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Research article

Arsenic tolerance and bioleaching from realgar based on response surface methodology by *Acidithiobacillus ferrooxidans* isolated from Wudalianchi volcanic lake, northeast China

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ABSTRACT

Background: Traditional methods of obtaining arsenic have disadvantages such as high cost and high energy consumption. Realgar is one of the most abundant arsenic sulphide minerals and usually treated as waste in industry. The aim of the present study was to screen an arsenic tolerant bacterium used for bioleaching arsenic from realgar.

Results: An acidophilic iron-oxidizing bacterium BYQ-12 was isolated from Wudalianchi volcanic lake in northeast China. BYQ-12 was a motile, rod-shaped gram-negative bacterium with an optimum growth at 30°C and pH 2.5. 16S rDNA phylogeny showed that BYQ-12 was a new strain of *Acidithiobacillus ferrooxidans*. The inhibitory concentrations (ICs) of arsenite and arsenate were 32 and 64 mM, respectively. A significant second-order model was established using a Box–Behnken design of response surface methodology (BBD-RSM) and it estimated that a maximum arsenic bioleaching rate (73.97%) could be obtained when the pulp concentration, pH and initial ferrous ion concentration were set at optimized values of 0.95% *w*/*v*, 1.74 and 3.68 g/L, respectively. SEM, EDS and XRD analyses also revealed that there was direct bioleaching besides indirect electrochemical leaching in the arsenic bioleaching system.

Conclusion: From this work we were successful in isolating an acidophilic, arsenic tolerant ferrous iron-oxidizing bacterium. The BBD-RSM analysis showed that maximum arsenic bioleaching rate obtained under optimum conditions, and the most effective factor for arsenic leaching was initial ferrous ion concentration. These revealed that BYQ-12 could be used for bioleaching of arsenic from arsenical minerals.

1. Introduction

Arsenic is often referred to as a metalloid or semi-metal that occurs in nature in two predominant oxidation states, namely arsenite and arsenate [1]. These arsenic species are classified chemically as inorganic or organic, and the organoarsenic can and does arise from inorganic arsenic via a series of reduction and oxidative/biomethylation processes [2]. Current and historical commercial uses of arsenic mainly include pharmaceuticals (anticancer agents and antibiotics), wood preservatives, agricultural chemicals (pesticides, herbicides, insecticides, cotton desiccants, defoliants, soil sterilants, and feed additives), and applications in the metallurgical, glass-making (decolorizing and fining agents), electronics (additive), and semiconductor (doping agent) industries [3,4]. Despite various rules and regulations that led to the decline of arsenic use in wood preservatives and agriculture industries, the world production of arsenic remains above 40,000 tons (t) in 2013. Traditional methods of obtaining arsenic include smelting and roasting of nonferrous metal ores or concentrates [5]. Though simple to put into practice, several disadvantages such as high cost, high energy consumption, and the difficulty in controlling the production of toxic gas have limited their further developments. Therefore, novel methods of efficient, eco-friendly and low cost for arsenic production are necessary.

As an alternative, bioleaching is generally less energy-intensive and less polluting than most non-biological procedures [6,7]. It is defined as the solubilization of specific metals from their ores either directly by the metabolism of chemolithotrophic sulfur- and/or iron-oxidizing bacteria or indirectly by the products of metabolism [8,9]. More than 200 minerals that contain arsenic are found in nature [6], among them realgar is one of the most abundant arsenic sulphide minerals and

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usually treated as waste in industry [9,10]. Acidophilic bacteria including *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans* and *Sulfobacillus sibiricu* are widely exploited in the bioleaching of realgar [9,11,12]. Among them, *A. ferrooxidans* is a chemolithotrophic acidophilic bacterium which takes in ferrous or reduced inorganic sulfur compounds as energy source. It is well documented that the isolate of *A. ferrooxidans* from China copper mine habitats shows a high resistance to arsenite (24 mM) and dimethylarsinic acid (32 mM) [11,13]. It is also reported that arsenic resistance genes are present on the chromosome of several *A. ferrooxidans* strains [14].

Although the arsenic tolerance of this bacterium has been considered one of the most important parameters in determining the efficiency of arsenical mine bioleaching process [11,15], there is still a lack of information concerning arsenic tolerance of indigenous thiobacilli found in volcanic lake. Additionally, bioleaching is also influenced by a series of other factors such as particle size, temperature, initial pH, energy source, and pulp density. Investigation of each factor separately would be very time consuming and the interactions between factors would not be discernible. Response surface methodology (RSM) is essentially a particular set of mathematical and statistical methods for experimental design and evaluating the effects of variables and searching optimum conditions of variables to predict targeted responses [16]. RSM has been successfully applied to the optimization of food processing, biochemical engineering and adsorption processes [17]. Although the process of arsenic leaching using *A. ferrooxidans* appears promising [9,11,12], there is no published report on arsenic bioleaching from realgar using the optimization of three independent variables with RSM and *A. ferrooxidans*.

In the present study, a novel strain of *A. ferrooxidans* was isolated from volcanic lake and characterized. Arsenic resistance of this bacterium was also investigated. To understand the role that *A. ferrooxidans* plays in mineral bioleaching, Box–Behnken response surface methodology was used to investigate and optimize the effects of pH, pulp density and ferrous ion concentration on the arsenic leaching from realgar. Additionally, the realgar samples before and after bioleaching were examined by SEM, EDS and XRD.

2. Materials and Methods

2.1. Culture medium

The modified 9 K liquid medium [per liter: 3 g $(NH_4)_2SO_4$, 0.1 g KCl, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \times 7H_2O$ and 0.01 g $Ca(NO_3)_2$, adjusted to pH 2.5 at room temperature using 5 M H_2SO_4 and autoclaved at 121°C for 15 min] supplemented with the filter (Φ0.22 μm)-sterilized ferrous iron (added as $FeSO_4 \times 7H_2O$, 30 g) was used for flasks enrichment and liquid culture. Furthermore, the modified 9 K-Fe-agarose solid medium (*i.e.* the modified 9 K liquid medium was solidified in sterile Petri dish with 0.4% agarose) was used for bacterial isolation.

2.2. Enrichment and isolation

The original liquid sample was collected from a volcanic lake in Wudalianchi, Heilongjiang province, China, situated at 48°39′19″ latitude and 126° 9′11″ longitudes. A 10 mL liquid sample was added aseptically to 250 mL flask containing 90 mL sterile modified 9 K-Fe medium with an initial pH of 2.5. The flasks were shaken in a horizontal shaker at 150 rpm at a temperature of 30°C. During incubation, the color of cultures gradually changed to weakly yellow and then to brown, showing the existence of iron-oxidizing bacteria in the cultures. After three sequential continuous subculture, the log-phase culture was diluted in serial 10-fold dilution steps up to 10^{-7}. The aliquots of 0.1 mL from 10^{-4} to 10^{-7} dilutions were spread onto the modified 9 K-Fe-agarose solid medium. The agar plates were inoculated for each dilution and incubated at 30°C for 10 d. A single colony was picked, recultivated and re-spread onto the 9 K-Fe-agarose solid medium. The isolation process was repeated five times to ensure strain purity. The pure culture obtained was designated strain BYQ-12.

2.3. Phylogenetic analysis of 16S rDNA

A. ferrooxidans BYQ-12 was cultured at pH 2.5, 30°C and 150 rpm. After oxidizing 90% of ferrous ions, bacterial culture reached the beginning of the stationary phase. Bacterial cells were harvested by centrifugation and washed with dilute sulfuric acid (at a pH of 2.0). Genomic DNA was extracted following the procedure reported by Bergamo et al. [18]. The16S rDNA gene was performed by a PCR machine (ABI 2720, Applied Biosystems, Foster City, CA) using the forward primer 27F (5′–AGAGTTTGATCCTGGCTCAG–3′) and the reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′). The 25 μL reaction mixture contained 1.0 μL template DNA (about 50 ng/μL), 12.5 μL of 2× PCR master Mix (Biomed, China), 9.5 μL of ddH_2O, 1.0 μL of forward and reverse primer respectively (5 μM). The thermal cycler protocol was an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 45 s with a final 10-min extension at 72°C.

The PCR product was purified with a PCR Product Recovery kit (Biomed, China), ligated into pGEM-T vector (Biomed, China), transformed into *Escherichia coli* Top10 competent cells (Biomed, China), and the inserts were amplified and sequenced with M13± universal primers on a vector. Sequence identification was initially estimated by using of the BLASTN facility of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/). Sequence alignment was performed using the Clustal X program and the trees were constructed the neighbor-joining method with MEGAR 6.0 software. Bootstrap analyses were conducted using 1000 replicates. The 16S rDNA gene sequence of isolated strain BYQ-12 was submitted to GenBank to get an accession number.

2.4. Optimum growth conditions

To determine the optimum pH and temperature for growth, BYQ-12 was grown in modified 9 K-Fe medium at 30°C and different initial pH values of 1.5, 2.0, 2.5, 3.0, and 3.5; or at initial pH 2.5 and different temperatures of 25, 30, 35, 40 and 45°C. The final cell density was about 1.0×10^7 cells/mL after inoculation. Batch cultivation of bacteria was performed in triplicate and incubated in Erlenmeyer flasks under shaking at 150 rpm and 30°C for 60 h. The loss of water content in the flasks due to evaporation was replenished with distilled water by weight method. Bacterial growth was estimated by dynamically monitoring ferrous ion oxidation [13].

2.5. Arsenic resistance experiment

Arsenic resistance of *A. ferrooxidans* BYQ-12 was studied using modified 9 K-Fe medium supplemented with different concentrations (8–128 mM) of arsenite ($NaAsO_2$) or arsenate ($NaHAsO_4$), adjusted to pH 2.5. All cultures were carried out in triplicate at 30°C on a gyratory shaker for approximately 60 h, during which the growth was monitored. Control experiments were also performed with same media without arsenic. Growth of the isolates was measured in terms of ferrous ion oxidation. The inhibitory concentration (IC) of arsenite or arsenate was determined following a procedure reported previously [13].

2.6. RSM-based bioleaching experiment

The realgar used in experiments was obtained from Shimen County of Hunan Province, China and purified by traditional methods. The raw realgar was ground and sieved to a 200 mesh size. Our previous study shows that the studied realgar consisted of realgar (97%, *w*/*w*) and

arsenolite (3%, *w*/*w*), and the amount of arsenic in the ore is about 68.0% (*w*/*w*) [19].

Bioleaching experiments were carried out in 250 mL Erlenmeyer flask containing 100 mL modified 9 K liquid medium at 150 rpm on a rotary shaker at 30°C for 25 d. The BYQ-12 bacterial suspension containing 1.5×10^8 cells/mL was used as a 10% (*v*/*v*) inoculum for bioleaching. During the experiments, make-up distilled water was added periodically to the flasks to compensate for evaporation loss; afterwards the pH of the solution was adjusted back to its initial value with 5 M H_2SO_4. Based on the single factor experiment (data not shown), a three factor three level Box–Behnken design (BBD) having one central point without any replicate was adopted using the statistical software Design-Expert (Version 8.0.6, Stat-Ease Inc., Minneapolis, USA) to investigate the best condition for arsenic bioleaching. Their range and levels were listed in Table 1.

The quadratic polynomial regression model described as Equation (1) was chosen for predicting the response variable in terms of the three independent variables:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{23} BC + \beta_{13} AC \quad (1)$$

where Y is the predicted response, β_0 intercept, β_1, β_2, β_3 linear coefficients, β_{11}, β_{22}, β_{33} squared coefficients, β_{12}, β_{23}, β_{13} interaction coefficients and A, B, C, A^2, B^2, C^2, AB, BC, AC are levels of the independent variables. The experimental results were investigated by applying the coefficient of determination (R^2), analysis of variance (ANOVA) and response plots. The significance level employed in the analysis was 5%. Moreover, a confirmatory experiment was done at optimal values predicted by the models.

2.7. Analytical procedure and methods

Ferrous ion concentration was determined by titrimetric method using $K_2Cr_2O_7$ with a diphenylamine as the indicator and ferrous ion oxidized percentage was calculated and expressed as the ratio of oxidized ferrous ion to initial ferrous ion in cultures [20]. The close correlation between the rates of ferrous ion oxidation and cell growth due to the bacterial property of utilizing ferrous ion as energy source for growth. The cell number was counted using a hemocytometer under an optical microscope and the rates of ferrous ion oxidation were analyzed simultaneously. The linear relationship between ferrous ion oxidation and cell growth was experimentally established and expressed as $Y = 1.68E-9X-0.067$ (Adj. $R^2 = 0.993$, $P < 0.001$). SEM (JSM-6701F, JEOL, Japan) was used to examine the morphology of the bacterial cells. Field-emission SEM (S-4800, Hitachi, Japan) equipped with an energy dispersive X-ray analyser (EDS) (S-4800, Hitachi, Japan) was used to characterize the initial sample powder and the final leaching residue. Inductively coupled plasma atomic emission spectrometer (ICP-AES, IRIS Advantage ER/S, Thermo Jarrell Ash, USA) with standard procedures was used to analyze the total arsenic in the bioleaching solutions. Arsenic bioleaching rate was calculated and expressed as the percentage of leached arsenic ion to initial arsenic ion in realgar. The phases of initial realgar sample and leached residues were determined by XRD (X'Pert Pro, PANalytical, Netherlands), using a diffractometer with a Cu target.

Table 1
Experimental plan based on BBD and the results of arsenic bioleaching.

RUN	Factors			Bioleaching rate (%)
	A: Pulp concentration (%)	B: pH	C: Initial ferrous ion concentration (g/L)	
1	0.50	1.75	1.00	19.36
2	0.50	2.00	3.00	21.84
3	0.50	1.75	5.00	58.85
4	0.50	1.50	3.00	21.32
5	1.00	1.50	5.00	37.63
6	1.00	2.00	1.00	18.77
7	1.00	1.75	3.00	72.45
8	1.00	1.50	1.00	20.84
9	1.00	1.75	3.00	73.04
10	1.00	2.00	5.00	33.91
11	1.00	1.75	3.00	72.29
12	1.50	2.00	3.00	17.86
13	1.50	1.50	3.00	23.41
14	1.50	1.75	5.00	34.39
15	1.50	1.75	1.00	42.55

3. Results and discussion

3.1. Isolation of A. ferrooxidans and its characteristics

It was found that after 3 d incubating of the lake water sample in the 9 K-Fe liquid medium at 30°C, the medium became reddish-brown due to the oxidation of ferrous ion to ferric ion by iron oxidizing bacteria. After 3 sequential subculturing, the appropriate dilutions of enrichment culture were spread on plates and incubated at 30°C. Round beige-colored colonies were developed on the plates after 7 d incubation, and turned to deep brown in the following 3 d (Fig. 1a). By repeating the above approach, a strain BYQ-12 was obtained.

It can be found that the colonies of the isolate BYQ-12 on solid medium showed smooth, round, slightly raised and a diameter of 1.0–2.0 mm after 10 d incubation at 30°C. The raised centers of the colonies were rust colored and surrounded by a yellow zone (Fig. 1a). The BYQ-12 cells were rod-shaped with a length ranging in 0.8–1.8 μm by a width of 0.5–0.6 μm, and occurred singly or rarely in pairs (Fig. 1b), which is very much similar with the *A. ferrooxidans* isolates obtained from acid mine water of different regions of China [15,20].

The strain BYQ-12 was then subjected to molecular identification. The PCR product of BYQ-12 was detected by 1.0% agarose gel electrophoresis (Fig. 1c). The approximately full-length 1.5 kb 16S rDNA was amplified and sequenced. The sequence has been deposited in the GenBank database under the accession number KP836245. The determined partial nucleotide sequence of 16S rDNA was used to find maximum similarity with the bacterial strains in the GenBank database. The phylogenetic relationships based on 16S rDNA sequences indicated that BYQ-12 is a member of the gamma subclass of the *Proteobacteria* and belongs to the genus *Acidithiobacillus*, species *ferrooxidans* (Fig. 1d). The 16S rRNA gene sequence of BYQ-12 shares an identity of 99.93% and 98.58% to that of *A. ferrooxidans* ATCC19859 and *A. ferrooxidans* ATCC23270.

3.2. pH and temperature for culture of isolate BYQ-12

Growth conditions such as culture pH and incubation temperature of *A. ferrooxidans* BYQ-12 were investigated. During 60 h of incubation, the cultures with different initial pH (1.5, 2.0, 2.5, 3.0, and 3.5) and different temperature (25, 30, 35, 40 and 45°C) gradually turned to turbid suspensions finally with reddish brown color. This suggested that strain BYQ-12 was able to survive in a wide pH range from 1.5 to 3.5, and exhibited a broad temperature range from 25 to 35°C.

Fig. 2a shows dynamic variations of ferrous ion oxidized percentage under five different pH values. There were more than 92.57% of ferrous ion oxidations noticed in the cases of initial pH 2.0 and 2.5 during the first 30 h of incubation. It can be seen that completion of ferrous ion oxidation in the cultivations with an initial pH 2.5 was earlier 12 h than those with an initial pH 2.0 (Fig. 2a). Results showed 2.5 as the optimum pH for growth of strain BYQ-12 at pH 30°C.

Most of the isolated strains of *A. ferrooxidans* are mesophilic with temperature optima 28 to 40°C. During 60 h of incubation, the lowest and highest percentages of ferrous ion oxidation (31.32% and 99.59%) were observed in the cultivations at 45 and 30°C, respectively

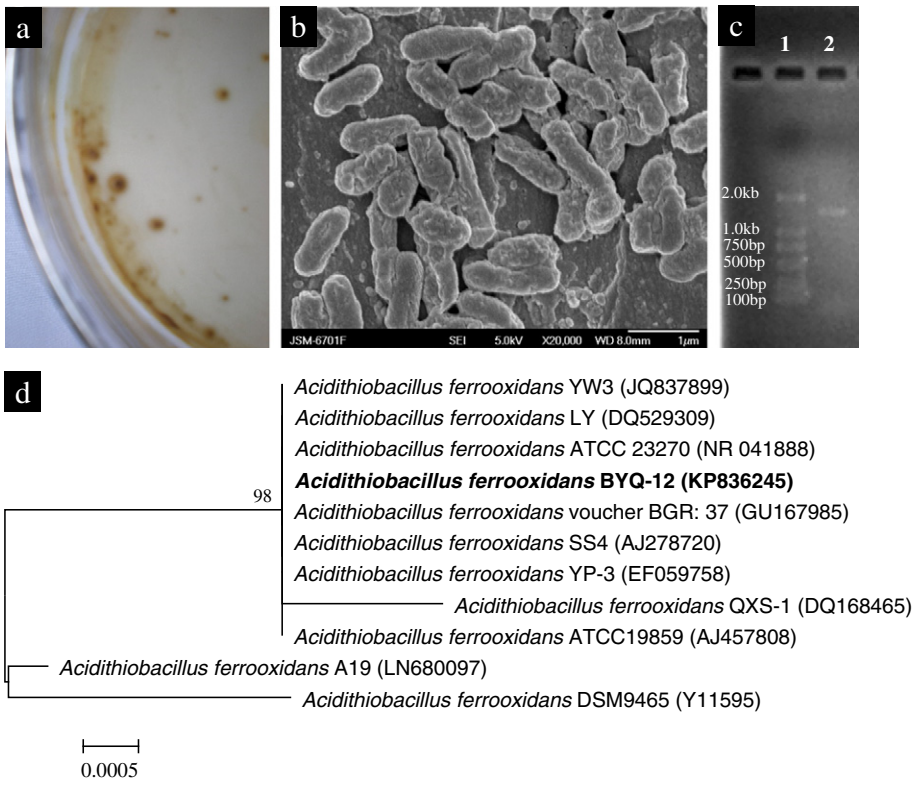

Fig. 1. Colony morphology, cell morphology, genomic DNA analysis and phylogeny of isolate BYQ-12. (a): colony morphology; (b): cell morphology; (c): 16S rDNA fragments amplified from the genomic DNA isolated from its pure cultures (Lane 1: BYQ-12 and lane 2: 10 kb DNA ladder); (d): phylogenetic tree derived from the 16S rDNA gene sequence of the strain BYQ-12.

(Fig. 2b). Although ferrous ion could be completely oxidized at 25, 30 and 35°C, the completion time of ferrous ion oxidation at 30°C was earlier 12 h than those at 25 and 35°C (Fig. 2b). Thus, the optimal temperature for growth of strain BYQ-12 was found to be 30°C under pH 2.5. It also revealed that 45°C as the maximum temperature for growth of strain BYQ-12 at pH 2.5. Our results are consistent with the findings of the previous study that the maximum temperature for bacterial growth was pH dependent, being 45°C over the pH range of 2.5 to 3.5 and temperature 35°C at a pH value of 1.5 [15].

3.3. Arsenic resistance of A. ferrooxidans

The growth of *A. ferrooxidans* was mainly reflected by variation of percent ferrous ion oxidized during the incubation period [13]. The growth responses of these bacteria at different concentrations of arsenite are given in Fig. 3a. At 8 mM, the growth of *A. ferrooxidans* was similar to that of the control; at 16 and 32 mM, no adverse effect on growth was observed. However, growth was completely inhibited at concentrations higher than 48 mM. The growth of *A. ferrooxidans* at 8, 16 and 32 mM of arsenate was similar to that of the control (Fig. 3b). However, a significant increase of the lag phase and delayed entrance in the stationary phase were observed at 64 mM and growth was completely inhibited at 96 and 128 mM (Fig. 3b).

In this study, the ICs of arsenite and arsenate have been determined in triplicate for *A. ferrooxidans*. Results revealed that the ICs of arsenite and arsenate were found to be 32 and 64 mM, respectively. This showed that arsenite is more toxic than arsenate. Similar results have been observed in a number of studies reported previously [21]. The resistance of BYQ-12 (from volcanic lake) to arsenic was higher than the *A. ferrooxidans* bacterium from mine habitats [11,15]. Arsenite and arsenate were the dominant arsenic species present in cultures of *A. ferrooxidans* for bioleaching of realgar [22]. Obviously, this bacterium BYQ-12 has potential application in arsenic leaching from the arsenical polymetallic sulfides.

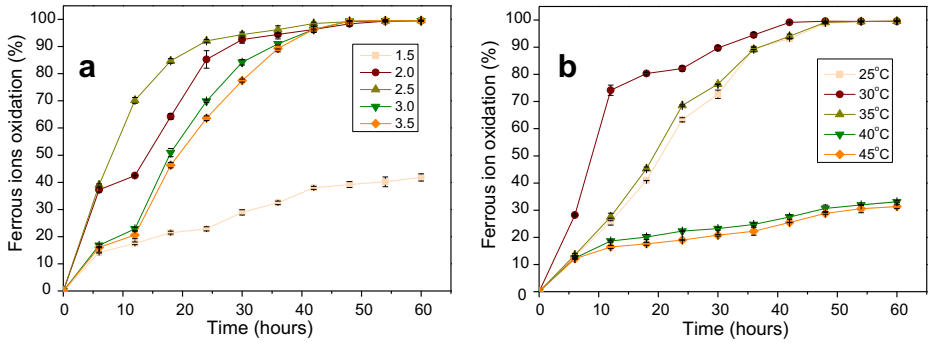

Fig. 2. Effects of pH and temperature on the growth of isolate BYQ-12. a: under different initial pH at temperature of 30°C; b: under different temperatures at an initial pH 2.5.

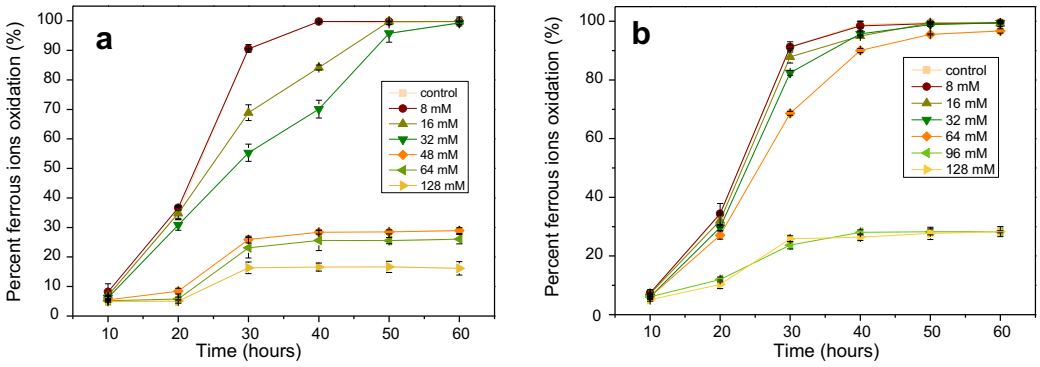

Fig. 3. Effect of arsenic concentration on the growth of isolate BYQ-12. (a): under different concentrations of arsenite; (b): under different concentrations of arsenate.

3.4. ANOVA of arsenic bioleaching

3.4.1. Model fitting

Table 1 lists the values of arsenic bioleaching rate after 25 d of incubation at each of the 15 combination of factor levels with the values ranging from 17.86% to 73.04%. The ANOVA results analyzed using Design Expert 8.0.6 are shown in Table 2. The accuracy and variability of the model is calculated with the coefficient of determination R^2 [23]. The value of the determination coefficient R^2 was found to be 0.99 (Table 2), close to unity, indicating a high validity of the model and a perfect coherence between experimental data and predicted value. The adjusted R^2 value of 0.99 implied that there is a 99% chance that the independent variables explain the variations in arsenic leaching efficiency and there is only a 1% chance that the variations in efficiency cannot be explained by the model.

The model F-value of 7268.26 implied the significance of the model, indicating that there is only a 0.01% chance that the "Model F-Value" in Table 2 results from noise. The "Lack of Fit F-value" of 0.35 implied the lack of fit is not significant relative to the pure error. There is a 79.63% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of indicated that the model fit the experimental result very well. Generally, the model adequacy checking is always necessary to examine the fitted model to ensure that it provides an adequate approximation to the true system, and to verify that none of the least squares regression assumptions are violated [24]. In arsenic bioleaching model, the adequate precision ratio of 217.03 indicated an adequate signal-to-noise ratio; a value greater than 4 is desirable.

The *P*-values less than 0.0500 indicates that the model terms are significant, whereas the values greater than 0.1000 are not significant. In Table 2, all the terms are significant according to *P*-values, indicating that a second order polynomial model is necessary to represent the data. Main effect plots (not shown) indicated that increase in the level of all the three factors-within the range studied-had a statistically significant effect on the rate of arsenic bioleaching after 25 d; increase in the level of these factors up to a certain (optimum) value led to an increase in the rate of arsenic bioleaching whereas further increases resulted in the opposite effect. The significant interaction among A (pulp concentration), B (pH) and C (initial ferrous ion concentration) revealed that the optimum level of one of these three factors in arsenic bioleaching process depends on the level of other two factors.

Table 2
ANOVA for the response surface model applied.

Source	Sum of squares	Degree of freedom	Mean square	F-value	*P*-value Prob > F
Model	6244.67	9	693.85	7268.26	<0.0001
A	1.25	1	1.25	13.08	0.0153
B	14.63	1	14.63	153.29	<0.0001
C	500.23	1	500.23	5240.01	<0.0001
AB	9.21	1	9.21	96.49	0.0002
AC	567.63	1	567.63	5946.06	<0.0001
BC	0.68	1	0.68	7.13	0.0443
A^2	1513.02	1	1513.02	15,849.2	<0.0001
B^2	3604.13	1	3604.13	37,754.13	<0.0001
C^2	679.21	1	679.21	7114.88	<0.0001
Residual	0.48	5	0.095		
Lack of fit	0.17	3	0.055	0.35	0.7963
Pure error	0.31	2	0.16		
Cor total	6245.15	14			

$R^2 = 0.99$, $R^2_{adj} = 0.99$, adequate precision = 217.03 (>4).

The coefficients of the regression equation were calculated and the following regression equation was obtained.

$$\text{Bioleaching rate } (\%) = 72.59 - 0.40A - 1.35B + 7.91C - 20.24A^2 - 31.24B^2 - 13.56C^2 - 1.52AB - 11.91AC - 0.41BC \quad (2)$$

Equation (2) showed that initial ferrous ion concentration (C) exert a positive linear effect. While a negative linear effect of pulp concentration and pH is also evident. Both quadratic and interactive terms had negative effects on arsenic bioleaching. It is obvious from Equation (2) that a greater amount of arsenic leaching results from a higher initial ferrous ion concentration but, at the same time, the negative quadratic effects and negative interactions between parameters should also be considered.

3.4.2. Optimization of bioleaching conditions

Arsenic can be leached from realgar via purely chemical and biologically-mediated extraction in microbial leaching system with ferrous ion. The purely chemical process can be expressed as follows [11]:

$$As_2S_2 + 14H_2O \rightarrow 2H_3AsO_3 + 2HSO_4^- + 10H^+ \quad (3)$$

$$2H_3AsO_3 + H_2O \rightarrow H_3AsO_4 + 2H^+ \quad (4)$$

And the biologically-mediated reaction can be described by the following equations [12]:

$$As_2S_2 + 3O_2 + 6H_2O \rightarrow 4H_3AsO_3 + 4S \quad (5)$$

$$2S + 3O_2 + H_2O \rightarrow 2SO_4^{2-} + 4H^+ \quad (6)$$

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \quad (7)$$

$$As_2S_2 + 6Fe^{3+} \rightarrow 2As^{3+} + 2S + 6Fe^{2+} \quad (8)$$

$$HAsO_2 + 2Fe^{3+} + 2H_2O \rightarrow H_3AsO_3 + 2Fe^{2+} + 2H^+ \quad (9)$$

The purely chemical process is slower and only dominates the overall arsenic bioleaching process in the early part when the bacterial population is going through their lag or acceleration phase of growth. The variation of leaching condition can lengthen the period of this early phase. Obviously, the biologically mediated arsenic bioleaching is also inherently a chemical process. However, this process directly depends on the rate of bacterial activity since arsenic bioleaching rate is limited by the concentration of ferric ion which is regenerated via biochemical activity of *A. ferrooxidans* BYQ-12.

To understand the interaction of the variables, the contour plots have been developed for the proposed empirical relation by keeping the other variables at their optimum levels as shown in Fig. 4. These response contours can help in the prediction of the response (bioleaching rate) for any zone of the experimental domain. It is clear from Fig. 4 that the arsenic bioleaching rate increased with the increase of pulp concentration, pH, and initial ferrous ion concentration to a certain value and then decreased. The statistically significant interaction between pulp concentration and pH can be explained by the increase in the contribution of the purely chemical leaching to the overall arsenic extraction, as a result of increase in pulp concentration. It has been reported that the rate of metal extraction is strongly influenced by the pulp concentration of the leach systems [25]. It can be seen from Fig. 4a the arsenic bioleaching rate increased with increasing pulp concentration from 0.50% to 0.95%, and then decreased slightly above 0.95% (*w*/*v*). In the low-range of pulp concentration (0.50%–0.95%), the realgar did not inhibit the growth of bacteria, the arsenic bioleaching rate increased from approximately 21.32 to 73.04%. However, the arsenic bioleaching rate descended to 23.41% when the pulp concentration increased to 1.50%. This phenomenon can be explained by the facts that, an increase in pulp concentration (exceed 0.95%), decrease the rates of oxygen/carbon dioxide transfer and also decrease the bacterial activity [26].

The variation of pH and initial ferrous ion concentration has dissimilar influences on the rate of arsenic leaching and that of bacterial growth. An increase in pH and a decrease in ferrous ion concentration both lead to an increase in the rate of arsenic leaching [25]. It is generally agreed that *A. ferrooxidans* has optimum pH for its activity. Therefore, any increase or decrease in pH away from the optimum value should lead to decrease in the rate of biologically-mediated arsenic extraction. The biologically-mediated leaching process is faster and dominates the overall arsenic leaching process in microbial leaching system. Thus, the pH which was predicted to result in the highest arsenic bioleaching rate should correspond to the pH which results in optimum activity of *A. ferrooxidans*. It has been reported that there is an optimum value of initial ferrous ion concentration for the growth and activity of *A. ferrooxidans* [11,12]. According to Fig. 4b and c, increase in initial ferrous ion concentration (in the range 1.00–3.68 g/L) leaded to an increase in arsenic bioleaching rate, whereas it decreased after passing the optimum value (3.68 g/L). This trend might be attributed to several reasons. Firstly, low concentrations of initial ferrous ion can promote the dissolution of realgar effectively by bacterial oxidation of ferrous ion to ferric ion [25]. Secondly, high concentrations of ferric ion which produced from high concentrations of initial ferrous ion by *A. ferrooxidans* could inhibit the bacterial activity, preventing the further oxidation of realgar [12]. Thirdly, the adsorption of soluble arsenic by *A. ferrooxidans* cells, ferric iron and its compounds resulted in the reduction of arsenic in system [15,21,25]. Finally, the sulfur layer formed in the reaction described by Equations (5) and (8), and the jarosite yielded in the reaction described by Equation (10) could accumulate on the surface of realgar, preventing direct bacterial action and/or ferric iron attack [11]. The equation of jarosite precipitation can be can be represented as follows [12]:

$$3Fe^{3+} + 2SO_4^{2-} + X^+ + 6H_2O \rightarrow X[Fe_3(SO_4)_2(OH)_6] + 6H^+ \quad (10)$$

where X^+ is a monovalent cation (generally As^{3+}, K^+, Na^+, NH_4^+ or H_3O^+).

The objective of this study was to determine the conditions of maximum arsenic bioleaching. Thus, by solving the Equation (2) which is statistically significant, the optimal values of pulp concentration, pH and initial ferrous ion concentration were estimated to be 0.95% *w*/*v*, 1.74 and 3.68 g/L, respectively. The predicted response at this point using Equation (2) is 73.97%.

In fact, there were many other ways to enhance the arsenic bioleaching rate besides optimization of physicochemical conditions mentioned-above. It has been documented that the domestication using arsenic or realgar can increase the bacterial activity, which would lead to the improvement of the arsenic bioleaching rate [11]. High-efficiency bacteria used for bioleaching can be obtained through UV irradiation or genetic manipulation [27]. Previous study indicated that the co-culture of *A. ferrooxidans* with other microbe can benefit the increase of the arsenic bioleaching rate [11]. The arsenic bioleaching rate was significantly improved in the presence of silver ion [9]. Additionally, the pretreatment of ore using ultrasound and microwaves might lead to the improvement of bioleaching rate [28,29].

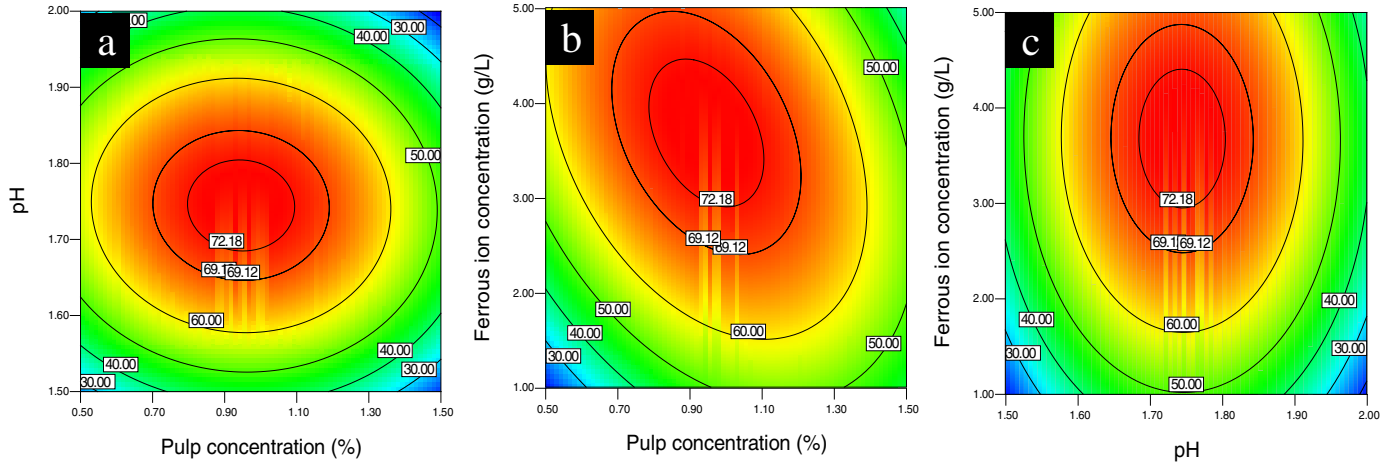

Fig. 4. Contour plots of the interactive effect for arsenic bioleaching. (a): effect of initial pH and pulp concentration at the constant initial ferrous ion concentration of 3.68 g/L; (b): effect of initial ferrous ion concentration and pulp concentration at the constant initial pH of 1.74; (c): effect of initial ferrous ion concentration and initial pH at the constant pulp concentration of 0.95%.

3.4.3. Confirmation testing

To test the validity of the optimized condition given by the model, an experiment was carried out in the optimal condition. The results showed that after 25 d, the arsenic bioleaching rate was 74.27%. Therefore, the model with an error margin of only 0.30% can fit the experimental data and acceptably is valid.

3.5. Characterization of bioleached realgar

The SEM micrograph showed that the realgar surface was smooth and clear before leaching (Fig. 5a). However, after interaction for 25 d, the surface of the mineral particles was rough and covered with a polyporous layer, and etched pits were also present (Fig. 5c). Large quantities of short rod-shaped bacteria, *i.e. A. ferrooxidans* cells, were attached or embedded in these etched pits, as there was direct bioleaching besides indirect electrochemical leaching in the bacterial bioleaching system. In addition, large amounts of deposits, in the form of porous floc, were seen on the realgar surfaces after 25 d of exposure to *A. ferrooxidans* (Fig. 5c). The generation of etch pits and deposits have also been demonstrated by many previous reports [11,19].

In order to analyze the components of realgar surface, EDS and XRD analysis of leached residues were conducted. The EDS analysis revealed that the major constituents of realgar was As and S before leaching (Fig. 5b) but changed to As, S, Fe and K after 25 d leaching (Fig. 5d). This might be attributed to the production of magnetite and/or jarosite [19]. The ratios of arsenic to sulfur for realgar decreased from 0.97 at the beginning of the experiment to 0.65 after 25 d leaching. This demonstrated that arsenic was bioleached prior to sulfur in this bioleaching process [11], and *A. ferrooxidans* can efficiently enhance the dissolution of realgar. The XRD patterns of the original mineral and bioleached realgar are displayed in Fig. 5e and f. The original mineral showed peaks of realgar and arsenic trioxide, but after 25 d of bioleaching, additional peaks of dimorphite, magnetite and jarosite emerged. This result was consistent with those of SEM and XRD analysis.

4. Conclusions

An acidophilic ferrous iron-oxidizing bacterium BYQ-12 was isolated from volcanic lake. The morphological, physiological and phylogenetic analyses indicated that it was identified as *A. ferrooxidans*. It can tolerate 32 mM arsenite and 64 mM arsenate which to our knowledge exceeds tolerance levels previously reported. The BBD-RSM analysis showed that maximum arsenic bioleaching rate was 73.97% under optimum conditions, and the most effective factor for arsenic leaching was initial ferrous ion concentration. SEM, EDS and XRD analyses confirmed the role of BYQ-12 on leaching. Results revealed that this strain would be of great potential in the application of arsenic bioleaching.

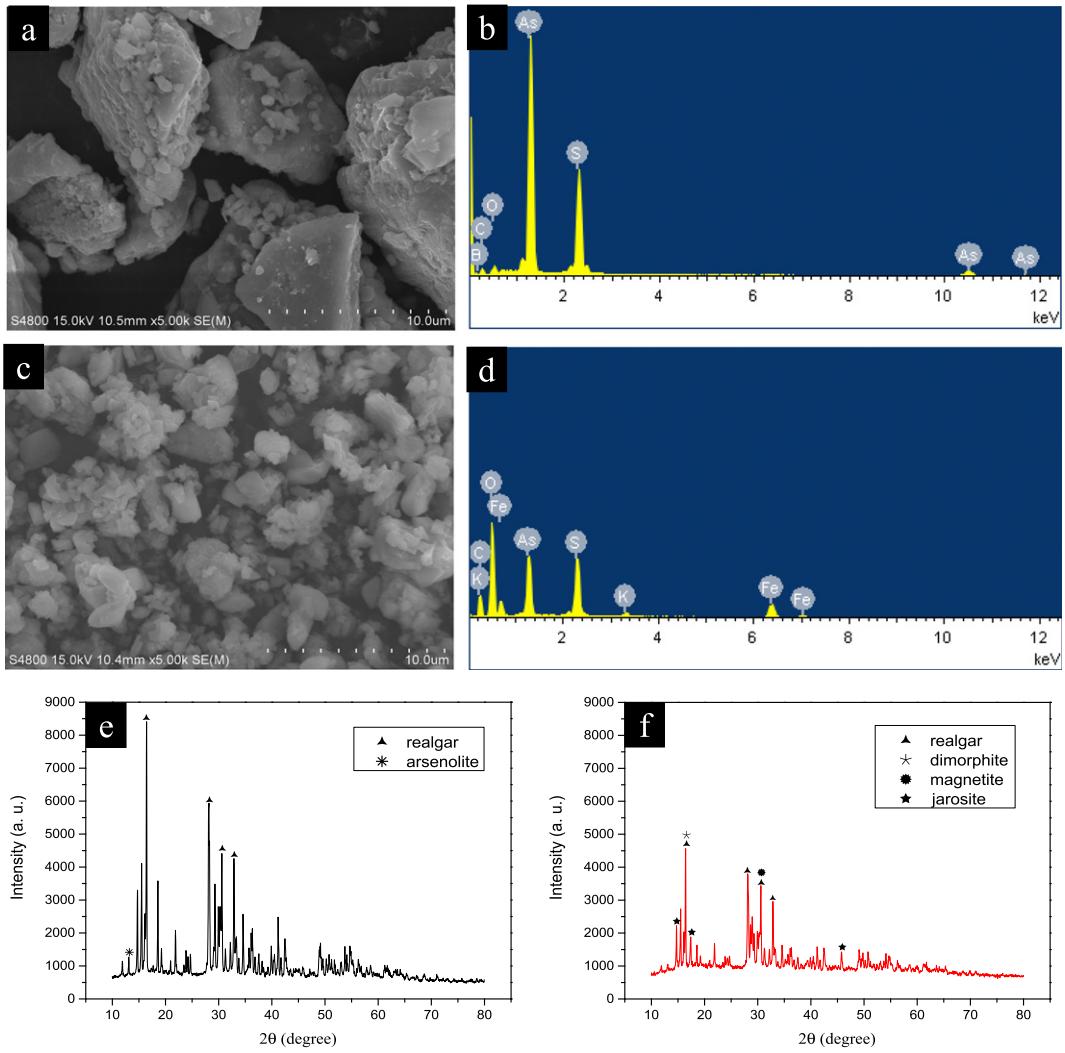

Fig. 5. SEM (a, c), EDS (b, d) and XRD (e, f) images of realgar before and after leaching with isolate BYQ-12. (a, b and e): raw realgar; (c, d and f): bioleached realgar.

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Conflict of interest statement

There are no conflicts of interest.

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