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Research article

Bioleaching of realgar nanoparticles using the extremophilic bacterium *Acidithiobacillus ferrooxidans* DLC-5

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ABSTRACT

Background: This paper presents micro- and nano-fabrication techniques for leachable realgar using the extremophilic bacterium *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) DLC-5.
Results: Realgar nanoparticles of size ranging from 120 nm to 200 nm were successfully prepared using the high-energy ball mill instrument. *A. ferrooxidans* DLC-5 was then used to bioleach the particles. The arsenic concentration in the bioleaching system was found to be increased significantly when compared with that in the sterile control. Furthermore, in the comparison with the bioleaching of raw realgar, nanoparticles could achieve the same effect with only one fifth of the consumption.
Conclusion: Emphasis was placed on improving the dissolvability of arsenic because of the great potential of leachable realgar drug delivery in both laboratory and industrial settings.
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1. Introduction

Realgar is a naturally occurring sulfide ore containing more than 90% tetraarsenic tetrasulfide (As_4S_4) [1]. It has a long history of being used in traditional Chinese medicine, and the earliest record of its use can be traced to *Shennong Ben Cao Jing*, which was one of the first books of medicaments written two thousand years ago in China [2]. Ancient Chinese doctors used realgar to treat certain diseases by the theory of combatting poison with poison [3]. Recently, it has been confirmed that realgar has an apparent antitumor effect in the treatment of many cancers *in vivo* and *in vitro*, especially in the treatment of chronic and acute promyelocytic leukemia [4,5,6]. However, as a mineral medicine containing arsenic, there remain many shortcomings, which must be addressed for the application of realgar. The main challenges to realgar application are low solubility, high toxicity, and poor bioavailability, which limit its utilization in the clinical settings [7, 8]. The conventional approach of processing realgar is by grinding it in acidic or alkaline liquid media or by grinding it into a fine powder using a high-energy ball mill instrument [9,10]. Unfortunately, the reality is that both these technologies are of high cost, low efficiency, and harmful to the environment and cannot overcome the fundamental disadvantages of the application of realgar [11]. Therefore, to achieve the goal of reducing toxicity and increasing its beneficial effects, choosing an appropriate and modern technique to prepare realgar is urgent and necessary.

Nanotechnology is one of the fastest growing and widespread technologies with great potential. In recent years, pharmaceutical industries have increasingly adopted the use of nanotechnology to prepare traditional Chinese medicine [5]. A previous study pointed out that the bioavailability of herbal drugs will be significantly enhanced over time [12]. Nanotechnology has also been used for the preparation of realgar. Nanorealgar powders were prepared using the high-energy ball mill instrument [13]. Compared with the traditional realgar powders, the absorption and distribution of nanorealgar particles are increased significantly [14,15]. Additionally, modification on the surface of nanopowders promotes

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the interaction between soluble arsenic and cancer-targeting sites, which improves the efficacy of realgar in the destruction of cancer cells [16]. Although realgar nanoparticles showed a significant anticancer effect, the process of grinding the two toxic substances pararealgar and arsenic trioxide enhanced the toxicity of the realgar [17]. More importantly, the agglomeration of nanoparticles in packing, loading, and storage is another disadvantage that limited the application of the nano-powders. Therefore, there is a need for further processing to prepare nanorealgar particles to overcome these shortcomings.

In recent decades, bioleaching has gradually advanced into the forefront of hydrometallurgy. This technology uses acidophilic bacteria such as *Acidithiobacillus ferrooxidans* (*A. ferrooxidans*) and *Acidithiobacillus thiooxidans* (*A. thiooxidans*) to leach sulfide ore [18]. After the process of bioleaching, the solubility of the realgar ore significantly increases [19]. These species of bacteria were isolated from acidic mine drainage by oxidizing ferrous ions to obtain the energy they need [20]. This kind of bacterial strain is highly resistant to arsenic regardless of whether it is organic (24 mM) or inorganic (32 mM) [21,22,23]. On the basis of this principle, we formulated a protocol to leach arsenic from micron-sized realgar using certain indigenous organisms such as *A. ferrooxidans* BY-3 [11,24]. A previous work conducted in our laboratory demonstrated that the solubility of realgar increases significantly as compared with that of traditional realgar powders. Moreover, this procedure can overcome the gastrointestinal irritation that rough realgar powders cause [25]. Furthermore, realgar bioleaching solution (RBS) showed an apparent antitumor effect in Sarcoma-180 Cells, HepG2 cells, *Caenorhabditis elegans* (*C. elegans*), and mice [26,27]. The antineoplastic mechanism of RBS includes cancer cell apoptosis through the generation of reactive oxygen species (ROS), which further accumulates in the cells. Additionally, pharmacological experiments confirm that the RBS has evident antineoplastic activity in the K562 cell strain and H22 cell strain, similar to that of realgar. Furthermore, the life expectancy of mice was extended, and the mutant of *C. elegans* was inhibited after treatment with RBS [28,29]. More importantly, RBS produces the same antitumor effect with less toxicity as that of other similar drugs such as raw realgar and arsenic trioxide [30]. Above all, realgar bioleaching is an efficient, ecologically safe, energy-saving, environment-friendly and characterized way to prepare realgar [11]. However, presently, it has been observed that the realgar particles in the bioleaching system are too large and form a passivation membrane on the surface of particles after bioleaching. This is a huge barrier for recycling the realgar particles, which is considered a waste of resources. Therefore, pretreatment in bioleaching technology is not perfect, and there will be a greater increase in extraction efficiency if an appropriate method to treat realgar particles is used before bioleaching. Hence, it is better to process realgar powder further before introducing them into a bioleaching system.

There is a complementary relationship between nanotechnology and bioleaching technology. On the basis on the above ideas, we confirmed that nanotechnology can further improve the efficiency of bioleaching, first, by using *A. ferrooxidans* to bioleach nanorealgar particles to decrease pararealgar and arsenic trioxide, which lead to the toxicity of nanorealgar powders, and second, by realgar nanoprocessing to improve the efficiency of bioleaching and the utilization of realgar and reduce the loss of realgar. Therefore, comprehensive technology is an innovative method and has an important theoretical and practical value. Thus far, there is still no systematic research on the use of *A. ferrooxidans* to bioleach nanorealgar powders. Therefore, research on bioleaching nanorealgar will not only improve the understanding of the mechanism of bioleaching realgar but also provide a safer and more effective clinical therapy.

2. Materials and methods

2.1. Microorganism

A pure culture of *A. ferrooxidans* DLC-5 (CCTCC NO: M2014362) was isolated and purified from an acidic mine drainage in Wudalianchi, Heilongjiang Province, P.R. China. 9 K medium, containing ferrous sulfate (44.69 g/L) and moderate realgar particles, was used to culture the strain of *A. ferrooxidans* DLC-5 throughout the experiments [31]. The bacteria were collected by centrifugation and resuspended in the medium without ferrous sulfate. A suspension containing 4.5×10^7 cells/mL was used as a 10% inoculum to ensure the same initial conditions for all experiments.

2.2. Realgar

Realgar ore was purchased from Shimen County of Hunan Province, P. R. China. X-ray powder diffraction (XRD) analysis was employed for the mineralogical identification of the drug. The raw realgar contained 3% arsenolite and 97% realgar. To obtain pure realgar, the raw realgar was grinded to 200-mesh size and suspended in 500 mL of 15% hydrochloric acid solution for 2 h. Then, it was washed with distilled water until the pH reached 7.0. Finally, realgar particles were dried in a drum wind drier at 40°C for 4 h.

2.3. Grinding experiment

Raw realgar particles were ground using high-energy milling equipment. The sample preparation method for high-energy milling experiment was as previously described [7,16]. The processed realgar powder was dispersed in 1.5X ultrapure water. The diameter of the grinding ball is 5 mm, 3 mm, and 1 mm. The proportion of each size is 5:3:1 mm = 1:2:7. The following milling conditions were used: ball-to-powder weight ratio of 1:5, 1:10, 1:15, and 1:20, respectively; rotation speed of the planet carrier of 200 r/min, 300 r/min, 400 r/min, 500 r/min, and 600 r/min; and milling times of 30 min, 60 min, 90 min, 120 min, and 150 min, respectively.

2.4. Bioleaching experiments

Bioleaching experiments were carried out in 250 mL Erlenmeyer flasks, and the bacteria were inoculated in 100 mL of medium containing 0.1 g of realgar. The influence of bioleaching time (40 h, 60 h, 80 h, and 100 h), temperature (25°C–40°C), initial pH (1.5–3.0, adjusted using 1.0 M H_2SO_4), bacterial population (5%–20%, *v*/v), and ferrous ion concentration (1.0–9.0 g/L) was analyzed. The medium was shaken at 150 r/min in an orbital shaking table, constantly.

2.5. Analytical procedures

Specific surface area was determined by the low-temperature nitrogen adsorption method. Gemini 2360 surface area analyzer (Micrometrics, USA) was used. Particle size distribution was determined using Malvern Mastersizer 2000 (Malvern Instruments Ltd., UK). Soluble arsenic concentrations were measured using a hydride generation-atomic fluorescence spectrometer (AFS-9700; Beijing Haiguang, China). The details of all the parameters are listed in Table 1. A calibration curve showed strong linearity ($r > 0.999$). The reagents used in sample processing and analysis were of analytical grade from Sinopharm Chemical Reagent Company, Shanghai, China. The digested solution consisted of two components: 5% ascorbic acid solution and 5% thiourea solution.

The surface properties of realgar particles were identified by gold coating and scanning electron microscopy (FESEM; JSM-6701F, Japan) operated at 5–10 kV. Additionally, an X-ray powder diffraction (XRD) instrument (X'Pert Pro MPD, Philips, The Netherlands) at 40 kV and

Table 1
Instrumental and operative conditions of AFS-970.

Instrument parameters	As
Software	AFS-9700
Photomultiplier voltage	220 V
Height of atomizer	8 mm
Flow of carrier gas (Ar)	400 mL min^{-1}
Lamp current	50 mA
Flow of sheath gas (Ar)	800 mL min^{-1}
Reading time	15 s
Delay time	1 s
Current carrying	5% HCl
Reaction liquid	2% KBH_4 + 0.5% NaOH
Measurement method	Standard curve method
Reading method	Peak area

40 mA and a high-resolution Raman spectrometer (Horiba-Jobin, Yvon, HR800) using a He–Ne laser (λ = 532 nm) as the excitation source were used. The bacteria were identified through an FT-IR spectrometer (NEXUS 670, Thermo Nicolet, USA) in a spectral band ranging from 400 cm^{-1} to 4000 cm^{-1}. The experimental data were analyzed by nonlinear mathematical models embedded in OriginPro 2017 software package (OriginLab, USA).

3. Results and discussion

3.1. Preparation of realgar nanoparticles

3.1.1. Effects of lapping speed

Fig. 1A shows that lapping speed had an apparent effect on particle size distribution. Under a speed condition of 200 r/min, the realgar particles had the largest particle size. The figure shows that more than 90% of the particles had size ranging from approximately 1.0 μm to 32 μm. Only 10% of particles had a size below 1.0 um. Under this speed condition, realgar particles did not reach the nano level. With increase in the speed, the diameter of realgar particles dwindled, especially in the group with an optimal speed of 600 r/min. After grinding, the size of the particles changed greatly. The proportion of nanorealgar

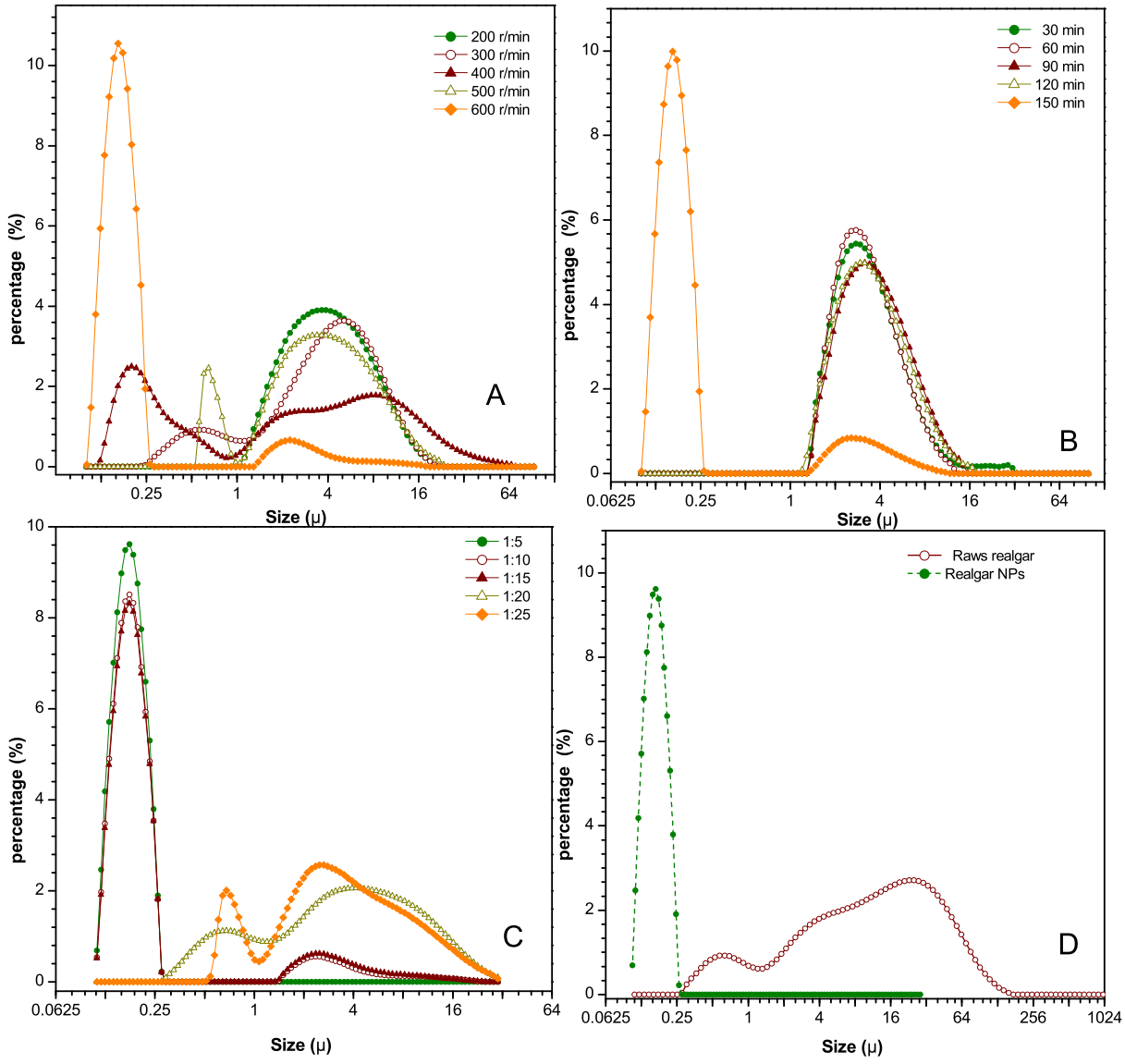

Fig. 1. Realgar NPs prepared using the high-energy ball mill instrument. (A). The effect of lapping speed during the preparation of realgar NPs (B). The effect of grinding time during the preparation of realgar NPs (C). The effect of ball-to-powder weight ratio during the preparation of realgar NPs (D). Comparison between realgar NPs and raw realgar powders.

accounted for approximately 91.5% of the total volume, and only approximately 8.5% of particles had a size larger than 1.0 μm.

3.1.2. Effects of grinding time

It can be seen in Fig. 1B that there is a slight effect of grinding time on the distribution of the realgar particle size and that the change in particle size distribution is not apparent when the grinding time is less than 120 min. However, it still can be concluded that with an increase in grinding time, the size of realgar particles becomes gradually small. A grinding time of 150 min is the best grinding time for the distribution of realgar particles. Under such operating conditions, the size of realgar powders is the smallest and the proportion of nanorealgar is the largest.

3.1.3. Effects of ball-to-powder weight ratio

Fig. 1C shows that ball percentage has an apparent effect when grinding the realgar by high-energy ball milling. The results show that the optimum ball-to-powder weight ratio was 1:5. Under this condition, the particle size ranges between 110 nm and 250 nm. Under other conditions, the distribution of realgar particles undergoes significant changes: there is a large amount of realgar powder attached to the surface of the grinding balls and the remaining realgar particles were precipitated at the bottom of the grinder when the ratio was 1:1. This phenomenon shows the limit of the grinding efficiency. When the ball ratio is more than 1:5, the proportion of realgar nanoparticles is gradually reduced because the realgar powders are not fully in contact with the grinding ball. This phenomenon is even more pronounced when the ratio is greater than 1:15.

3.1.4. Preparation of realgar nanoparticles under optimum parameters

It can be seen from Fig. 1D that after grinding with the high-energy ball mill instrument, the realgar particles obtained in this experiment are all distributed between 0.1 μm and 0.26 μm. The smallest size of the realgar powder was 0.115 μm and the largest was 0.26 μm. SEM image of the realgar nanoparticles (Fig. 2A) revealed that the particle size is evenly rounded and uniform. SEM image (Fig. 2C and D) demonstrated that the size of the particles was smaller and smoother than those before.

3.2. Bioleaching of realgar nanoparticles

3.2.1. Effects of time on bioleaching

Fig. 3A shows that there is no significant difference between the 10 h period and the 20 h period. The total arsenic concentration was 92 mg/L. When the bacteria were added to the bioleaching process, the total arsenic content increased significantly. Other data showed that the total arsenic content in the 0 h group was 80 mg/L, and the content in the 30 h group was 73 mg/L. Finally, the arsenic concentration in the 40 h group was the lowest at 69 mg/L.

3.2.2. Effects of initial pH on bioleaching

There was a large amount of precipitate in the 9 K medium when the pH value was greater than 2.0. That is because the Fe^{2+} precipitates in a

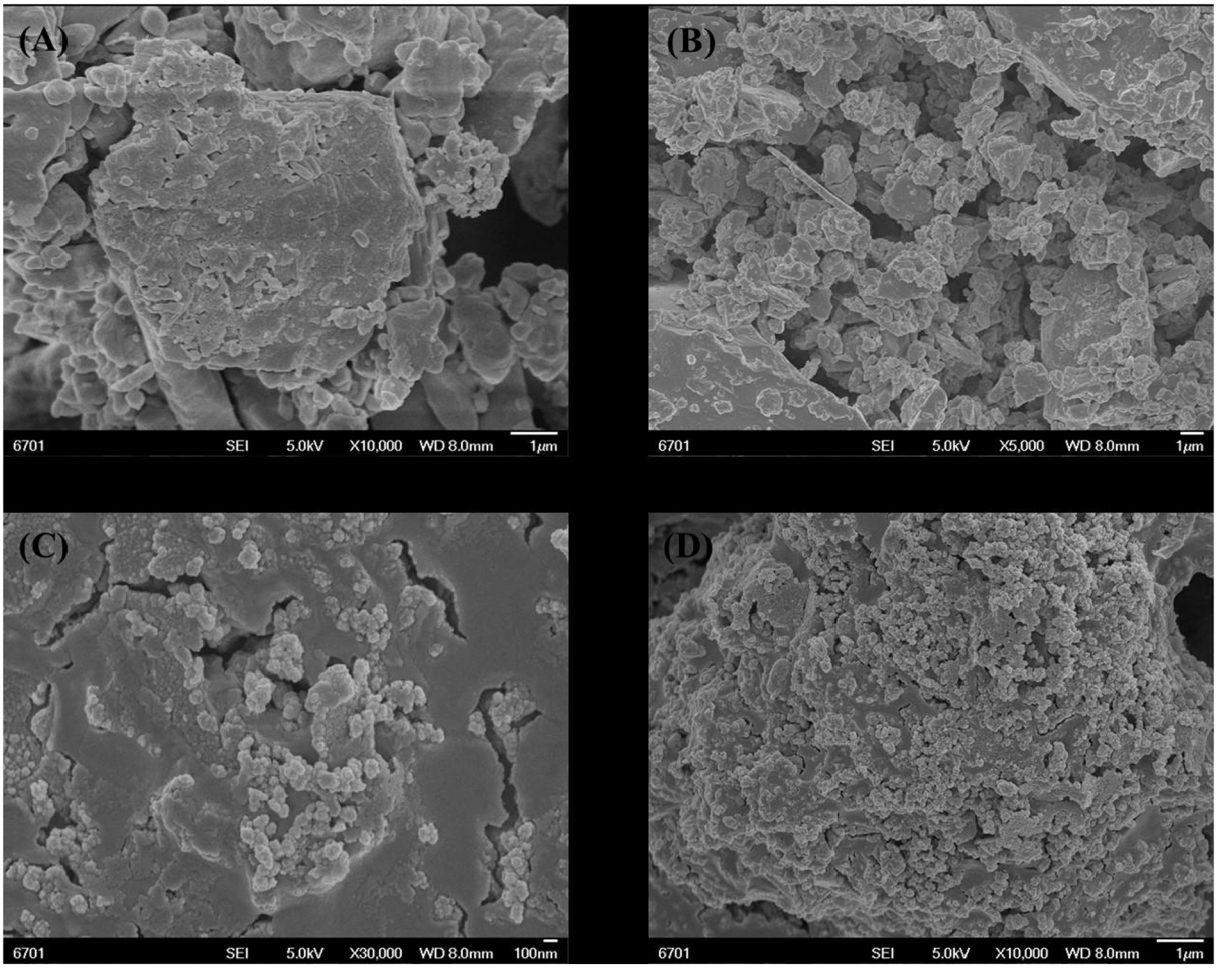

Fig. 2. A SEM image of the surface of the realgar NPs reacting with *A. ferrooxidans* DLC-5. Before bioleaching: (A). Raw realgar powder (scale bar = 1 μm) (B). Raw realgar NPs (scale bar = 1 μm) After bioleaching: (C). realgar NPs (scale bar = 100 nm) (D). realgar NPs (scale bar = 1 μm).

high pH value solution, which decreases the Fe^{2+} concentration in solution. Considering that Fe^{2+} is the energy source for the growth of bacteria, a large amount of ferrous precipitation limits the growth of bacteria. This was verified from the results shown in Fig. 3B. Arsenic concentration reduced with an increase in pH. The final data were collected after 100 h of bioleaching. The concentration of arsenic in the solution of pH 3.0, 2.0, and 1.5 was 80 mg/L, 100 mg/L, and 150 mg/L, respectively. Therefore, a culture of pH 1.5 is more suitable than other pH for bacterial survival and, therefore, improves the efficiency of bioleaching of realgar.

3.2.3. Effects of temperature on bioleaching

A. ferrooxidans DLC-5 is a moderate thermophilic bacterium. Therefore, the most suitable temperature for bacterial growth is approximately 30–35°C. Fig. 3C confirms this with the experimental conditions selected at 25–40°C. It can be seen from the experimental results that the total arsenic content in the solution is 90 mg/L at 40°C. At 25°C and 35°C, the total arsenic content was similar but showed an increasing trend with a total arsenic concentration of 120 mg/L at 35°C. It shows that 35°C is the most suitable temperature for the growth of *A. ferrooxidans* DLC-5.

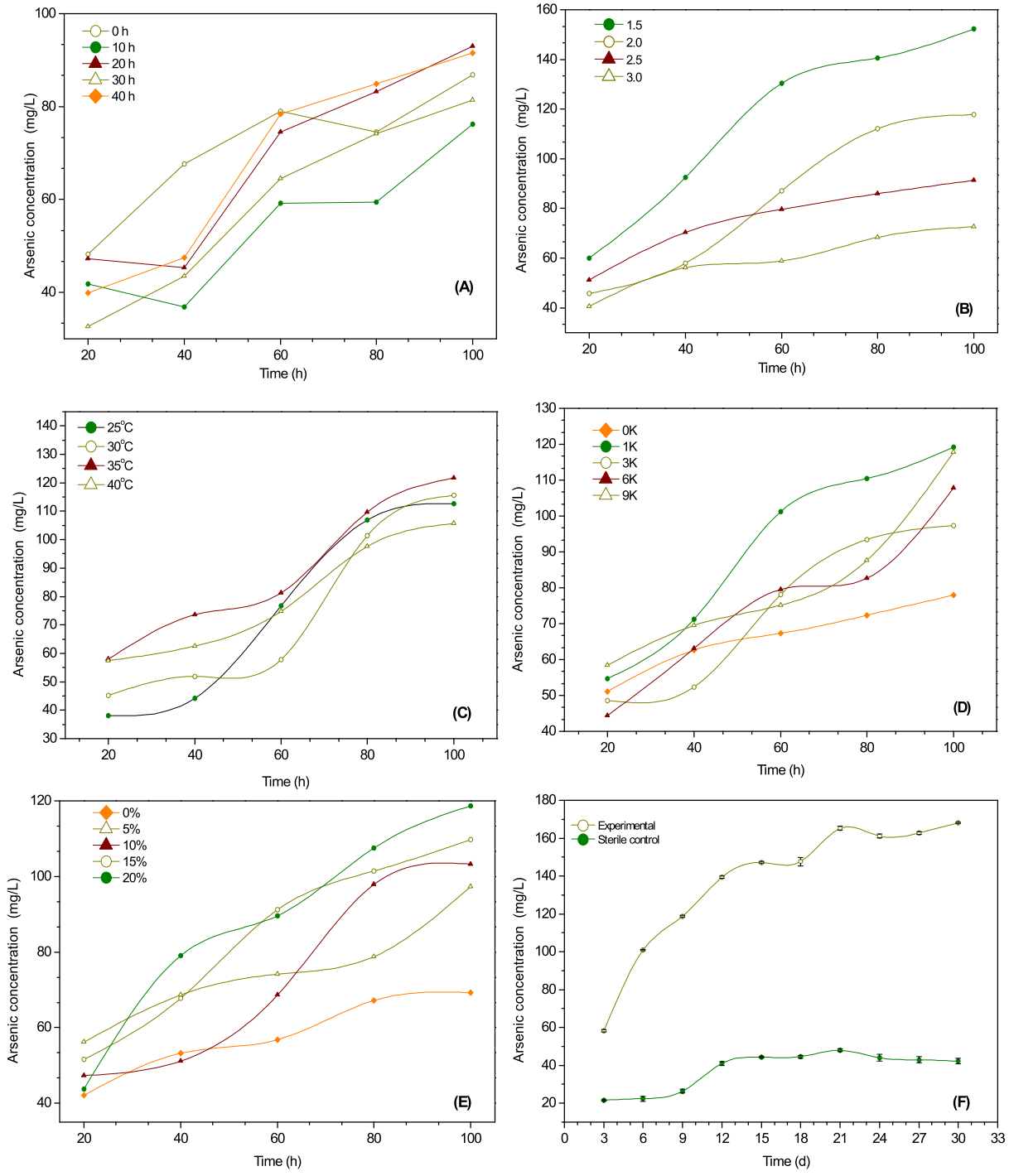

Fig. 3. Arsenic concentration in the bioleaching experiment. (A). The effect of the realgar addition time on the bioleaching of realgar NPs (B). Effects of the initial pH value on the bioleaching of realgar NPs (C). Effects of temperature on the bioleaching of realgar NPs (D). Effects of Fe^{2+} concentration on the bioleaching of realgar NPs (E). Effects of inoculum concentration on the bioleaching of realgar NPs.

3.2.4. Effects of Fe^{2+} concentration on bioleaching

Fig. 3D indicates that when the Fe^{2+} concentration is greater than 1 g/L, the increase in Fe^{2+} concentration correlates with a decrease in the total arsenic content from 120 mg/L to 90 mg/L. The results suggest that *A. ferrooxidans* efficiently enhances realgar dissolution. Without bacteria, the total arsenic concentration of the solution was significantly lower, only 60–70 mg/L. The arsenic concentration reached 120 mg/L in the group with a 1 g/L inoculum after bioleaching for 100 h. A previous study found that a large amount of ferrous in the solution co-precipitates with arsenic and form an iron compound, jarosite, which gradually reduces the total arsenic concentration [32].

Fig. 5 shows Raman and XRD spectra, which have given powerful evidence. Both these spectra confirmed that excess ferrous in the medium generates a large amount of jarosite in the bioleaching system. Fig. 5A and B have similar character peaks that range from 200 to 400 cm^{-1}. Those peaks are the typical characteristic of realgar [33]. However, four Raman characteristic lines are apparent at 430 cm^{-1}, 450 cm^{-1}, 1023 cm^{-1}, and 1121 cm^{-1} in Fig. 5C. They are due to the jarosites [34]. Similarly, the typical peak of XRD is presented in Fig. 5F, and those peaks are not found in Fig. 5 D and E. Those peaks have proved that the medium contained jarosite, magnetite, and ferric sulfide after bioleaching [25,34,35,36]. Therefore, when the Fe^{2+} concentration is greater than 1 g/L, an increase in the Fe^{2+} concentration gradually decreases the total arsenic content from 120 mg/L to 90 mg/L.

3.2.5. Effects of inoculum concentration on bioleaching

A. ferrooxidans DLC-5 has a moderate tolerance for arsenic, and that is why the bacteria can live in the medium with realgar. A previous study demonstrated that the bacteria in the medium absorb free arsenic ions in the medium, which leads to a slightly decreased arsenic concentration [37]. In this research, the bacteria were collected and evaluated by FT-IR, and the spectrum is shown in Fig. 6. It indicated the main functional groups on the surface of bacteria involved in the arsenic biosorption. The peaks at 3413, 2926.7, 1653, 1537, 1452.4, 1236, and 1079.8 cm^{-1} in the FT-IR spectrum of *A. ferrooxidans* DLC-5 after bioleaching were identified as the —OH, —NH, —CH_2, C═O stretching, —NH stretching, —CH, —SO_3 stretching, and —CN stretching vibration groups, respectively. There was no apparent shift when compared with the control group. Therefore, the surface morphology of *A. ferrooxidans* DLC-5 did not undergo a major change in the presence of realgar in the medium. When comparing arsenic concentrations, it was observed that with an increase in bacterial inoculum, the bioleaching rate showed an increasing trend. The concentration of total arsenic in the solution without bacteria was only 60 mg/L. It was evident that the concentration of total arsenic in other groups remarkably increased, especially in the 20% group. The concentration was above 120 mg/L, and in the other groups, it was 90 mg/L, 100 mg/L, and 110 mg/L. These results indicate that the highest total arsenic concentration was 120 mg/L and support *A. ferrooxidans* as an indispensable element in the bioleaching of realgar.

3.3. Mathematical models for the bioleaching of realgar nanoparticles

3.3.1. Linear model for the bioleaching of realgar nanoparticles

After 30 d, the surface of realgar nanoparticles that underwent bioleaching changed significantly when compared to realgar nanoparticles. SEM images (Fig. 2C and D) demonstrated that the size of the particles was smaller and smoother than that before.

$$X_i = mp\frac{M_{As_4S_4}}{M_{As}}\left[C_i\left(V_0 - \sum_{i=1}^{n-1} V_i\right) + \sum_{i=1}^{n-1} C_i V_i\right] \quad \text{[Equation 1]}$$

where, X_i is the realgar conversion of the realgar nanoparticle sample (i = 1, 2, … n), m is the mass of charged solids (g), p is the realgar purity (91%), M_{As4S4} is the molar mass of arsenic (74.922 g/mol), M_{As} is the molar mass of realgar (213.97 g/mol), C_i is the arsenic concentration of the ith sample (g/L), V_0 the initial volume of the leaching solution (L), V_i is the volume of leaching solution taken from the ith sample (L), and n is the total number of samples.

The conversion of realgar nanoparticles is represented by two different symbols in Fig. 4(A). After 30 d, bioleaching the arsenic conversion showed an apparent difference between the experimental group and sterile control group. The conversion of realgar nanoparticles after 30 d in *A. ferrooxidans* DLC-5 cultures was higher (26.38%) than that in the sterile control group (7.53%). This proves that *A. ferrooxidans* DLC-5 plays an important role in enhancing the conversion of realgar nanoparticles under the examined conditions. Equation 1] of the lines of best fit and the correlation factors (R^2) are listed in Table 2. The correlation coefficients of the linear model in the experimental group (0.8472) and sterile control group (0.5479) were

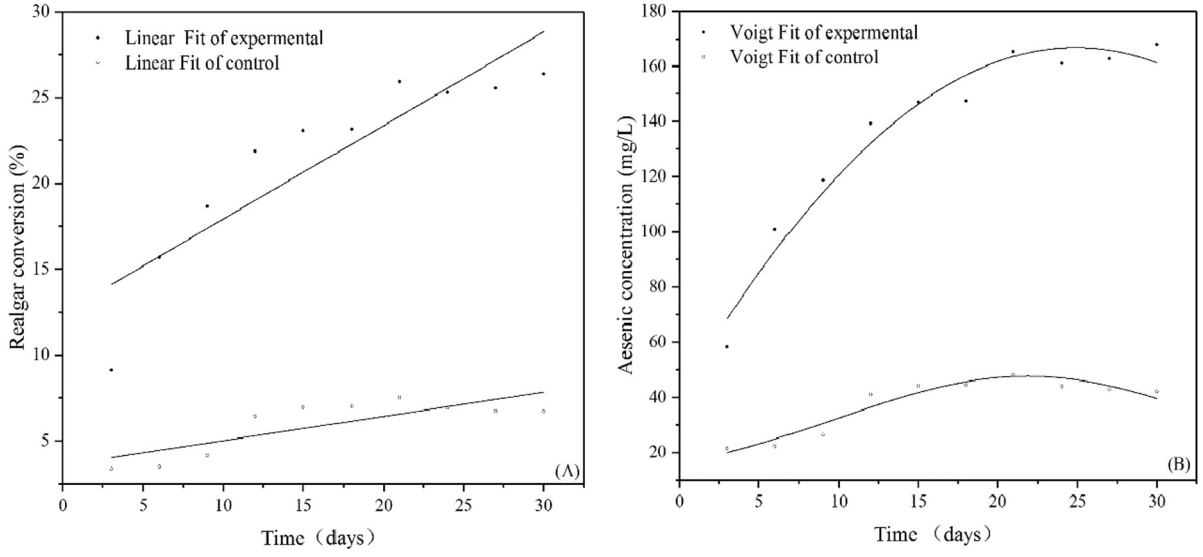

Fig. 4. Mathematical models for the bioleaching of realgar NPs. (A). Linear mathematical models for the bioleaching of realgar NPs (B). Nonlinear mathematical models for the bioleaching of realgar NPs.

Table 2
Linear and best-fit nonlinear equations and correlation factors (R-square) for realgar conversion.

Group	Linear model		Nonlinear model			
	Best-fit equation	R-square	Best-fit equation	Reduced chi- square	Adjusted R-square	Fit-status
1	Y = 15.53 + 0.4063x	0.8472	Eq. Equation 2]	72.86556	0.92087	Succeeded
2	Y = 3.606 + 0.1412x	0.6489	Eq. Equation 2]	14.57311	0.99696	Succeeded

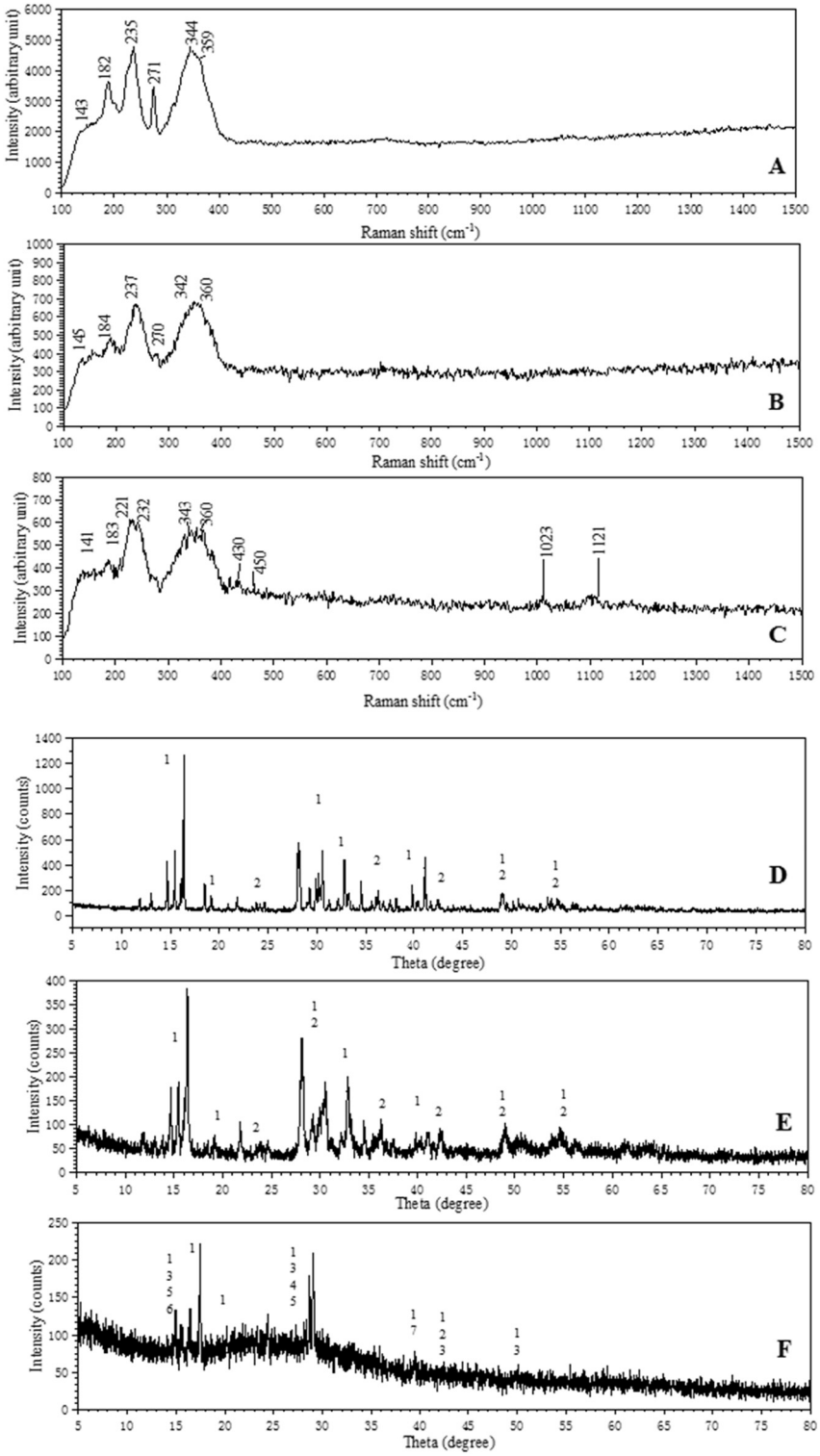

Fig. 5. Raman spectra and Powder XRD patterns of various realgar preparations. (A). Raman spectrum of raw realgar (B). Raman spectrum of realgar NPs (C). Raman spectrum of realgar NPs after bioleaching (D).Powder XRD patterns of raw realgar (E). Powder XRD patterns of realgar NPs (F). Powder XRD patterns of realgar NPs after bioleaching (b) *A. ferrooxidans* DLC-5 in the 9 K medium.

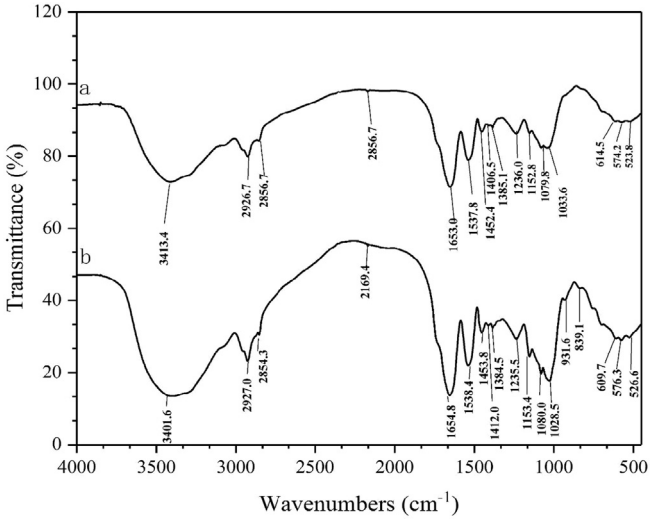

Fig. 6. Difference in FT-IR spectra of the surface of *A. ferrooxidans* DLC-5. The FT-IR spectra were recorded using an FT-IR device (NEXUS 670; Thermo Nicolet, USA) over the wave number range of 500 cm^{-1} to 4000 cm^{-1} under ambient conditions. (a): Pristine: *A. ferrooxidans* DLC-5 in the medium without realgar NPs. (b): As tolerance: *A. ferrooxidans* DLC-5 in the medium with realgar.

low (R^2 < 0.990). The conversion *Xi* was not appropriate for the experimental data.

3.3.2. Nonlinear model for the bioleaching of realgar nanoparticles

$$y = y_0 + A\frac{2}{\pi^{3/2}}\frac{\ln 2}{w_L}\frac{w_L}{w_G^2}\int_{-\infty}^{\infty}\frac{e^{-t^2}}{\left(\sqrt{\ln 2}\frac{w_L}{w_G}\right)^2+\left(\sqrt{4\ln 2}\frac{x-x_c}{w_G}-t\right)}dt \quad \text{[Equation 2]}$$

This nonlinear model for the bioleaching of realgar nanoparticles is used, where, y_0 is the offset; *A* is the peak area; and W_L, W_G, and X_c are the Lorentzian width, Gaussian width, and center of the parabola, respectively. The best-fit nonlinear equations and correlation factors are listed in Table 2. The chi-square statistic was used to test whether the observed distribution differed from the theoretically expected frequencies. The reduced chi-square values of the nonlinear model for the experimental group and sterile control group were 72.87 and 14.57, respectively. The adjusted R^2 values of the nonlinear model for the experimental group and sterile control group were 0.92 and 0.997. These results show that the experimental group had the maximal reduced chi-square and minimal adjusted R^2 values.

The fit status code, 100, of the nonlinear model indicated that the fit succeeded for the experimental group and sterile control group. The fit of those two groups succeeded, and the chi-square value was reduced until the tolerance was reached. The conversion of realgar is represented by two different bioleaching symbols in Fig. 4(A). The concentration of arsenic increased gradually, especially in the first 20 d of bioleaching. However, improvement in arsenic concentration became slower in the final 10 d.

4. Conclusion

In this study, realgar nanoparticles were successfully prepared using the high-energy ball mill instrument. *A. ferrooxidans* DLC-5 was used to bioleach realgar nanoparticles. This study found that the solubility of realgar nanoparticles increased significantly and that realgar nanoparticles were more conducive to bioleaching than traditional bioleaching. Therefore, this technology can reduce the physical and chemical adsorption at the last stage of traditional bioleaching, hence making bioleaching technology more advanced.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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