, Cd, etc.). The ineffective treatment of AMD will cause it to flow into the soil surface, thus generating water pollution and soil acidification. Furthermore, heavy metals in wastewater continue their enrichment, resulting in excessive heavy metal content, endangering animals and plants, and causing direct or indirect harm to human health [4–6].
The neutralisation method is often used for treating AMD, where CaCO$_3$ and Ca(OH)$_2$ are used to enhance the pH of wastewater [7,8]. However, the resulting hydroxide precipitate has poor stability and is highly affected by the solution’s pH. In addition, the pH required for Fe$^{2+}$ precipitation is much higher than that for Fe$^{3+}$ hydrolysis precipitation [9]. In this context, the neutralisation method produces the wastage of raw materials and increases treatment expenses. Unlike biological oxidation, non-biological oxidation is difficult to conduct at a pH less than 5 [10]. Acidithiobacillus (A.) ferrooxidans is an obligate chemolithotroph that can yield energy from the oxidation process of Fe$^{2+}$ and reduce sulphides for its growth metabolism, thus increasing the oxidation rate of Fe$^{2+}$ by $10^5$–$10^6$ times [11]. Fe$^{3+}$ creates secondary high-iron minerals via biomineralisation, effectively removing Fe$^{2+}$, Fe$^{3+}$ and SO$_4^{2-}$ from wastewater. Moreover, the generated secondary high-iron minerals can eliminate numerous heavy metals from the water by adsorption or co-precipitation, which is critical for environmental improvements [12].

A large amount of humus in nature contains various functional groups, such as hydroxyl, carboxyl and quinone. These functional groups can develop complex or chelate with ions in a solution or material, thereby affecting the migration of metal ions [13,14]. The fulvic acid in humus has a small molecular weight and active chemical properties [15]. It can dissolve in water under acid-base conditions and has complicated functional groups [16]. Fulvic acid has been reported to adsorb mineral particles and metal ions, affecting the surface chemical properties of minerals [17]. Liu et al. [18] demonstrated that fulvic acid can facilitate the reduction of Fe/As under microbial mediation. Using fulvic acid as an electron carrier offers an alternative pathway for the biological reduction of Fe/As without direct contact between microorganisms and AS-containing iron oxides. Functional groups represented by hydroxyl and carboxyl groups are responsible for specific chemical properties or reactions within molecules while actively promoting electron transfer and playing a crucial role in reactions. Li et al. [19] researched the kinetic process of humic acid degradation of di-n-butyl phthalate (DnBP) in soil and the impact of soil’s microbial activity. Adding humic acid could enhance the degradation of DnBP in soil and promote basic respiration. It also increased the activities of catalase and dehydrogenase in soil. In an earlier investigation, this study’s research group discovered that the biological oxidation rate was significantly increased when the concentration of fulvic acid was less than 0.4 g/L. However, the activity of A. ferrooxidans was enhanced considerably when the concentration of fulvic acid was 0.2 g/L [20]. Furthermore, the shear force of solutions is reportedly different with different rotation speeds [21]. At low rotation speeds, fulvic acid increase the oxidation rate of organisms, and how does it affect the formation of secondary high-iron ore? These questions are worth exploring. Zhang et al. [22] examined the distribution characteristics and sources of coloured dissolved organic matter (CDOM) in Hongze Lake and found that these primarily included terrigenous inputs and endogenous productions. This finding can explain the source of dissolved organic matter in natural water. AMD can enter natural water bodies and produce variations in the water’s pH, ion composition and content, microbial community composition and species richness index. Hence, this experiment initially focussed on the pollution of natural water bodies by AMD. The physical and chemical indexes of unpolluted natural water bodies were instrumental in revealing the optimal A. ferrooxidans culture conditions. This information can potentially be applied to enhance the treatment of AMD in the environment. In this study, the effects of different concentrations of fulvic acid on the pH, Fe$^{2+}$ oxidation rate, total iron (TFe) precipitation rate, morphology of secondary high-iron minerals and mineral facies of the biosynthesis system were investigated by simulating the disturbance of water flow with varying rotation speeds in a constant temperature incubator. This experiment would mimic the biochemical behaviour of similar organic matter in water; thus, findings may provide a theoretical foundation for treating AMD.
2. Materials and Methods

2.1. Test Materials

Analytically pure (98%) fulvic acid was purchased from Hefei BASF Biotechnology Co., Ltd. (Hefei, China). *A. ferrooxidans* (ATCC23270) was obtained from Beijing Baocang Biotechnological Company Limited (Beijing, China). The isolation base of this strain was AMD. A 20× concentration of 9K medium was prepared as follows: 2.0 g of KCl, 60 g of (NH₄)₂SO₄, 0.2 g of Ca(NO₃)₂·4H₂O, 10 g of K₂HPO₄ and 10 g of MgSO₄·7H₂O were dissolved in 1 L of deionised water. H₂SO₄ was used to adjust the pH to 2.5, as needed.

*A. ferrooxidans* resting cells were prepared as follows: *A. ferrooxidans* was inoculated (20%, volume ratio) in a 9K culture medium. In all, 12.5 mL of a 20× concentration of 9K medium was added to a conical bottle measuring 500 mL. A total of 11.12 g of FeSO₄·7H₂O was added to each conical bottle, and the mixture’s volume was adjusted to 250 mL by adding deionised water. At this time, the concentration of Fe²⁺ was 160 mmol/L (8960 mg/L). The initial pH was set at 2.5, and the total volume of the reaction system was 250 mL. The inoculated medium was cultured on a shaker at a constant temperature (28 °C) and speed (180 r/min). Culturing was stopped at the late exponential growth stage (around two to three days). Subsequently, the culture solution was filtered to remove sediments, and the filtrate was centrifuged at 10,000× g (4 °C, 10 min) of centrifugal force to collect the bacteria. The collected bacteria were washed thrice with the acidic solution at a pH of 1.5 (H₂SO₄ preparation) to remove ion impurities. The bacterial solution derived from the 250-mL culture system was resuspended in 5 mL of an acidic solution (pH: 2.5) generated by adding H₂SO₄. At this point, the bacterial solution is concentrated to 50 times the original. The resulting solution was a concentrated *A. ferrooxidans* solution. The concentration of the resulting bacterial solution was approximately 4×10¹¹ cells/mL.

2.2. Experimental Setup

Fulvic acid was prepared as follows: 2.5 g of fulvic acid was weighed and added into a 250-mL volumetric bottle to create a 10 mg/mL solution. Two rotation speeds were set: 180 r/min and 100 r/min. In a series of 500-mL conical bottles, 12.5 mL of a 20× concentration 9K media was added to each conical bottle measuring 500 mL. In all, 11.12 g of FeSO₄·7H₂O was added to each conical bottle, and the mixture’s volume was adjusted to 250 mL by adding deionised water. Here, the concentration of Fe²⁺ was 160 mmol/L (8960 mg/L). Subsequently, fulvic acid solutions with different concentrations (0, 0.2 and 0.4 g/L) were prepared and recorded as “FA-0 g/L”, “FA-0.2 g/L” and “FA-0.4 g/L”, respectively. The FA-0 g/L solution did not contain fulvic acid and is defined as a “Control” in all plots. Song et al. [23] demonstrated that *A. ferrooxidans* oxidises Fe²⁺ at the fastest rate at a rotation speed of 180 r/min and produces the highest mineral quality when the culture system’s pH is 2.0–3.0. Among these, the activity of *A. ferrooxidans* at a pH of 2.5 was better than that at a pH of 3.0. Therefore, the pH in this experiment was adjusted to 2.5 (±0.02) with 1:1 H₂SO₄ (v/v), and each treatment was inoculated with 1 mL of the concentrated bacterial solution. The final volume of solutions was 250 mL, and triplicates were used for each group. The conical flasks were placed on a shaker at a constant temperature (28 °C) and a rotation speed of 180 r/min or 100 r/min. Samples were taken at regular intervals to determine the pH along with the Fe²⁺ and Fe³⁺ concentrations of the solution. The sampling times were as follows: 12, 24, 36, 48, 60 and 72 h. The sediments in each system were filtered with filter paper after the experiment. These sediments were rinsed with sulphuric acid (pH: 1.5) to remove soluble ions and rinsed with deionised water to eliminate acids remaining on the surface of solids from the previous step. The cleaned sediments were dried at 60 °C for 24 h and weighed. The resulting dried mineral was crushed before passing through a nylon screen with a mesh size of 200 and then set aside.

2.3. Measurement Method

The pH of solutions was measured using a pH meter (model PHS-3C), and the Fe²⁺/total Fe contents were measured using a UV visible spectrophotometer and the o-
phenanthroline colorimetric method [24]. The Fe$^{2+}$ oxidation rate was calculated as follows:

$$Fe^{2+} (%) = \left( \frac{Fe_{\text{initial}}^{2+} - Fe_{t}^{2+}}{Fe_{\text{initial}}^{2+}} \right) \times 100,$$

where $Fe_{\text{initial}}^{2+}$ denotes the initial Fe$^{2+}$ concentration, and $Fe_{t}^{2+}$ is the Fe$^{2+}$ concentration at $t$ (hours) of reaction time.

$$TFe(\%) = \left( \frac{TFe_{\text{initial}} - TFe_{t}}{TFe_{\text{initial}}} \right) \times 100,$$

where $TFe_{\text{initial}}$ represents the initial iron concentration, and $TFe_{t}$ is the iron concentration at $t$ (hours) of reaction time.

The sediment mineral phase was determined using an X-ray diffractometer (X’Pert 3 Powder). The test conditions were as follows: tube current = 150 mA, tube voltage = 50 kV, step length = 0.02°, scanning interval = 10–80° (2θ), scanning rate = 5°/min, and Cu target (curved crystal monochromator). The internal groups of minerals were determined via Fourier transform infrared (FTIR) spectroscopy (Frontier FTIR). The mixture ratio of the sample and KBr was 1–2%, and the tablet was pressed and placed into the FTIR Spectroscopy for testing. The experimental data were analysed using the Origin mapping software.

3. Result and Analysis

3.1. Effect of Fulvic Acid on pH with Different Rotation Speeds

*A. ferrooxidans* catalysed the oxidation of Fe$^{2+}$ to synthesise secondary high-iron minerals. The pH value first increased and then decreased. This trend could be roughly divided into two stages. In the first stage, Fe$^{2+}$ was oxidised to Fe$^{3+}$ and H$^{+}$ was consumed. In the second stage, schwertmannite or jarosite was developed by Fe$^{3+}$ biomineralisation developed.

The reaction process is explained as follows [25]:

1. Oxidation of Fe$^{2+}$ to Fe$^{3+}$ involves the consumption of H$^{+}$.

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

2. Hydrolysis of Fe$^{3+}$ to schwertmannite or jarosite and release of H$^{+}$.

$$8Fe^{3+} + SO_4^{2-} + 14H_2O \rightarrow Fe_8O_8(OH)_{6}SO_4(\text{schwertmannite}) + 22H^+ \quad (2)$$

$$M + 3Fe^{3+} + 2SO_4^{2-} + 6H_2O \rightarrow MFe_3(SO_4)_2(OH)_{6} (\text{jarosites}) + 6H^+ + M = (NH_4^+, K^+, H_3O^+) \quad (3)$$

The above two processes had no obvious macro order, and changes in pH were a comprehensive reflection of these two processes.

Figure 1 depicts the pH changes in each secondary high-iron mineral synthesis system at different rotation speeds. The rotation speed significantly affected the pH changes during Fe$^{2+}$ oxidation and Fe$^{3+}$ hydrolysis. When the rotation speed was 180 r/min, the pH of the control, FA-0.2 g/L, and FA-0.4 g/L groups initially increased from 2.5 at 0 h to 2.67, 2.68 and 2.59, respectively, at 12 h, and then decreased to 2.31, 2.16 and 2.19, respectively, at 48 h. At the end of the experiment, the pH decreased to 2.18, 2.05 and 2.07, respectively. When the rotation speed was 100 r/min, the pH of the blank and FA-0.2 g/L groups increased from 2.5 at 0 h to 2.68 and 2.70 at 12 h, respectively. In contrast, the pH of the FA-0.4 g/L group peaked to 2.67 at 24 h. At the end of the experiment, the pH of the systems decreased to 2.25, 2.14 and 2.19, respectively. Overall, after the experiment, the higher the rotation speed, the lower the pH under the same conditions. The FA-0.2 g/L system exhibited the fastest pH decline at different rotation speeds.
was higher than that of the control and the FA-0.4 g/L system. The results demonstrated
the oxidation rate of Fe\(^{2+}\) can be enhanced when the concentration of fulvic acid was
increased. For instance, in the FA-0.2 g/L system, when the rotation speed was 180 r/min, Figure 3a reveals that Fe\(^{2+}\) was completely oxidised after 48 h of reaction, the FA-0.2 g/L system had the highest oxidation rate. For instance, at 24 h, the Fe\(^{2+}\) oxidation rate of the FA-0.2 g/L system reached 54.7%, whereas the oxidation rates in the control and FA-0.4 g/L systems were 25.2% and 27.8%, respectively. At 36 h, the Fe\(^{2+}\) oxidation rate of the FA-0.2 g/L system reached 95.1%. In contrast, the oxidation rates in the control and FA-0.4 g/L systems were 45.8% and 78.5%, respectively. When the rotation speed was 100 r/min, Fe\(^{2+}\) was completely oxidised in the control, FA-0.2 g/L, and FA-0.4 g/L systems at 72 h, 60 h and 72 h, respectively. The oxidation rate of Fe\(^{2+}\) was significantly increased after 12 h. Moreover, the oxidation rate of the FA-0.2 g/L system was higher than that of the control and the FA-0.4 g/L system. The results demonstrated that the oxidation rate of Fe\(^{2+}\) can be enhanced when the concentration of fulvic acid was 0.2 g/L. Under the same concentration of fulvic acid, the oxidation rate of Fe\(^{2+}\) at 100 r/min was lower than that at 180 r/min. A low rotation speed was not conducive to the oxidation of Fe\(^{2+}\). It is worth noting that even at a low rotation speed, a fulvic acid concentration of 0.2 g/L was still conducive to the rapid oxidation of Fe\(^{2+}\) by A. ferrooxidans.

Figure 1. Changes in the pH of biosynthetic secondary high-iron mineral systems.

3.2. Effect of Fulvic Acid on the Oxidation Rate of Fe\(^{2+}\) at Different Rotation Speeds

The formation of secondary high-iron minerals largely depends on the effective oxidation of Fe\(^{2+}\). Changes in the Fe\(^{2+}\) oxidation rates under different rotation speeds for the control. The FA-0.2 g/L, and FA-0.4 g/L biocatalytic secondary high-iron mineral synthesis systems are shown in Figure 2. When the rotation speed was 180 r/min, the Fe\(^{2+}\) in the control, FA-0.2 g/L, and FA-0.4 g/L systems were completely oxidised within 48 h. In the first 12 h of the reactions, the Fe\(^{2+}\) oxidation rate of the FA-0.2 g/L system increased significantly and reached 13.6%. It was higher than that of FA-0.4 g/L and the control. At 12, 24 and 36 h of reaction, the FA-0.2 g/L system had the highest oxidation rate. For instance, at 24 h, the Fe\(^{2+}\) oxidation rate of the FA-0.2 g/L system reached 54.7%, whereas the oxidation rates in the control and FA-0.4 g/L systems were 25.2% and 27.8%, respectively. At 36 h, the Fe\(^{2+}\) oxidation rate of the FA-0.2 g/L system reached 95.1%. In contrast, the oxidation rates in the control and FA-0.4 g/L systems were 45.8% and 78.5%, respectively. When the rotation speed was 100 r/min, Fe\(^{2+}\) was completely oxidised in the control, FA-0.2 g/L, and FA-0.4 g/L systems at 72 h, 60 h and 72 h, respectively. The oxidation rate of Fe\(^{2+}\) was significantly increased after 12 h. Moreover, the oxidation rate of the FA-0.2 g/L system was higher than that of the control and the FA-0.4 g/L system. The results demonstrated that the oxidation rate of Fe\(^{2+}\) can be enhanced when the concentration of fulvic acid was 0.2 g/L. Under the same concentration of fulvic acid, the oxidation rate of Fe\(^{2+}\) at 100 r/min was lower than that at 180 r/min. A low rotation speed was not conducive to the oxidation of Fe\(^{2+}\). It is worth noting that even at a low rotation speed, a fulvic acid concentration of 0.2 g/L was still conducive to the rapid oxidation of Fe\(^{2+}\) by A. ferrooxidans.

Figure 2. Changes in the Fe\(^{2+}\) oxidation rate of biosynthetic secondary high-iron mineral synthesis systems.
3.3. The Effect of Fulvic Acid on the TFe Precipitation Rate at Different Rotation Speeds

Fe$^{3+}$ hydrolytic mineralisation leads to the development of secondary hydroxy-sulphuric acid high-iron minerals in soluble iron and SO$_4^{2-}$ in the culture system, which can remove soluble iron and SO$_4^{2-}$ from liquid phases. Among these, the TFe precipitation rate, which reflects how much soluble iron is eliminated, is an important index. The higher the TFe precipitation rate, the more iron is removed from the liquid phase, and the better the effect of Fe$^{3+}$ and SO$_4^{2-}$ removal in the culture system. Figure 3 illustrates the variations in the TFe precipitation rate in the control, FA-0.2 g/L and FA-0.4 g/L secondary high-iron mineral synthesis systems at 180 r/min and 100 r/min. For both the 180 r/min and 100 r/min conditions, the TFe precipitation rate in the first 12 h was less than 5%. It was significantly improved during the subsequent culture process. After incubation at 180 r/min for 48 h, Fe$^{2+}$ was completely oxidised in the FA-0.2 g/L and FA-0.4 g/L systems. Figure 3a shows that the TFe precipitation rate was similar at this time. At the end of the experiment, the TFe precipitation rate of the former system was 32.9%, and the latter system was 34.3%. The TFe precipitation rate of the control was significantly lower than that of the fulvic acid systems. The TFe precipitation rates of the FA-0.2 g/L and FA-0.4 g/L systems were 29.6% and 26.9%, respectively, after 72 h of incubation at 100 r/min. The TFe precipitation rate of the former was slightly higher than the latter’s. It should be noted that the TFe precipitation rates of the FA-0.2 g/L and FA-0.4 g/L systems were significantly different at the different rotation speeds. For instance, in the FA-0.2 g/L system, when the rotation speed was 180 r/min, Figure 3a reveals that Fe$^{2+}$ was completely oxidised after 48 h of culture. According to Figure 3, the TFe precipitation rate was 21.0% at this time and 32.9% at the end of the experiment. When the rotation speed was 100 r/min, the TFe precipitation rate was 29.6% when the Fe$^{2+}$ was completely oxidised (at the end of the experiment). The precipitation rate of TFe in the system with a rotation speed of 180 r/min was higher than that in the system with a rotation speed of 100 r/min. However, the precipitation rate of TFe in the system with a rotation speed of 180 r/min was lower than that at the end of the experiment. The hydrolysis of Fe$^{3+}$ also requires sufficient time to produce a greater amount of secondary high-iron minerals.

![Figure 3](image-url)

(a) 180 r/min  
(b) 100 r/min

Figure 3. Changes in the TFe precipitation rate of biosynthetic secondary high-iron mineral synthesis systems.

3.4. The Effect of Fulvic Acid on the Morphology of Secondary High-Iron Minerals at Different Rotation Speeds

Figure 4 shows the morphology of secondary high-iron minerals in the control, FA-0.2 g/L and FA-0.4 g/L systems at the end of the experiment. When the rotation speed was 180 r/min, the secondary high-iron minerals were evenly dispersed at the...
bottom of the bottle. The secondary high-iron minerals collected in the control, FA-0.2 g/L, and FA-0.4 g/L systems in the form of a yellow-orange powder. At the end of the experiment, with a rotation speed of 100 r/min, the Fe$^{2+}$ in the system was completely oxidised. Importantly, the secondary high-iron yellow-orange coloured minerals adhered to the bottom of the bottle. The collected minerals were large particles with only a small amount of powder. When the rotation speed was low, the water’s shear face was insufficient, and the secondary high-iron minerals sunk to the bottom of the bottle under the influence of gravity, adhering to the bottle’s wall.

Figure 4. Minerals in the final state of the reaction within the biosynthetic secondary high-iron mineral synthesis systems. ((a). FA-0 g/L. (b). FA-0.2 g/L. (c). FA-0.4 g/L).

3.5. The Effect of Fulvic Acid on the Mineral Phase of Secondary High-Iron Minerals at Different Rotation Speeds

X-ray diffraction is an effective means of identifying crystalline and amorphous minerals. Figure 5 illustrates the X-ray diffraction patterns of secondary high-iron minerals obtained in the current experiment. The primary strong diffraction peaks in the X-ray diffraction patterns of the secondary high-iron minerals in each culture system were similar. The main diffraction peaks were high when the rotation speed was low, indicating high mineral crystallinity. Schwertmannite is an amorphous material [26]. Based on the standard spectrum, there were eight standard broad peaks; the main broad peak was 36.15°. In the XRD pattern, the wide peak was not significant, and the jarosite had a sharp diffraction peak at this position. It is challenging to infer the existence of schwertmannite from Figure 5. However, it has been reported that pure schwertmannite is reddish-brown, whereas jarosite is yellow [27,28]. Based on Figure 4, the colour of the minerals in each system was yellowish-orange. Thus, it can be inferred that schwertmannite and jarosite were present in these systems.
Figure 5. XRD spectra of secondary high-iron minerals.

3.6. The Effect of Fulvic Acid on the Functional Groups of Secondary High-Iron Minerals at Different Rotation Speeds
3.6. The Effect of Fulvic Acid on the Functional Groups of Secondary High-Iron Minerals at Different Rotation Speeds

Figure 6 shows the FTIR spectra of the secondary high-iron minerals obtained from the experiment. The absorption peak at 3393 cm\(^{-1}\) corresponds to the stretching vibration peak of -OH, the absorption peak at 1635 cm\(^{-1}\) corresponds to the deformation vibration peak of H-O-H, and the absorption peak at 1432 cm\(^{-1}\) corresponds to the vibration peak of N-H. This result indicates the presence of ammonioiarosite. In total, 1191 cm\(^{-1}\) and 1087 cm\(^{-1}\) are the \(v_3\) stretching vibration absorption peaks of the SO\(_4^{2-}\) group, and 1004 cm\(^{-1}\) is the deformation vibration peak of the OH group. 630 cm\(^{-1}\) is the \(v_4\) stretching vibration absorption peak of the SO\(_4^{2-}\) group, and 510 cm\(^{-1}\) and 474 cm\(^{-1}\) are the vibration peaks of the FeO\(_6\) octahedron [29,30]. It is worth noting that Na\(^+\), NH\(_4^+\), and other monovalent cations can replace K\(^+\) in jarosite. It has been reported that K\(^+\) has 40 times the sulphate-forming capacity of NH\(_4^+\) [31]. This present study shows that jarosite was also present in the precipitate. A small amount of carphosiderite may also be present because H\(_3\)O\(^+\) can replace the cations in jarosite. The FTIR spectra for each experiment were similar. Combined with the XRD analysis and the appearance of the precipitate, it can be speculated that the precipitate was a mixture of jarosite, ammonioiarosite, carphosiderite and schwertmannite.

![FTIR spectra](image)

**Figure 6.** FTIR spectra of the secondary high-iron minerals.

4. Discussion

4.1. Effects of Rotation Speed and Fulvic Acid Concentration on Fe\(^{2+}\) Oxidation by A. ferrooxidans

At different rotation speeds, 0.2–0.4 g/L of fulvic acid promoted the oxidation of Fe\(^{2+}\) in the experimental system. This outcome is attributed to quinone, carboxyl, hydroxyl and other active groups in fulvic acid. Fulvic acid can be used as an electron shuttle, which is conducive to electron transfer [32]. The above groups can affect the regulation of extracellular electron transfer in microorganisms and the electron transfer capacity (ETC) of fulvic acid. In a sense, the ETC of fulvic acid depends on the characteristics and abundance of these groups [18]. In addition, the carboxyl group in fulvic acid can form complex or chelate with the cations in the solution. Due to the rapid oxidation rate of Fe\(^{2+}\), existing data do not reflect the phenomenon of complexation between Fe\(^{2+}\) and fulvic acid. The oxidation rate in the system with a fulvic acid concentration of 0.4 g/L was lower at 24 h and 36 h than that of the control and the fulvic acid concentration system of 0.2 g/L at 100 r/min. Fulvic acid has an inhibitory effect on bacterial activity. Song et al. [33] reported that a certain concentration of low molecular weight organic acids (such as formic acid, acetic acid, and propionic acid) exerted an inhibitory effect on the activity of A. ferrooxidans. The above studies show that the carboxyl and phenolic hydroxyl groups in fulvic acid also demonstrate inhibitory effects on the activity of A. ferrooxidans. When the
rotation speed was 180 r/min, this phenomenon was not significant. It can be speculated that the high rotation speed increased the dissolved oxygen and CO$_2$ concentrations in the solution, effectively alleviating the toxic effect of fulvic acid on $A. \text{ferrooxidans}$. The oxidation rate of Fe$^{2+}$ was also low when the rotation speed was low. This can be explained as follows: (1) The dissolved CO$_2$ in water is the carbon source of $A. \text{ferrooxidans}$, and O$_2$ is an electron acceptor. When the rotation speed is low, the dissolved oxygen and CO$_2$ concentration in water are reduced. (2) A certain amount of $A. \text{ferrooxidans}$ was adsorbed or encapsulated during the precipitation of secondary high-iron minerals [34]. Dong et al. [35] used dissolved schwertmannite and undissolved schwertmannite to deoxidise Fe$^{2+}$, and the Fe$^{2+}$ oxidation rate of the former system was significantly improved. In the case of an insufficient shear force of water, secondary high-iron minerals adhered to the bottle’s bottom, and the free $A. \text{ferrooxidans}$ in the solution decreased, leading to a decrease in the Fe$^{2+}$ oxidation rate. When the rotation speed was high, the secondary high-iron minerals were uniformly dispersed in the solution, which helped improve the Fe$^{2+}$ oxidation rate. Secondary high-iron minerals attached to the bottle’s bottom affect the pH, Fe$^{2+}$ oxidation and TFe precipitation of the solution to a certain extent.

4.2. The Effects of Rotation Speed and Fulvic Acid Concentration on the Formation of Secondary High-Iron Minerals

Zhang et al. [36] explored the effects of multiple factors, including rotation speed, on the formation of secondary minerals in $\text{Leptospirillum ferrodiazotrophum}$. At low speeds, the homogeneity of the culture medium was lower, oxygen levels were lower, and the conditions could not meet the bacteria’s rapid growth and metabolism needs. Increasing the speed of the culture media made the material mixing uniform and increased the oxygen content, which enhanced the growth and metabolism speed of the bacteria. However, excessively high speeds would intensify the collision and friction between the bacterial cells and the material, destroying the bacterial cell precursors and affecting the bacteria’s activity and the mineral formation rate. These results can elucidate the influence of rotation speed on generating secondary minerals by $A. \text{ferrooxidans}$. The higher the precipitation rate of TFe, the more secondary iron minerals are obtained and the stronger the removal ability of Fe and SO$_4^{2-}$ in wastewater. The results demonstrated that when Fe$^{2+}$ was not completely oxidised, the higher Fe$^{2+}$ oxidation rate in the system at the same rotation speed resulted in a higher precipitation rate. The increase in the TFe precipitation rate during this period is primarily attributed to the rise in Fe$^{3+}$ supply. After the complete oxidation of Fe$^{2+}$ at 180 r/min, the TFe precipitation rate of the FA-0.4 g/L system was higher than that of the FA-0.2 g/L system after some time, indicating that fulvic acid had a promoting effect on the TFe precipitation rate. When the rotation speed was low, secondary high-iron minerals adhered to the bottle’s bottom due to insufficient shear force. Secondary high-iron minerals can be used as ‘seeds’ to facilitate the formation of secondary high-iron minerals [37]. In Figure 3, the low precipitation rate of TFe at 100 r/min was attributed to reduced Fe$^{2+}$ supply. In the early stage of the experiment, the lack of seed stimulation led to a reduction in the mineralisation rate. According to the morphology of secondary high-iron minerals at the end of the experiment and the XRD and FTIR analyses, the obtained secondary high-iron minerals are a mixture of jarosite and schwertmannite. It is noteworthy that when the rotation speed was low, the diffraction peak intensity of the secondary high-iron minerals was slightly higher, indicating that the crystallinity of the secondary high-iron minerals was improved. Studies have demonstrated that in secondary high-iron mineral synthesis systems, the metastable precursor phase is first formed and finally transformed into a stable crystalline phase [38]. Liu et al. [39] obtained schwertmannite after 12 h, whereas jarosite appeared after 48 h. It can be inferred that when the rotation speed is high, the secondary high-iron mineral synthesis system provides a large amount of Fe$^{3+}$ and preferentially generates schwertmannite. At the end of the experiment, several schwertmannite minerals have not yet changed to jarosite, and the proportion of schwertmannite minerals increases; hence, the measured mineral diffraction peak intensity is relatively weak.
5. Conclusions

AMD has become the focus of environmental governance due to its high acidity and high content of heavy metal ions. Novel biological treatment methods can reduce the environmental load and cause economic benefits. Microbial oxidation-chemical neutralisation processes use *A. ferrooxidans* to oxidise Fe²⁺ in the water to Fe³⁺ before the chemical neutralisation process. This action causes Fe³⁺ to precipitate at a lower pH and eliminates the part of the soluble iron via microbial mineralisation. Therefore, adding the microbial treatment process to the front end of the conventional neutralisation process can considerably reduce the amount of neutralisation reagents, the load and the amount of sediment produced. This study investigated the effects of the fulvic acid concentration and rotation speed on the oxidation of Fe²⁺ by *A. ferrooxidans* to produce secondary minerals by shaking flask experiments to simulate water disturbances under natural conditions. The findings offer a theoretical foundation for practical engineering applications. The primary findings of this paper are as follows:

(1) When the concentration of fulvic acid was 0.2 g/L, the biological activity of *A. ferrooxidans* was enhanced at different rotation speeds, which enhanced the oxidation of Fe²⁺ by *A. ferrooxidans*, increased the precipitation rate of TFe, and eliminated more harmful substances in the water.

(2) When the rotation speed of the secondary iron mineral synthesis system was low, *A. ferrooxidans* was wrapped by secondary iron minerals and adhered to the bottom of the flask; this was not conducive to the biological oxidation of *A. ferrooxidans*, reducing the TFe precipitation rate and the efficiency of pollutant removal.

(3) When the rotation speed of the secondary high-iron mineral synthesis system was low, the primary diffraction peak of the XRD pattern of the secondary high-iron minerals was enhanced, and the mineral crystallinity was high. However, the obtained secondary high-iron minerals were still a mixture of jarosite and schwertmannite.

(4) Appropriately increasing the concentration of fulvic acid in the culture system and strengthening the hydraulic disturbance help remove soluble iron from the solution. The more secondary high-iron minerals are produced, the more soluble iron is removed and the higher the TFe precipitation rate is. As a result, more pollutants are removed.

6. Further Outlook

Start with the chemical formula of secondary high-iron minerals. Secondary high-iron minerals contain a large amount of Fe³⁺ and SO₄²⁻, and Fe³⁺ and SO₄²⁻ are ions that enter the water through metal sulphide ore. *A. ferrooxidans* mediate the generation of secondary high-iron minerals, which is also a process in which Fe³⁺ and SO₄²⁻ in AMD are largely removed. The resulting secondary minerals can be separated from water bodies and have economic value. Compared to the chemical methods, *A. ferrooxidans* treatment of AMD is a method with lower cost, less environmental pressure, and higher economic benefit than chemical method.

Second, talk about the addition of fulvic acid. The fulvic acid used in this experiment is a representative component of humus in nature. Adding fulvic acid to the culture medium on the one hand is to simulate the composition and acidity of AMD, on the other hand is to simulate the humus formed by natural organic matter through biochemistry and other effects into the water through a variety of channels—there is a certain amount of humus in natural water. It is a real problem in industrial production that the natural water body is mixed with pollutants from mines under the premise of containing humus. The experimental results and corresponding experimental conditions are compared to the actual situation of industrial production, it can not only provide reference for the water quality before and after the treatment of AMD, but also provide new thinking for many treatment methods.

Third, the technology of AMD treatment in China is maturing and there is still a lot of room for progress. Chemical neutralisation method has been widely used in our country. If a biological treatment process is added in the front section of chemical neutralisation
process, the microorganisms represented by *A. ferrooxidans* will be used in the process. Fe
and SO$_4^{2-}$ in AMD could be removed by biologically mediated formation of secondary
hydroxy sulphuric acid high-iron minerals, which could reduce the subsequent processing
load and enhance the treatment effect.

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