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Simplification of the *Acidithiobacillus ferrooxidans* Culture Process for Expanding the Field of Biomachining

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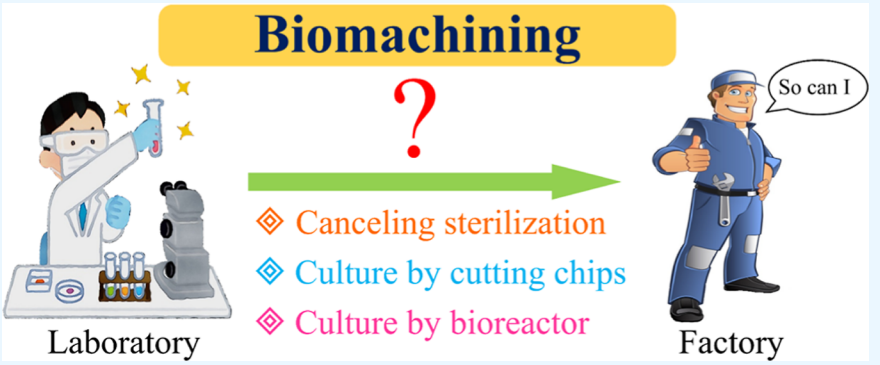

ABSTRACT: Biomachining is an eco-friendly metal processing method with broad application potential. Nevertheless, the bacterial culture methods that are currently involved in biomachining require the intensive use of chemical reagents, especially $FeSO_4$, specialized equipment, and professional-level skills in the field of biology. Herein, the differences between two cultures with and without sterilization were evaluated. *Acidithiobacillus ferrooxidans* was cultured with iron instead of $FeSO_4$ in the culture medium. The chemical and biochemical parameters of the culture were analyzed by studying the area of exposed iron and continuously regulating the pH. Eliminating the sterilization and sterile inoculation of the medium is feasible for culturing *A. ferrooxidans*. The key to achieving a high bacterial density in culture with iron was to maintain the solution pH. The possibility of mass culturing *A. ferrooxidans* with steel cuttings was evaluated in a custom bioreactor, and the bacterial concentration reached 9×10^7 cells/mL.

1. INTRODUCTION

Biomachining is a method of processing metals by using microorganisms capable of continuous catalytic oxidation.[1] This technique is very similar to chemical processing, with the capacity to process complex surfaces without damaging the machined surface layer. Biomachining is considered more environmentally friendly and stable than chemical machining.[2] Due to these advantages, biomachining is a very promising processing method. With the efforts of scholars in various fields, biomachining has developed rapidly in recent years.

The bacterium most widely used in biomachining is *Acidithiobacillus ferrooxidans* (hereafter referred to as *A. ferrooxidans*).[3] *A. ferrooxidans* is an extreme bacterium that is rod-shaped, 1−2 μm in length, and approximately 0.5 μm in thickness. This bacterium requires specific living environment conditions. The metabolic activity of bacteria is the main energy source for biomachining; thus, the growth and reproduction of bacteria greatly influence the processing performance.[4] In one study,[5] an orthogonal experiment was conducted, and increasing the *A. ferrooxidans* concentration improved both the material removal rate (MRR) and stability of the instantaneous MRR. Therefore, a reliable and efficient bacterial culture method is considered a fundamental requirement of biomachining.

Many researchers have studied different culture methods for promoting the stable and efficient growth of *A. ferrooxidans*. To increase the concentration of bacteria, various adsorptive materials, such as bacterial cellulose,[6] cotton gauze,[7] jarosite,[8] and foam,[9] have been introduced to immobilize bacteria. Bioreactors have been used to increase the amount of bacterial culture and improve automatic management.[2,10] *A. ferrooxidans* was mixed with *Acidithiobacillus thiooxidans* to synergistically increase oxidation susceptibility and durability in different strains.[11,12] *A. ferrooxidans* has been shown to have a high survival rate when preserved under specific parameters.[13,14]

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Specifically, the pH plays an important role in the active locomotion of *A. ferrooxidans*.[15]

Nevertheless, the complexity of the entire culture process, including culture media preparation, sterilization, aseptic inoculation, and constant temperature incubator operation, has not been significantly reduced. *A. ferrooxidans* is generally cultured in a liquid medium composed of aqueous solutions of several inorganic salts. These salts must include a large amount of $FeSO_4$ to provide energy for *A. ferrooxidans*; in addition, the compositions of different media all differ slightly.[16] $FeSO_4$ cannot be sterilized by autoclaving due to its instability at high temperatures, which further increases the complexity of sterilization. According to previous reports,[17−21] manufacturing is the most widely used application of biomachining. However, most of the manufacturers do not have an understanding of *A. ferrooxidans* or possess relevant professional operation skills. Therefore, the lack of skills necessary for *A. ferrooxidans* culture is an important factor hindering the development of biomachining.

Furthermore, pollution reduction and treatment are important subjects in academia. Zhou et al.[22] generated dual-active materials to treat micropollutants in complex water. Zheng et al.[23] designed and synthesized a novel 2D nanocomposite to adsorb heavy metals. This study revealed that a large amount of metal cuttings is produced daily in machining workshops, which is usually treated as waste. Most of these metal cuttings involve iron alloys with high iron contents. This iron can be oxidized into Fe^{2+} and Fe^{3+} by *A. ferrooxidans* to generate energy. Therefore, these metal cuttings can theoretically be used to replace $FeSO_4$ in media, which is conducive to the utilization of waste resources.

In this paper, attempts were made to simplify the culture of *A. ferrooxidans* and replace $FeSO_4$ in the culture media with steel cuttings to improve the cleanliness of the process. The feasibility of cancellation via sterilization and sterile inoculation was verified. Pure iron blocks with different areas of exposure were designed to culture bacteria. The differences in the cell density, ion concentration, and weight loss characteristics of iron blocks in culture with pure iron at the same pH were recorded. The reasons for these differences were discussed from the perspectives of thermodynamics and chemical reactions. Based on these results, the possibility of culturing *A. ferrooxidans* in a custom-prepared bioreactor with steel cuttings was demonstrated.

2. MATERIALS AND METHODS

2.1. *A. ferrooxidans* Strain and Media. A pure *A. ferrooxidans* strain, which was originally withdrawn and isolated from acidic pit water in an iron mine in China, was used in this study. A scanning electron microscopy (SEM) image of *A. ferrooxidans* is shown in Figure 1. Before observation, the cells were fixed with glutaraldehyde and dried.

The strain was continuously cultured for three generations to ensure stable growth before the formal test was performed. The culture medium used in this experiment was liquid 9K medium supplemented with several mineral salts. The compositions and concentrations of these compounds are shown in Table 1. The initial pH was adjusted to 1.8 with H_2SO_4.

2.2. Sterilization, Inoculation, and Culture Conditions. The medium was divided into two parts for sterilization. The trace elements were sterilized by autoclaving at 121 °C for 15 min. One component of the energy source—$FeSO_4$ aqueous solution—was disinfected with 0.22 μm filter paper and mixed during inoculation. The inoculation process was carried out in a sterile environment. The volume ratio of inoculum to fresh medium was 1:4. The inoculated medium was first cultured in 100 mL batches in a 250 mL Erlenmeyer flask and then placed in a thermostatic incubator shaker at a temperature of 30 °C and speed of 160 rpm. During the culture process, the pH of the solution was not adjusted. When the amount of Fe^{2+} in solution decreased, the current generation of *A. ferrooxidans* increased. The culture fluid was used as the next generation of inoculum.

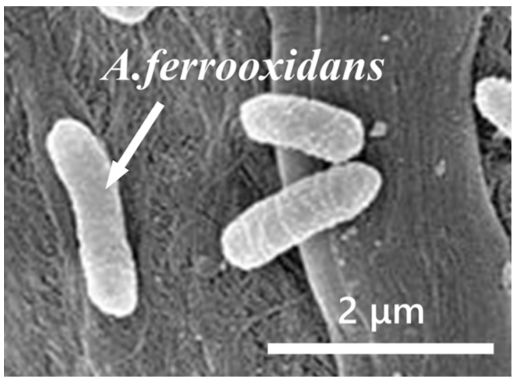

Figure 1. SEM image of *A. ferrooxidans*.

Table 1. Composition of 9K Medium

group	name	qty. (g/L)
trace elements	$(NH_4)_2SO_4$	3
	$MgSO_4{\cdot}7H_2O$	0.5
	KCl	0.1
	K_2HPO_4	0.5
	$Ca(NO_3)_2{\cdot}4H_2O$	0.01
energy source	$FeSO_4{\cdot}7H_2O$	24.83

2.3. Culturing *A. ferrooxidans* without Sterilization. The stimulation of the medium in the experimental group was terminated, and aseptic procedures were performed. The chemical reagents were quantitatively dissolved in distilled water to create the medium. The medium preparation and inoculation processes were carried out in an ordinary laboratory, which was not cleaned or disinfected. Compared with conventional sterilization and sterile inoculation processes, the differences in *A. ferrooxidans* growth resulting from the two culture processes were studied. The cell density, pH, and Fe^{3+} concentration of the cultures were measured as the average values of three parallel groups.

2.4. Iron in Place of $FeSO_4$ in 9K Medium. An iron block (99.9% purity) was used instead of $FeSO_4$ in 9K medium to verify the feasibility of culturing *A. ferrooxidans* with iron. The effect of increasing the area of exposed iron in the culture solution on *A. ferrooxidans* growth was studied. Iron blocks of increasing size were inlaid in polystyrene resin (φ25 × 7 mm) to ensure a constant exposed area, as shown in Figure 2a. The selected resin was corrosion resistant and not hygroscopic; although trace metal ions were deposited on the surface of the resin, the amount of deposition was 2 orders of magnitude lower than that of metal corrosion. Therefore, the weight loss of the entire sample is considered to be due to the amount of the iron block removed. The exposed surfaces were polished to obtain relatively uniform surface parameters (Sa under 100 nm). Then, the samples were rinsed with distilled water and

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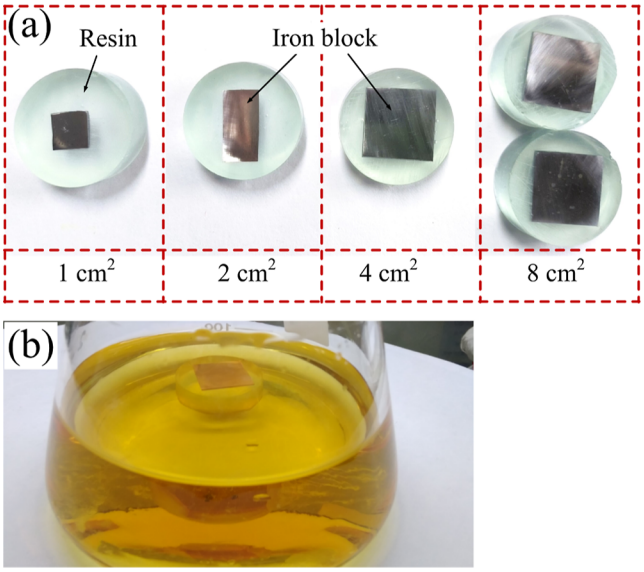

Figure 2. (a) Increasing area of exposed iron and (b) placement of iron in the culture.

ethanol (analytical reagent) in an ultrasonic cleaner and dried in an oven at 60 °C. The iron block was independently placed on the bottom of a 250 mL flask with 80 mL of 9K medium containing trace elements and 20 mL of inoculum (Figure 2b). Afterward, the block was placed in a thermostatic incubator shaker at a temperature of 30 °C and a speed of 160 rpm.

The effect of adjusting the pH of the culture solution on *A. ferrooxidans* growth was studied throughout the culture process. In addition to the experimental group in which the pH was not adjusted during the culture process, the experimental group in which the pH was periodically adjusted was included. The incubator shaker was stopped every 3 h, and the pH values at those times were measured and adjusted to 1.8. Then, the iron blocks were removed, rinsed, and dried as described above, after which the weight loss was measured. The Fe^{3+} and Fe^{2+} concentrations and bacterial density were determined.

2.5. Observation and Analytical Determinations. The weights of the iron blocks were measured with an electronic balance machine (BSM-220, Yousheng, d = 0.0001 g). The roughness of the exposed surfaces before biomachining was measured by an optical 3D surface profilometer (Super View W1, CHOTEST). The pH of the solution was measured with a pH meter (PH-100B, Lichen), which was calibrated with standard buffer solutions before use. SEM micrographs of *A. ferrooxidans* and precipitates were acquired using a scanning electron microscope (JSM-6510, JEOL). X-ray diffraction (XRD) analyses were carried out using an X-ray diffraction system (D8 Advance Series II, Bruker). The details of measuring the Fe^{3+} and Fe^{2+} concentrations and bacterial densities were described in previous works.[2,5]

3. RESULTS

3.1. Culture of *A. ferrooxidans* by Fe^{2+} with and without Sterilization. Figure 3a–c shows the variations in bacterial density, Fe^{3+} concentration, and pH during the culture process with and without sterilization of the medium. The figures show that the curves of the three parameters almost coincided. The bacterial densities decreased gradually in the first 6 h before rapidly increasing (Figure 3a). The variation trends of bacterial densities are generally the same as those in the literature.[24] The difference is that a decrease in bacterial density does not appear during early culture in the literature. This is probably because the sampling interval in the literature is too long (10 h), and some details of the variations are missing. The initial Fe^{3+} concentration was approximately 1.2 g/L after inoculation but before culture, after which the concentration steadily increased to 6 g/L (Figure 3b). The pH

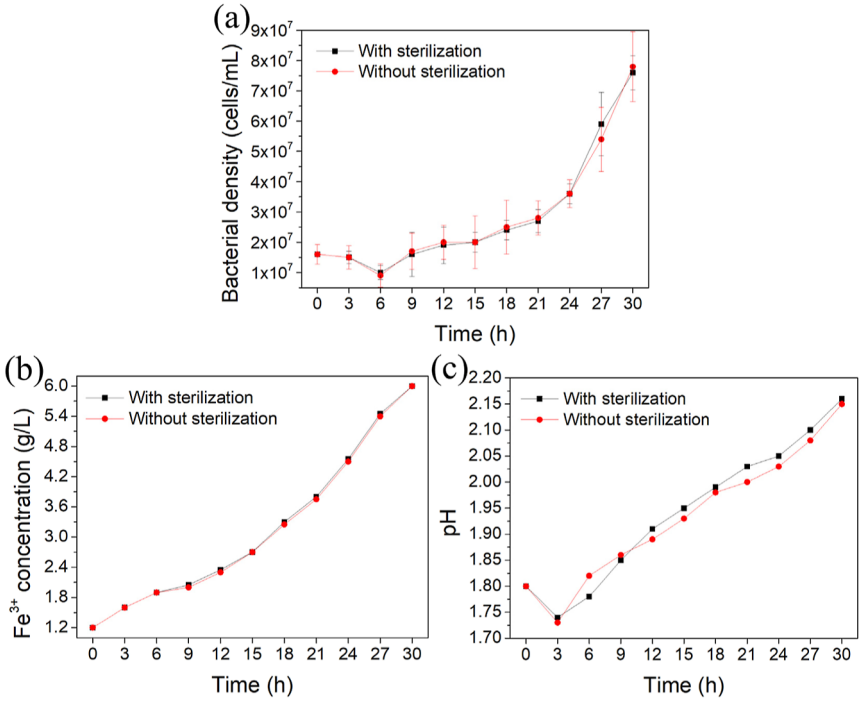

Figure 3. (a) Bacterial density, (b) Fe^{3+} concentration, and (c) pH of the culture with time for the groups with and without sterilization.

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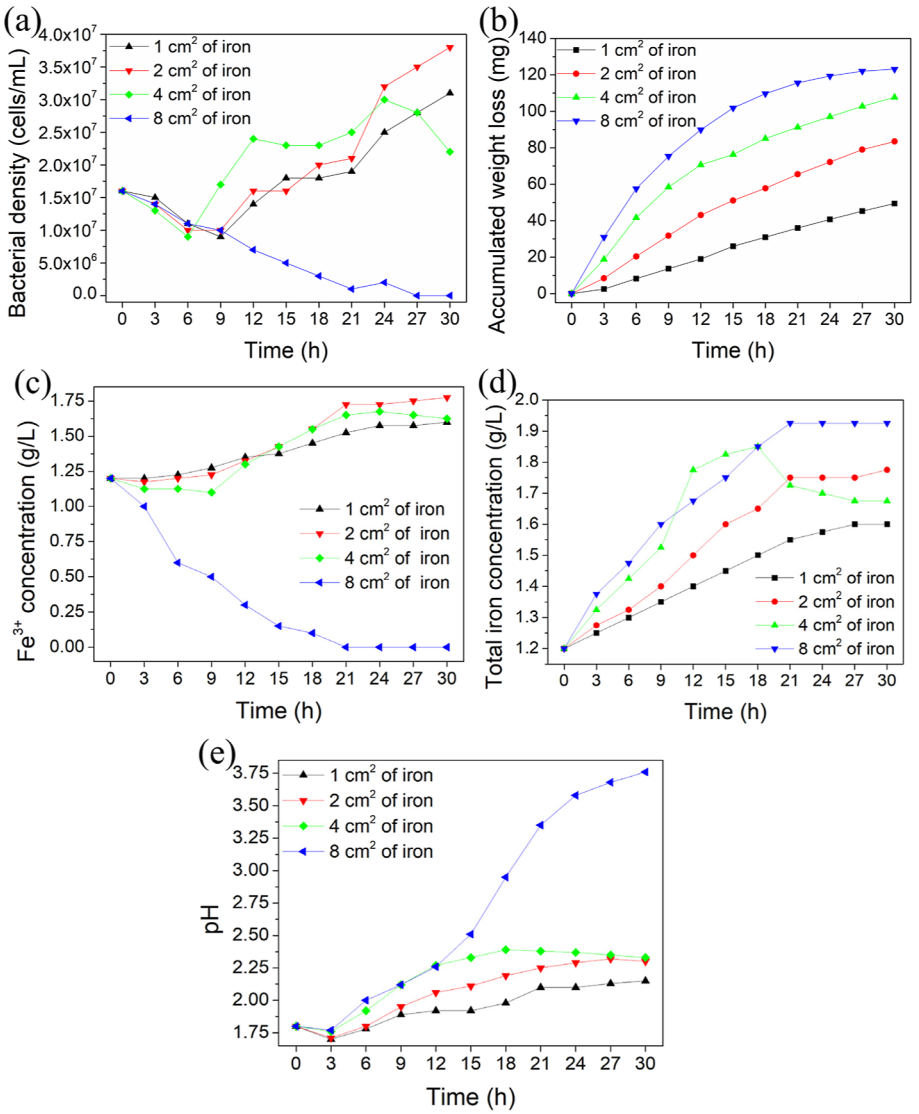

Figure 4. Without adjusting the pH, the variations in (a) bacterial density, (b) accumulated weight loss, (c) Fe^{3+} concentration, (d) total iron ion concentration, and (e) pH in the culture of *A. ferrooxidans* using iron with different exposed areas.

decreased significantly to approximately 1.74 in the first 3 h and then gradually increased to approximately 2.15 (Figure 3c).

3.2. Culture of *A. ferrooxidans* with Iron without Adjusting the pH. Figure 4a–e shows the variations in the characteristics of cultured *A. ferrooxidans* in 9K media, in which $FeSO_4$ was replaced with iron blocks with gradually increasing areas of exposure.

As shown in Figure 4a, the bacterial densities all continued to decrease during the first 6 h, which was the same trend as that observed for the bacteria cultured with Fe^{2+}. The use of small exposed areas (1 and 2 cm^2) led to a continuous upward trend. The bacterial density decreased until the end of the 8 cm^2 exposure period. Moreover, the bacterial density of the group with an exposed iron area of 4 cm^2 first increased and then fluctuated.

Figure 4b clearly shows that the accumulated weight loss of iron with the different areas of exposure increased with time. In these four groups of experiments, the greater the exposed area of iron, the greater the final cumulative weight loss. The weight loss rates of iron blocks with small exposed areas were greater than those with large exposed areas.

As shown in Figure 4c, the Fe^{3+} concentrations of the cultures with exposed iron areas of 1, 2, and 4 cm^2 were initially stable, then increased, and finally stabilized. The concentration of Fe^{3+} in the culture with an exposed iron area of 8 cm^2 did not follow the trend of the concentrations in the other three groups and continued to decrease from the beginning of the experiment to zero after 21 h.

The variations in the total iron ion concentrations, including the Fe^{2+} and Fe^{3+} concentrations, with time were studied (Figure 4d). The total iron ion concentration first generally increased and then gradually decreased toward the end of the process.

Similar to the traditional culture method, the pH of the fluid cultured with iron decreased in the first 3 h and then gradually increased to more than 2.15 (Figure 4e). Overall, the larger the exposed iron area, the greater the pH value. Interestingly, the pH of the group with an exposed iron area of 8 cm^2 increased to 3.76 at the end of the experiment.

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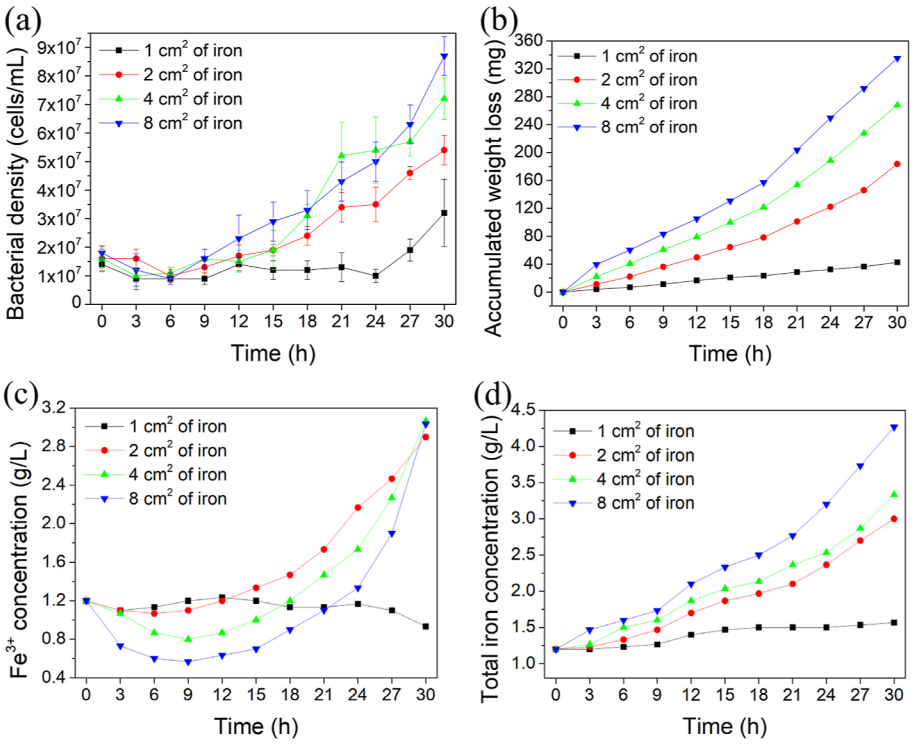

Figure 5. Variations in (a) bacterial density, (b) accumulated weight loss, (c) Fe^{3+} concentration, and (d) total iron ion concentration in the culture of *A. ferrooxidans* with different areas of iron exposure with changing pH.

3.3. *A. ferrooxidans* Culture with Iron and pH Adjustment. When adjusting the culture fluid pH, the variations in the characteristics of culturing *A. ferrooxidans* with iron blocks with stepwise increases in the exposed area are shown in Figure 5a–d.

Figure 5a shows that the bacterial concentration decreased in the first 6 h and then clearly increased until the end of the culture. According to the four curves, the greater the exposed area of iron, the faster the increase in the bacterial concentration. After 30 h of culture, the highest bacterial concentration obtained was 8.7×10^7 cells/mL in an artificially constant pH environment, which was significantly greater than that obtained without actively adjusting the pH (3.8×10^7 cells/mL). Moreover, the larger the exposed area of iron, the faster the growth of bacteria.

Figure 5b clearly shows that the cumulative weight loss of iron in the four experiments increased with time. The weight loss rates of the iron blocks with exposed areas of 2, 4, and 8 cm^2 slightly increased with time, while that of the iron block with an exposed area of 1 cm^2 showed a linear relationship with time. Similar to the experiments without pH readjustment, the greater the exposed area of iron, the greater the final cumulative weight loss.

Figure 5c shows that the Fe^{3+} concentrations in the cultured fluids with exposed iron areas of 2, 4, and 8 cm^2 first decreased and then rapidly increased. The Fe^{3+} concentration with an exposed iron area of 1 cm^2 was relatively stable. Moreover, the larger the exposed area of iron, the faster the decrease in the Fe^{3+} concentration in the culture fluid during the initial stage of culture.

The change curve of the total iron concentration in the culture fluid with time (Figure 5d) was very similar to that of the cumulative weight loss of the iron block (Figure 5b). The total iron concentration in the experimental groups with exposed iron areas of 2, 4, and 8 cm^2 slightly increased with time, and the total iron concentration in the experimental group with an exposed iron area of 1 cm^2 showed a linear curve. Therefore, the greater the area of exposed iron, the greater the total iron ion concentration in the culture fluid.

4. DISCUSSION

4.1. Consequences of Canceling Sterilization of Culture Medium. According to previous experiments, *A. ferrooxidans* can grow in a simple bioreactor without aseptic treatment in an ordinary machining laboratory,[18] and the culture can be used as an inoculum to successfully culture the next generation. In addition, the strain in this study was isolated from mine pit water in the field, and bacterial pollution in the environment is significant. Therefore, the growth and reproduction of *A. ferrooxidans* can clearly proceed as usual in environments affected by other bacteria.

As shown in Figure 3a–c, under sterilized and nonsterilized conditions, the curves of bacterial concentration, Fe^{3+} conversion rate, and pH value with time are almost coincident, indicating that the growth and metabolism rates, ion conversion efficiency, and pH of the culture solution are not significantly affected by microorganisms in the environment in this experiment. In general, the bacteria in the air of laboratories and factories are mainly autotrophic organisms that use light energy or heterotrophic microorganisms that consume organic matter. Most of these microorganisms cannot tolerate acidic environments with low pH values. In this study, *A. ferrooxidans* is considered an extremophile. The pH of the growth environment solution is usually between 1.5 and 2.5, and no organic matter is present in the environment. The microorganisms in the environment die soon after mixing into the culture medium. Therefore, the growth of *A. ferrooxidans* is

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hardly affected by microorganisms in the laboratory or factory environment.

4.2. Iron Culture of *A. ferrooxidans*. *A. ferrooxidans* proliferates rapidly via cell division. When the environmental conditions are suitable (appropriate pH, sufficient energy, and low metabolite concentration), the cell concentration increases exponentially. However, when environmental conditions are not conducive to growth (in this experiment, mainly at the end of the culture process, the pH increases, the Fe^{2+} ion concentration decreases, and the Fe^{3+} ion concentration increases), the speed of bacterial reproduction decreases rapidly, the metabolic activity of bacteria slows rapidly, some bacteria become dormant, and some bacteria gradually die.

In this study, the inoculation solution was derived from the culture medium at the end of the last generation of culture, and the metabolic activity of the bacteria in the inoculation solution decreased. Then, the activity levels of the bacteria gradually increased. However, this process is relatively slow and is called the lag phase. In this study, the lag phase of *A. ferrooxidans* growth was 6−9 h after inoculation (Figures 3a, 4a, and 5a). The cell concentration decreases during this period and then increases rapidly. After the lag phase, the bacterial concentration exponentially increased (Figures 3a and 5a), which is called the logarithmic phase. The figure shows that the culture processes were stopped in the logarithmic growth phase in this study. Nevertheless, in the complete growth curve, the growth of *A. ferrooxidans* undergoes a stationary phase and a declining phase.[25]

During the iron culture process, *A. ferrooxidans* obtained energy from the catalytic oxidation reaction for survival (eq 1). Equation 1 shows that Fe^{3+}, which is an oxidizing agent, can react with solid iron in solution (eq 2). Therefore, microbiological processes and chemical reactions form a cycle. However, the reaction rate may cause an ion imbalance due to various factors, such as an overly low *A. ferrooxidans* concentration or an overly large area of iron exposed in the solution. Therefore, the production rate of Fe^{3+} is lower than its consumption rate. In the group with an exposed iron area of 8 cm^2, due to the large area of reaction, the rate of eq 2 is higher than that of eq 1, resulting in a low concentration of Fe^{3+} in the solution (Figures 4c and 5c).

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

$$Fe^0 + 2Fe^{3+} \rightarrow 3Fe^{2+} \quad (2)$$

Without adjusting the pH, the pH decreased significantly in the third hour (Figures 3c and 4e), which may be due to the influence of culture temperature. The original pH in this study was directly measured after inoculation. The original temperature of the inoculation solution is usually close to room temperature (approximately 20 °C), and the temperature increases to 30 °C after 3 h of culture. The pH meter used in this study is based on the principle of H^+ electrode potential, and the potential value follows the Nernst equation (eq 3).

$$E = E^0 - \frac{2.303RT}{F}pH \quad (3)$$

In this formula, E is the electrode potential, E^0 is the standard electrode potential [0.925 V vs standard hydrogen electrode (SHE)], R is the gas constant, T is the absolute temperature of the culture fluid, and F is Faraday's constant. Therefore, based on the measurement principle, a higher temperature resulted in a greater electrode potential (E) and lower measured pH.

The H^+ ions in the solution are consumed and continuously regenerated, and the entire process is a complex thermodynamic process. Equation 1 shows that the catalytic oxidation of Fe^{2+} by *A. ferrooxidans* involves H^+ consumption. Conversely, H^+ is produced in the hydrolysis reaction between Fe^{3+} and H_2O in solution. The process can be divided into three stages (eqs 4−6)). In the first stage, hydrolysis is dominant. Only after the first stage when hydrolysis reaches saturation do the second and third stages begin. The amount of H^+ produced by hydrolysis is generally less than the amount of H^+ consumed during the catalytic oxidation of Fe^{2+}. Overall, if not readjusted, the pH gradually increases, as shown in Figures 3c and 4e.

$$Fe^{3+} + H_2O \rightarrow Fe(OH)^{2+} + H^+ \quad (4)$$

$$Fe(OH)^{2+} + H_2O \rightarrow Fe(OH)_2{}^+ + H^+ \quad (5)$$

$$Fe(OH)_2{}^+ + H_2O \rightarrow Fe(OH)_3 \downarrow + H^+ \quad (6)$$

In the traditional culture process, the Fe^{2+} from the culture medium is continuously transformed into Fe^{3+} by *A. ferrooxidans* and accumulates in the culture medium. In this study, the Fe^{3+} concentration continuously increased to 6 g/L by the end of the experiment (30 h) (Figure 3b). However, when cultured with iron, Fe^{3+} continues to oxidize iron (Fe^0) for consumption (eq 2), and the rate of increase in the Fe^{3+} concentration is much lower than that of traditional culturing processes. The lower the concentration of Fe^{3+}, the slower the hydrolysis reaction. Specifically, the lower the H^+ production, the faster the pH increases. This finding revealed that the pH of the culture fluid supplemented with iron increased rapidly without pH adjustment. The larger the exposure area of iron, the faster the pH increases (Figure 4e). An overly high pH is harmful to the growth and metabolism of *A. ferrooxidans* and, in severe cases, can lead to the death of many *A. ferrooxidans* cells (Figure 4a). Similar conclusions have also been reported in the literature.[26]

In addition, a high pH can lead to considerable precipitation in the culture medium. Figure 6a shows an image of the precipitate after 30 h of culture with iron without readjusting the pH. It has also been reported that the pH has been increased by adding NaOH to the biomachining liquid so that the metal ions in the solution precipitate.[27] The morphology of the precipitate and the type of compounds were confirmed. The precipitates were observed and analyzed by SEM and XRD after filtering and drying, as shown in Figure 6b,c. The figure shows that the precipitate has a very uniform granular shape. According to the XRD standard pattern, the diffraction peak of the precipitate is consistent with that of Joint Committee on Powder Diffraction (JCPDS) #22−0827 (jarosite, $KFe_3(SO_4)_2(OH)_6$), and no other special diffraction peaks appear. The main component of the precipitate is jarosite, which is consistent with the literature.[28,29]

The results of iron culture showed that the maximum *A. ferrooxidans* density was 3.8×10^7 cells/mL (Figure 4a) when the pH of the culture was not changed. This value is significantly lower than that of the traditional culture method, where the Fe^{2+} concentration was 7.8×10^7 cells/mL (Figure 3a). However, the maximum *A. ferrooxidans* concentration increased to 8.7×10^7 cells/mL (Figure 5a) when the pH was adjusted during culture. Moreover, compared with those at which the pH was not adjusted, the amount of dissolved iron

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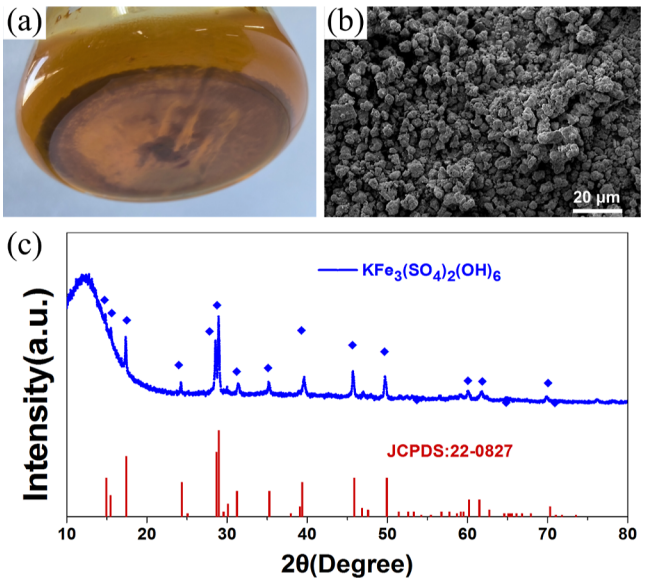

Figure 6. (a) Precipitate at the bottom of the culture fluid, (b) SEM image of the precipitate, and (c) XRD pattern of the precipitate.

and the concentration of Fe^{3+} significantly increased when the pH was adjusted regularly during the culture process. The bottom of the culture medium no longer exhibited obvious precipitation. Moreover, the highest obtained concentration of *A. ferrooxidans* was comparable to that of the traditional culture method with Fe^{2+}. The parameters (the obtained *A. ferrooooxidans* concentration, cumulative removal of iron, and concentration of Fe^{3+} in the solution) had positive relationships with the area of exposed iron. The experimental results show that the use of iron to culture *A. ferrooxidans* is feasible, provided that the pH is periodically adjusted during the culture process.

4.3. Culture of *A. ferrooxidans* by Steel Cuttings in a Bioreactor. Steel cuttings, which are treated as waste in mechanical processing, contain mainly iron. To facilitate the utilization of waste, the simple bioreactor shown in Figure 7a

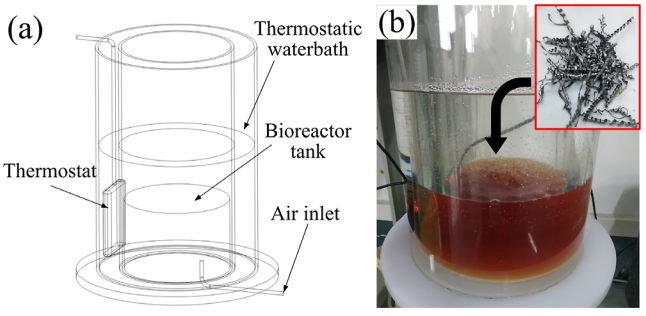

Figure 7. (a) Schematic of the bioreactor. (b) Photograph of the steel cutting chips and culture system.

was used to facilitate the large-scale culture of *A. ferrooxidans*. The outer ring of the bioreactor has an automatic thermostatic water tank that can adjust the temperature. An intake pipe is at the bottom of the bioreactor tank, which can continuously supply air through an air pump with an adjustable flow rate.

The American Iron and Steel Institute (AISI) 1045 steel is commonly used in the manufacturing industry. In this study, AISI 1045 steel drill cuttings were used as an energy source for culturing *A. ferrooxidans*. Combined with the above results, the culture parameters were as follows: 10 g of drill cuttings, 2.4 L of 9K medium, and 0.6 L of inoculum directly poured into the bioreactor without sterilization. The solution pH was set to 1.8, and the pH was adjusted every 3 h during the culture process. The thermostatic water bath temperature was set to 30 °C, and the flow rate of the inlet pipe was set to 1.2 L/min (Figure 7b). When the drilling cuttings in the culture medium were completely corroded and the concentration of Fe^{3+} ions accounted for 90% of the total iron ion concentration, the culture process was stopped. Then, the culture fluid was used as an inoculum to continue to culture the next generation. After 5 consecutive generations of culture experiments, the bacterial concentration reached 9×10^7 cells/mL.

5. CONCLUSIONS

1. Eliminating the sterilization and sterile inoculation of the medium is feasible for culturing *A. ferrooxidans*. The solution temperature significantly affects the measurement of the pH by the electrode.
2. Compared with the pH during culturing with Fe^{2+}, the pH of the culture fluid increased faster during culturing with iron. The main reason for this phenomenon is that the Fe^{3+} converted by bacteria is reduced to Fe^{2+} by the iron in the solution, significantly decreasing the intensity of the hydrolysis of the Fe^{3+} necessary to produce H^+.
3. If the pH is not readjusted during culturing, a large amount of granular jarosite is produced at the bottom of the culture solution due to the high pH. The key to obtaining a high bacterial density in the culture with iron is to maintain the pH of the solution.
4. Culturing *A. ferrooxidans* in a custom bioreactor with steel cuttings while regularly adjusting the pH during the culture process is feasible.

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Notes

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