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Full Length Research Paper

Enhanced Fe^{2+} oxidation by mixed culture originated from hot spring: Application of response surface method

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For maximum oxidation efficiency and minimum amount of jarosite, a total of 30 experimental runs were conducted and the experimental data fitted to the experimental quadratic model. The analysis of variance (ANOVA) demonstrated that the model was highly significant. Three dimensional plots were illustrated to depict the interaction between all the factors. The experimental results indicated that, the optimal conditions for the desired objects were 1.3, 7 g/l, 141 rpm and 35°C for the media initial pH, Fe^{2+} ion concentration, agitation rate and temperature, respectively. Using the regression model, a maximal oxidation efficiency of 55% and minimum jarosite precipitation of 0.34 g/l were achieved with the consideration of the optimal condition for the parameters at the end of 26 days of operation. The experiment was carried out under optimal condition and resulted in efficient oxidation and jarosite amount of 52% and 0.32 g/l, respectively. Similar values were predicted by the model. According to these results, the response surface methodology was not only applied to determine the significance information on the interaction between the factors, but also was conducted at optimal conditions for the desired objectives. It was also concluded that, the mixed culture isolated from the hot spring had the potential to oxidize ferrous to ferric irons.

Key words: Biooxidation, response surface method, jarosite, biodesulfurization, process optimization.

INTRODUCTION

Oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) in the presence of microorganism is a quite useful process which is employed in many industrial processes. This biological process have widely been used for the treatment of acid mine drainage and bioleaching processes (Rawlings et al., 2003; Long et al., 2004). Also, it plays a major role in the regeneration of ferric iron (Fe^{3+}) as an oxidizing agent used in the process of desulphurization of sour gases and coal (Juszczak et al., 1995; Acharya et al., 2001; Nestor et al., 2001; Drogui et al., 2005; Daoud and Karamanev, 2006; Watling, 2006; Akcil et al., 2007). Hydrogen sulfide is a colorless, extremely toxic and flammable atmospheric pollutant which is a strict risk to human health and safe environment. Besides human threat, H_2S can cause major corrosion in industrial facilities. It occurs naturally in gases from volcanoes, sulfur hot springs, undersea vents, swamps and as a product of the biological degradation of organic matter (Lomans et al., 2002). Considerable amount of hydrogen sulfide are also generated and emitted from industrial activities, including petro-chemical refining, food processing, dye production, fuel treatment, wastewater treatment, natural gas processing, tanneries and viscose rayon manufactures (Guoqiang et al., 1994; Yang and Allen, 1994; Chung et al., 1996). Dissolved sulfide ions (S^{2-} and HS^{-}) in wastewater stream and also H_2S in tail gases are emitted into the environment. Traditional physico-

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chemical methods such as incineration, absorp-tion and chemical oxidation have been used for treatment of sour gas (Sorokin, 1994; Acharya et al., 2001; Mesa et al., 2004). These processes are relatively high energy intensive (Malhotra et al., 2002), high chemical and disposal cost and are also, associated with the release of by-products such as sulfur dioxide (Nemati et al., 1998). The biodesulphorization process has a number of advantages such as, less process costs and simple maintenance compared to the conventional sour gas treatment processes (Elvidge and Blitz, 1992; Gallup, 1996). They can operate at mild pressure and ambient temperature. Thus, the use of bacteria in oxidation of various mineral sulfides (hydrogen sulfide) has received significant commercial interests. Removal of H_2S from gas stream first involves chemical absorption of sour gas with a ferric sulfate solution. The solution absorbs H_2S and then it is oxidized to the sulfur form, while the ferric sulfate is reduced to ferrous sulfate as it is shown in the following reaction (Nemati et al., 1998). Elemental sulfur can be removed easily by solid-liquid separation (Mousavi et al., 2006).

$$H_2S + Fe_2(SO_4)_3 \rightarrow S_{\perp} + 2FeSO_4 + H_2SO_4 \qquad (1)$$

Secondly, bacteria are used to catalyze the oxidative reaction, thus, the Fe^{2+} produced in the first step were converted to Fe^{3+} as absorbing solution (Malhotra et al., 2002). In batch culture, several factors are influential in the biooxidation of ferrous ions by the related bacteria. It was reported that the media pH was quite effective in biooxidation process. Since, increasing the medium pH was counteracted with the hydrolysis of ferric iron (Daoud and Karamanev, 2006), it is clear that pH was effective on the hydrolysis of the reactions. The oxidation of inorganic substances such as ferrous sulfate release electrons which provide the bacteria with the essential energy for the metabolic activities such as growth and reproduction. Aerobic cultures used oxygen as the terminal electron acceptor, via acceptance of electrons through the cell wall from the chemical oxidation reactions (Mishra et al., 2005).

Jarosite formation

The overall oxidation of ferrous to ferric iron is catalyzed by bacteria via the following reaction:

$$4Fe^{+2} + O_2 + 4H^+ \xrightarrow{Bacteria} 4Fe^{+3} + 2H_2O \qquad (2)$$

According to the stated reaction, there was consumption of hydrogen ions; consequently the pH of the medium initially increased. However, the pH increase was counteracted by the hydrolysis of ferric iron which is in competition with the reaction by the given products such as basic ferric hydroxysulphates with the formula $MFe_3(SO_4)_2(OH)_6$,

where, $M = K^+, Na^+, NH_4^+, Ag^+$ or H_3O^+ (Jensen and Webb, 1995). The dark yellow hydroxysulphate precipitates are known as jarosites. The mixed bacteria obtained from the hot spring were grown in medium which contained a high concentration of NH_4^+ when compared with the other nutrients. Therefore, the jarosites mostly produced are ammoniojarosites with the formula of $NH_4Fe_3(SO_4)_2(OH)_6$. It follows the following chemical reaction (Liu et al., 2009):

$$3Fe^{+3} + NH_4^+ + 2SO_4^{2-} + 6H_2O \rightarrow NH_4Fe_3(SO_4)_2(OH)_6 + 6H^+ \qquad (3)$$

Jarosite formation is a very important phenomenon that is observed in many bacterial cultures. Jarosite mediated ferrous oxidation by the microorganism has negative influence on many applications, such as biodesulphurization of natural gas, coal biodesulphurization and bioleaching. Some of these effects includes the dissipation of ferric iron used as absorbent for hydrogen sulfide, decrease in bacterial absorbability of nutrients and reduction of the rate of bioleaching due to deactivation of the cell surface. In order to increase efficiency, the jarosite formation should be minimized.

In this study, the ability of microorganism isolated from hot spring for the oxidation of ferrous to ferric ions was investigated. The effects of media initial pH, Fe^{2+} ion concentration, agitation rate and temperature were evaluated by means of central composite design (CCD) under response surface methodology (RSM) using design expert software. Furthermore, a numerical optimization methodology was applied to optimize the biooxidation efficiency of ferrous to ferric ions along with minimal jarosite precipitation. The bacteria used in this study were aerobic, so the cell consumed oxygen for the biooxidation process.

MATERIALS AND METHODS

Microorganism and media

The iron oxidizing bacteria used in this work was isolated from Ramsar hot spring (Ramsar, Iran). The growth medium composition in 1 L solution was 132 mg $(NH_4)_2SO_4$, 53 mg $MgCl_2.6H_2O$, 27 mg KH_2PO_4 and 147 mg $CaCl_2.2H_2O$. Trace metal solution (1 ml) was made up of 62 mg $MnCl_2.2H_2O$, 68 mg $ZnCl_2$, 64 mg $CoCl_2.6H_2O$, 31 mg H_3BO_3, 10 mg Na_2MoO_4, and 67 mg $CuCl_2.2H_2O$. Distilled water was added to make it up to 1L solution. Ferrous ions as ferrous sulfate with five different concentrations were used as the substrate.

Experimental procedure

The experiments were carried out in a 250 ml Erlenmeyer flask that contained 100 ml of the basal media. The media were autoclaved and inoculated with a fresh seed culture where the size of inoculum was 5% (v/v). Batch culture was conducted in an incubator-shaker (Stuart, S1500, UK). The concentrations of Fe^{+3} and Fe_{tota} in the flasks were initially and also daily measured. The oxidation efficiency of ferrous iron and the amount of formed jarosite at the end of 26 days of the operation was considered for the performance of 30 designed experiments. This selection (26 days) gave more perfect results since the oxidation efficiency did not go to completion in any of the samples at the end of 26 days. Furthermore, the oxidation efficiency in some samples was more than 50%, thus the oxidation efficiency and amount of jarosite was accurately compared for all of the samples at the time. However, the results were compared with each other to obtain the desire and maximal objective. After 26 days of incubation, the flasks were removed from the shaker in order to measure the jarosite precipitation.

Analytical methods

For the jarosite precipitation measurements, firstly, the liquid in each flask was filtered using a vacuum flask and filter paper (pore size 25 µm). The solids that accumulated on the filter were then, returned back to the flask by washing them with distilled water. This was done in order to mix the filtered jarosite that were attached to the wall of the flask (Daoud and Karamanev, 2006). Then, approximately 15 ml of 50% H_2SO_4 were added to each flask in order to dissolve the jarosite (including the suspended culture and the biomass attached to the flask wall). Finally, the total iron concentration was measured using the sulfosalycilic method (Karamanev et al., 2002) and the volume of mixture in each flask was measured using graduated cylinders in order to get the total mass of jarosite. Jarosite precipitates were identified by the means of Shimadzu IR-460 fourier transform infrared spectroscopy.

The analysis of ferrous and ferric iron concentrations in the withdrawn samples was carried out by sulfosalycilic acid as a color indicator (Karamanev et al., 2002). Red-colored ferric–sulfo Salicylate complex indicated the concentration of Fe^{3+} in the solution. Ammonia was added. 5-sulfosalicylic acid (SSA) formed a yellow complex with all the iron ions, which gives a total iron concentration in the sample solution (Karamanev et al., 2002). The concentrations of Fe^{3+} and Fe_{total} were determined by spectrophotometer (Unico, 2100, USA) at wave lengths of 500 and 425 nm, respectively. The differences between concentrations of total iron and Fe^{3+} ions were used to obtain the Fe^{2+} ion concentration in the solution. The pH was monitored by pH meter (HANA, 211, Romania). All chemicals used in the study were of analytical grade and supplied by, Merck (Damestart, Germany).

Experimental conditions and optimization by response surface method

The traditional method of a tentative optimization involves one-factor-at-a-time approach, which means changing one variable at a time but keeping the others constant. This method is impractical to perform experiments with every probable combination of test variables because of the large number of experiments required (Myers et al., 2009). It is also a boring, cumbersome and time-consuming process because it needs a large number of parameters to be taken into account. In addition, it does not consider the effect of interactions of various parameters. Response surface methodology (RSM) is a statistical model, which can be used as an alternative approach for understanding complex processes and for describing interactions between different process parameters (Khuri and Cornell, 1987). The RSM consists of a group of empirical technique employed for the evaluation of the relationship existing between a cluster of controlled experiments factors using quantitative data obtained from properly designed experiments (Khuri and Cornell, 1987). It measures responses according to one or more selected criteria by solving many equations simultaneously.

According to the design, the total number of combinations was $2^k + 2k + n_0$ where, k is the number of independent variables and n_0 is the number of repetitions of the tests at the center point. Optimization studies were carried out by means of RSM using central composite design (CCD). In the primary experiments, it was found that 4 essential variables including Fe^{2+} concentrations, pH, temperature and agitation rate were quite effective in bio-oxidation process. Based on the importance of these four parameters on oxidation efficiency and jarosite amount, researches were planned. It was observed that these parameters were very influential on the objective function. Thus, RSM was used to demonstrate the interactions of these four parameters. The importance of other factors on bio-oxidation process may not be very crucial in bio-oxidation; however, certain degree of influences may be exerted by other factors. For simplicity, other variables were neglected.

The chosen independent parameters used in this study were coded according to Equation (4):

$$x_i = \frac{X_i - X_0}{\Delta x} \quad (4)$$

Where, x_i is the dimensionless coded value of the i^th independent variable, X_0 is the value of X_i at the center point and Δx is the step change value. Knowing that the variation of parameters does not follow linear form, second order polynomial model was chosen for simplicity of optimization of the process parameters. It is often customary to use quadratic 2nd order equation to observe any nonlinear function (Daneshi et al., 2010; Amini and Younesi, 2009). The manner of the system defined by the empirical second-order polynomial model is represented in Equation (5):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=1}^{k} \beta_{ij} X_i X_j + \varepsilon \quad (5)$$

Where, Y is the predicted response, $X_i, X_j, \ldots, X_k$ are the input variables which affect the response Y, $X_i^2, X_j^2, \ldots, X_k^2$ are the square effects, $X_i X_j, X_i, X_j X_k$ are the interaction effects, β_0 is the intercept term, $\beta_i\ (i = 1,2, \ldots, k)$ is the linear coefficients, $\beta_{ii} (i = 1,2, \ldots, k)$ is the squared coefficients, $\beta_{ij}\ (i = 1,2, \ldots, k; j = 1,2, \ldots, k)$ is the interaction coefficients and ε is a random error (Ghorbani et al., 2008).

The optimum values of the selected variables were achieved by solving the regression equation at the desired values of the responses and also by analyzing the response surface plots. Each of the parameters in the design was coded at five different levels ($-\alpha$, –1, 0, 1, $+\alpha$; where $\alpha = 2$). All variables were taken at a central coded value considered as zero. The range and the levels of the variables investigated in this study are given in Table 1. A 2^4 full factorial central composite design for four independent parameters each at five levels with eight star points and six replicates at the centre points was employed to fit a quadratic model in which 30 experiments were required for this procedure (Table 2). A total of 24 experimental runs were carried out in triplicates and the average values were recorded. The last 6 remaining values (rows 25 to 30) were automatically given by the software as repeated for the 6 sets of experiments and all of the individual values are recorded in Table 2.

At the end of 26 days of operation, the oxidation efficiency and jarosites amounts were considered as the dependent variables or responses (Y_1 and Y_2). Design expert 7.01 (Stat-Ease Inc., Minneapolis, MN, USA) software was used for the regression and graphical examination of the experimental results. Central composite

Table 1. Experimental ranges and levels of the independent variables.

Independent variable	Range and level				
	$-\alpha$ (-2)	-1	0	+1	$+\alpha$ (+2)
Media initial pH (X_1)	1	1.5	2	2.5	3
Fe^{+2} concentration, g/l (X_2)	2	4	6	8	10
agitation rate, rpm (X_3)	80	110	140	170	200
Temperature, ℃ (X_4)	25	30	35	40	45

design (CCD), which is the most commonly used method under RSM design, was applied to optimize the parameters.

RESULTS AND DISCUSSION

Identification of jarosites

The jarosites precipitate in the medium contained bacteria which were collected and then, air dried naturally. Jarosites were identified by the means of Fourier transform infrared spectroscopy (FTIR). Figure 1 depict that the absorption peak in the region of 2850-3550 cm^{-1} was attributed to the O-H stretching and N-H stretching of NH_4^+; the absorption peak of 1631 cm^{-1} were ascribed to the N-H bending. The ϑ_4 ϑ_3, ϑ_2 and $\vartheta_1$vibration of SO_4 were represented in the wave numbers of 607, 1122, 984 and 899 cm^{-1}, respectively; the vibration peaks of FeO_6 octahedron were observed in the wavenumbers of 510 cm^{-1}. Therefore, the stated results proved that the precipitates that was bio-mediated was aminojarosites.

Process optimization

RSM is a sequential procedure with an initial goal to conduct the experiments rapidly and effectively towards the vicinity of the optimum. This method is appropriate when the optimal region for operating the process has been identified. In this study, four independent variables of media initial pH, Fe^{2+} ion concentration, agitation rate and temperature were chosen to optimize the oxidation of ferrous to ferric ions along with minimum amount of formed jarosite.

The results of the second-order response surface model fitted in the form of analysis of variance (ANOVA) are given in Table 3. ANOVA was required to test the significance and adequacy of the model. Furthermore, the statistical significance of the quadratic model was evaluated by the F-test ANOVA. The significance of each coefficient was determined by F-values and P-values. The values of "Prob > F" of less than 0.05 indicated that the model terms are significant. According to Table 3, for oxidation efficiency, the coefficients for the main and the square effects were highly significant in comparison with the interaction effects. For jarosite, the main effects of the variables of X_1X_2, X_1X_3, X_1X_4 which represent the interaction effects and the square effects of X_1^2, X_3^2, X_4^2 were the significant model terms.

The accuracy of the fit of the model was also checked by the multiple correlation coefficients (R^2). R^2 value always varies from 0 to 1. It shows how much variability in the observed response value can be explained by the experimental parameters and their interactions. If the R^2 value is closer to 1, the model would be strong and it can predict the response better (Khuri and Cornell, 1987). In this case, the values of R-squared for oxidation efficiency and jarosite (R^2= 0.9911 and 0.9927, respectively) and the adjusted R-squared (adj.R^2 =0.9828 and 0.9859, respectively) indicated a high correlation between the observed and predicted values (Table 4). The predicted R-squared (pred.R^2) of 0.9630 and 0.9649 for oxidation and jarosite, respectively, were in reasonable agreement with R^2 and adjusted R^2. The actual value and predicted values are summarized in Table 2. The average error was less than 0.5%. This means the predicted values were quite close to the actual value with R^2 values of close to unity. Moreover, the coefficient of variance for oxidation efficiency and jarosite (C.V. =2.25% and 6.95%, respectively) was low, which implies significant precision and reliability of the experimental data (Can et al., 2006). Adequate precision, measures the signal to noise ratio and a ratio greater than four is desirable. The signal to noise ratio of 38.113 and 44.751 for oxidation efficiency and jarosite, respectively, were high, indicating adequate precision and reliability for the experiments carried out. The examination of the fit summary output advocated that the quadratic model was statistically significant for the response and consequently was used for further analysis. The relationships between the oxidation efficiency and jarosites were responses and the independent variables in coded units were expressed by the regression equation of Y_1 and Y_2, respectively (Table 4).

Table 5 shows ANOVA for the response surface quadratic model. However, the model F-value of 119.5 and 145.74 and values of probability (< 0.0001) indicated that, the model terms were significant for oxidation efficiency and jarosite. The mean squares resulted by

Table 2. Full factorial central composite design matrix of four test variables in coded and real values along with the observed responses.

Run number	Independent variable								Oxidation efficiency (%)		Jarosites (g/l)	
	Coded values ^{a}				Real value				Experimental value	Predicted value	Experiment al value	Predicted value
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4				
1	-1	-1	-1	-1	1.5	4	110	30	42	41.58	0.34	0.33
2	1	-1	-1	-1	2.5	4	110	30	32	31.63	2.35	2.44
3	-1	1	-1	-1	1.5	8	110	30	50	50.29	0.80	0.79
4	1	1	-1	-1	2.5	8	110	30	41	40.08	2.50	2.46
5	-1	-1	1	-1	1.5	4	170	30	40	39.79	0.16	0.10
6	1	-1	1	-1	2.5	4	170	30	28	28.58	1.70	1.80
7	-1	1	1	-1	1.5	8	170	30	49	49.75	0.60	0.67
8	1	1	1	-1	2.5	8	170	30	38	38.29	2.00	1.93
9	-1	-1	-1	1	1.5	4	110	40	47	46.79	0.70	0.81
10	1	-1	-1	1	2.5	4	110	40	38	37.08	2.50	2.44
11	-1	1	-1	1	1.5	8	110	40	56	55.25	1.40	1.31
12	1	1	-1	1	2.5	8	110	40	45	45.29	2.40	2.50
13	-1	-1	1	1	1.5	4	170	40	45	45.75	0.50	0.55
14	1	-1	1	1	2.5	4	170	40	35	34.79	1.70	1.75
15	-1	1	1	1	1.5	8	170	40	55	55.46	1.20	1.15
16	1	1	1	1	2.5	8	170	35	44	44.25	1.90	1.92
17	-2	0	0	0	1.0	6	140	35	52	51.63	0.13	0.15
18	2	0	0	0	3.0	6	140	35	30	30.46	3.10	3.03
19	0	-2	0	0	2.0	2	140	35	34	34.46	0.75	0.64
20	0	2	0	0	2.0	1	140	35	53	52.63	1.20	1.26
21	0	0	-2	0	2.0	6	80	35	43	44.46	2.40	2.38
22	0	0	2	0	2.0	6	200	35	43	41.63	1.60	1.57
23	0	0	0	-2	2.0	6	140	25	38	37.96	1.00	0.99
24	0	0	0	2	2.0	6	140	45	49	49.13	1.50	1.46
25	0	0	0	0	2.0	6	140	35	50	50.50	0.90	0.92
26	0	0	0	0	2.0	6	140	35	50	50.50	1.00	0.92
27	0	0	0	0	2.0	6	140	35	51	50.50	0.90	0.92
28	0	0	0	0	2.0	6	140	35	49	50.50	0.90	0.92
29	0	0	0	0	2.0	6	140	35	51	50.50	0.80	0.92
30	0	0	0	0	2.0	6	140	35	52	50.50	1.00	0.92

(a), X_1; media initial pH; X_2, Fe^{2+}concentration; X_3, agitation rate; X_4, temperature.

dividing the sum of the squares of each of the two sources of variation, means the model and the error variance, by the respective degrees of freedom (d.f.). In addition, the insignificant value of lack of fit (more than 0.05) indicated that the quadratic model was valid for the maximum biooxidation of ferrous ions and minimum jarosite formation by the originated mixed culture from hot spring.

Effect of pH and Fe^{2+} ion concentration on oxidation efficiency and jarosite formation

An effort was made to improve the oxidation efficiency of ferrous to ferric ion associated with minimal jarosite formation. The effect of different levels of media initial pH and Fe^{2+} ion concentration on biooxidation efficiency of ferrous ion and jarosites was predicted from the three-

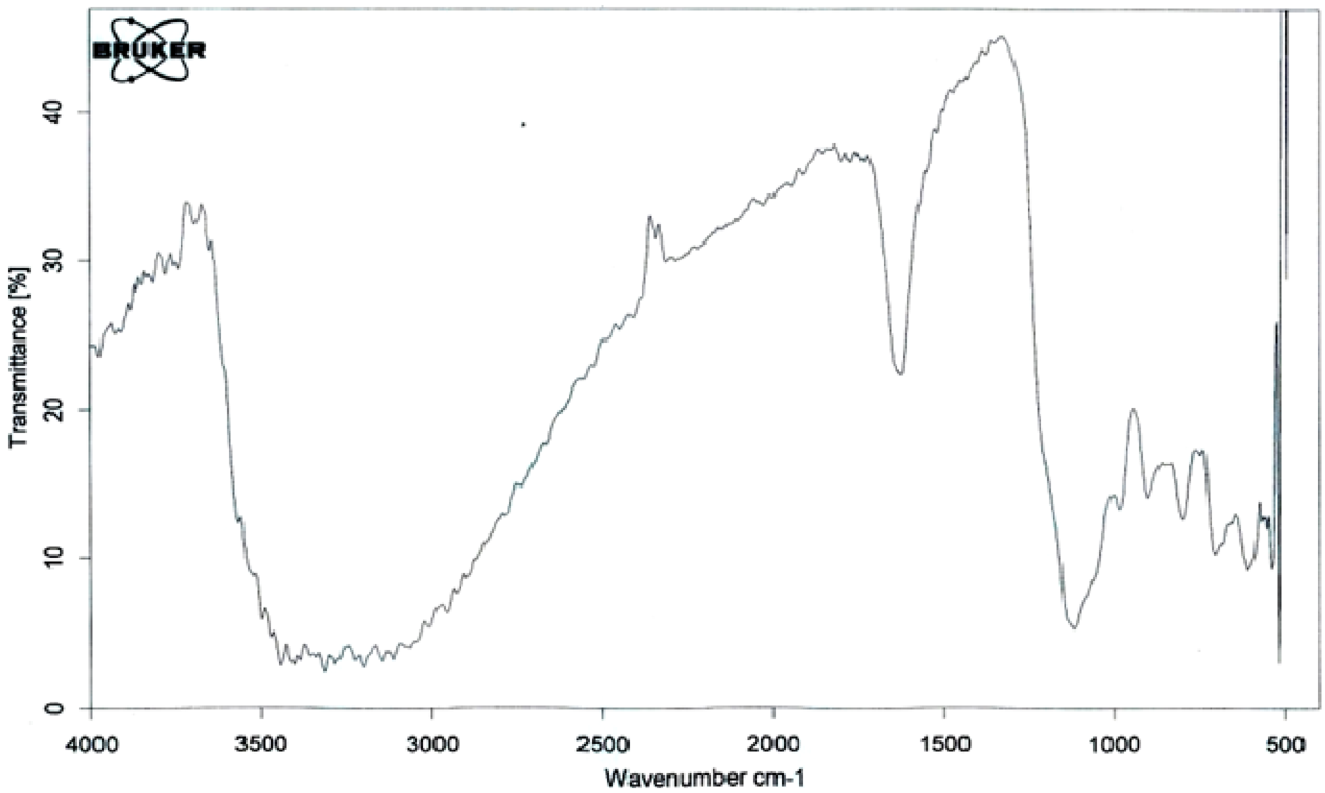

Figure 1. FTIR of iron precipitates mediated by the isolated microorganism from the hot spring.

Table 3. Regression analysis using the 2^4 factorial central composite designs.

Model term [a]	Parameter estimates [b]		Standard error		F-value		P-value	
	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Intercept	50.50	0.92	0.41	0.038				
X_1	-5.29	0.72	0.20	0.019	675.80	1456.56	<0.0001	<0.0001
X_2	4.54	0.16	0.20	0.019	497.81	68.52	<0.0001	<0.0001
X_3	-0.71	-0.20	0.20	0.019	12.11	113.67	0.0034	<0.0001
X_4	2.79	0.12	0.20	0.019	188.09	39.58	<0.0001	<0.0001
X_1X_2	-0.062	-0.11	0.25	0.023	0.063	22.38	0.8054	0.0003
X_1X_3	-0.31	-0.10	0.25	0.023	1.57	20.38	0.2292	0.0004
X_1X_4	0.063	-0.12	0.25	0.023	0.063	27.79	0.8054	<0.0001
X_2X_3	0.31	0.027	0.25	0.023	1.57	1.35	0.2292	0.2632
X_2X_4	-0.062	9.375E-003	0.25	0.023	0.063	0.16	0.8054	0.6908
X_3X_4	0.19	-0.011	0.25	0.023	0.57	0.21	0.4636	0.6524
X_1^2	-2.36	0.17	0.19	0.018	154.22	90.77	<0.0001	<0.0001
X_2^2	-1.74	8.229E-003	0.19	0.018	83.47	0.22	<0.0001	0.6479
X_3^2	-1.86	0.26	0.19	0.018	95.89	224.35	<0.0001	<0.0001
X_4^2	-1.74	0.077	0.19	0.018	83.47	19.01	<0.0001	0.0006

[a] The degree of freedom for all the model terms was 1. X_1, X_2, X_3, X_4 are the main effects; $X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, X_3X_4$ are the interaction effects; $X_1^2, X_2^2, X_3^2, X_4^2$ are the square effects; [b] Y_1, oxidation efficiency; Y_2, Jarosite.

Table 4. ANOVA results for response parameters

Modified equations with significant terms [a)]	R^2	Adjusted R^2	Predicted R^2	Adequate Precision	SD [b)]	CV, %[c)]	PRESS [d)]
$Y_1 = +50.50 - 5.29X_1 + 4.54X_2 - 0.71X_3 + 2.79X_4 - 0.062X_1X_2 - 0.31X_1X_3 + 0.063X_1X_4 + 0.31X_2X_3 - 0.062X_2X_4 + 0.19X_3X_4 - 2.36X_1^2 - 1.74X_2^2 - 1.86X_3^2 - 1.74X_4^2$	0.9911	0.9828	0.9630	38.113	1.00	2.25	62.16
$Y_2 = +0.92 + 0.72X_1 + 0.16X_2 - 0.20X_3 + 0.12X_4 -$ 0.12 $X_1X_4 + 0.027X_2X_3 - 9.375\times 10^{-3}X_2X_4 - 0.011X_3$	0.9927	0.9859	0.9649	44.751	0.092	6.95	0.62

[a)] X_1: media initial pH; X_2: Fe^{2+} ion concentration; X_3: agitation rate; X_4:temperature; Y_1: oxidation efficiency; Y_2: jarosite

[b)] SD: Standard deviation

(C) CV: Coefficient of variation

[d)] PRESS: Predicted residual error sum of squares

Table 5. ANOVA results for response surface quadratic model.

Source of variation	**SS** [a)]		**DF** [b)]		**MS** [c)]		**F-value**		**P-value**	
	Y_1[d)]	Y_2[e)]	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2	Y_1	Y_2
Model	1663.75	17.45	14	14	118.84	1.25	119.50	145.74	<0.0001	<0.0001
Residual	14.92	0.13	15	15	0.99	8.552E-003				
Lack of fit	9.42	0.10	10	10	0.94	9.994E-003	0.86	1.76	0.6114	0.2759
Pure error	5.50	0.028	5	5		5.667E-003				
Total	1678.67	0.18	29	29						

(a), SS: sum of squares; (b), DF: degrees of freedom; (c), MS: mean square; (d), Y_1: oxidation efficiency; (e), Y_2: jarosite.

dimensional (3D)-response surface plot as shown in Figure 2. The 3D-reponse surface plot is a graphical representation of the objective responses of the regression model. In each plot, the effects of the two variables were depicted, while other variables were held at zero level (Figure 2 to 7). Here, it was used to illustrate the main and interactive effects of the independent parameters on the biooxidation efficiency and jarosite. Because of the consumption of H^+ in the beginning of the process, the pH of the aqueous media initially increased. However, increase in pH was counteracted by the hydrolysis of ferric iron (Daoud and Karamanev, 2006). Therefore, it was obvious that the pH of the system had significant effect on the extent of the oxidation and amount of the formed jarosites. In this study, media initial pH values were adjusted to the range of 1 to 3 and were used to examine the effect of pH on the biooxidation and the amount of jarosite precipitation. In addition, Fe^{2+} ion concentrations were varied from 2 to 10 g/l. Figure 1a represents the combined effects of the independent variables (media initial pH and Fe^{2+} ion concentration) on the oxidation of ferrous ion. From this plot, the oxidation efficiency increased with increase in media initial pH ranging from 1 to 1.43 as well as with Fe^{2+} ion concentration ranging from 2 to 10 g/l. Nevertheless, pH values higher than 1.44 reduced the oxidation efficiency. On the other hand, the oxidation efficiency was increased with the increase of Fe^{2+} ion concentration ranging from 2 to 10 g/l. However, the maximal biooxidation efficiency of 56.52% was achieved at initial pH of 1.43 and Fe^{2+} ion concentration of 8.68 g/l, while the other variables were set at the middle value. The estimated P-value (0.8054) for the interaction of these two factors was high, which indicated high interaction. Increase oxidation efficiency by increasing Fe^{2+} ion concentration caused by increasing the substrate, which was encountered by the active sites of bacteria in the medium made the processes of adsorption to be carried out more sufficiently. Higher values of the media initial pH did not result in higher oxidation efficiency. A reduction in oxidation efficiency by increasing pH from 1.43 to 3 demonstrated that the acidic condition had significant influence on the proper growth

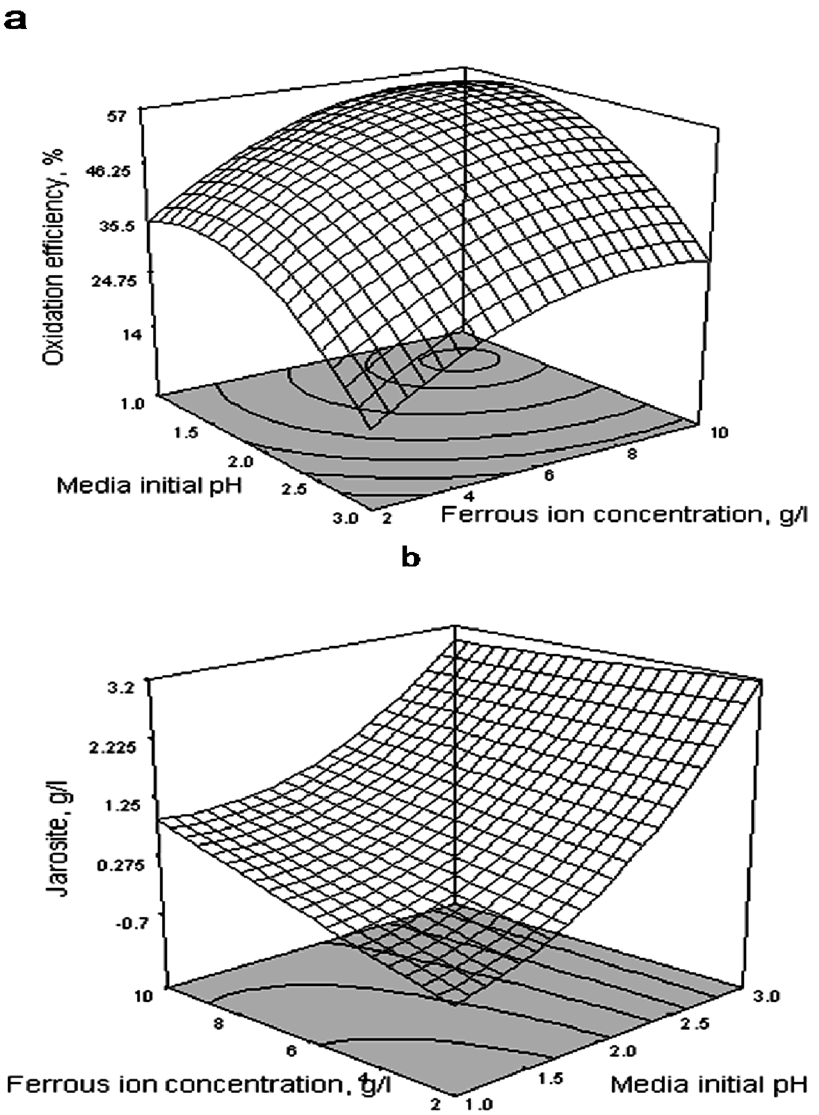

Figure 2. A 3D-response surface plot of the effect of media initial pH and Fe^{2+} ion concentration on (a) oxidation efficiency and (b) jarosite formation. The other variables were set at zero levels.

of the bacteria. Figure 2b illustrate the 3D-respo surface plots of the jarosite as a function of the amounts of two variables (media initial pH and Fe^{2+} ion concentration) keeping the other variable constant at its respective zero value. It was shown that the lowest amount of both variables resulted in the minimal value of jarosite (zero). However, an increase in the amount of jarosite by increasing media pH, resulted to more Fe^{3+} precipitates, which occurred at high pH. The low P-value (0.0003) showed significant interaction between the two variables.

Effect of agitation rate and media initial pH on oxidation efficiency and jarosite formation

A relationship between agitation rate and media initial pH considering their effect on oxidation efficiency is shown in Figure 3a. According to this plot, the oxidation efficiency increased with increase in the agitation rate ranging from 80 to 136.52 rpm as well as with media initial pH ranging from 1 to 3. This behavior was as a result of direct relationship between increases in the agitation rate value and more oxygen consumption by the bacteria. However, agitation rate values higher than 136.52 rpm reduced the oxidation efficiency, because of the mass transfer limitation of oxygen to the bacteria. The maximal oxidation efficiency for the optimum agitation rate of 136.52 rpm was found to be 53.48%. On the other hand, the oxidation efficiency was decreased with increase in the media initial pH ranging from 1 to 3 and its optimal value was 1.44. The high P-value (0.2292) showed insignificant interaction between the agitation rate and media initial pH.

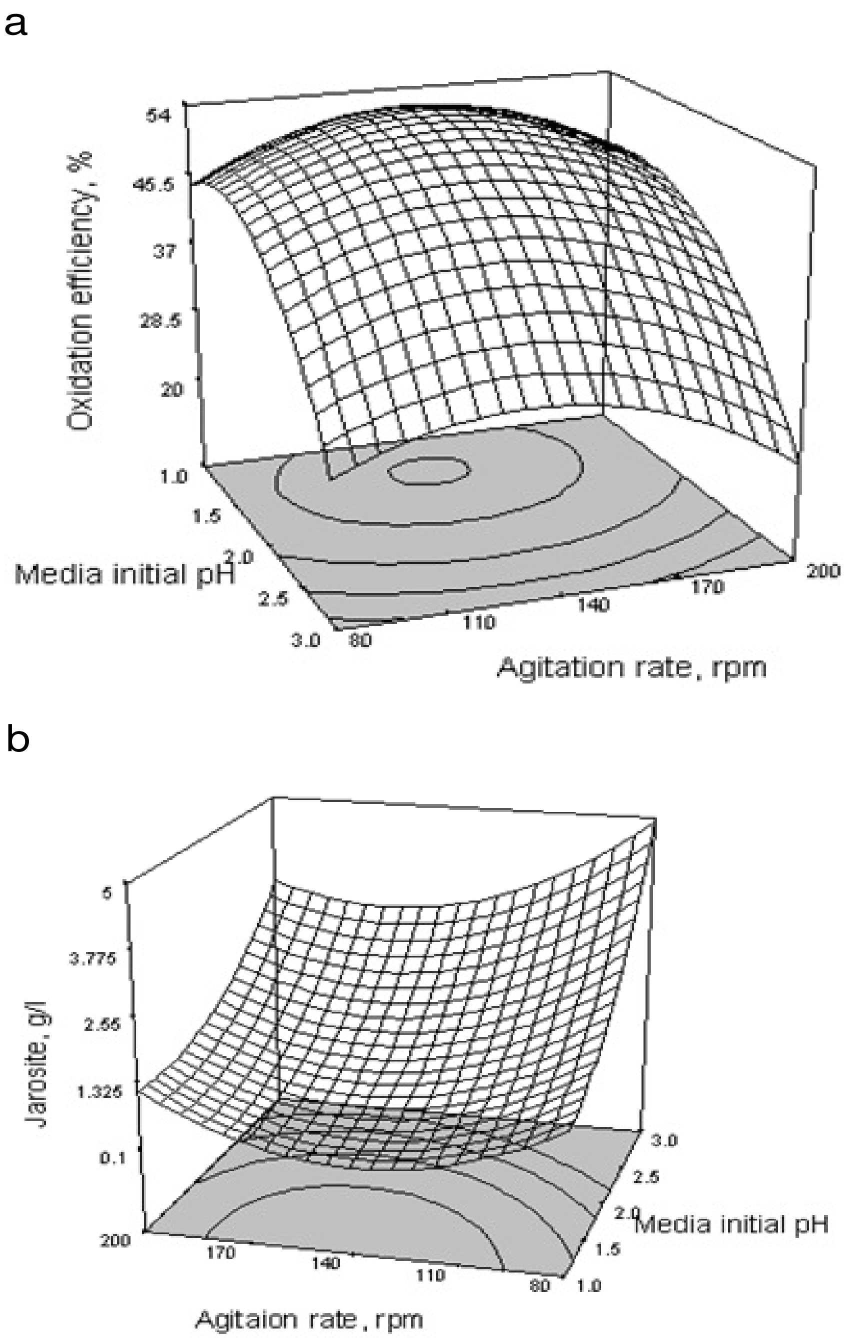

Figure 3. A 3D- response surface plot of the effect of the media initial pH and agitation rate on (a) oxidation efficiency (b) jarosite formation. The other variables were set at zero levels.

on biooxidation efficiency. Figure 2b depicts the effects of agitation rate and media initial pH on the amount of jarosite formation in the medium. The minimum amount of jaosite (zero) was achieved at the lowest value of pH corresponding to 1 and agitation rate value of 140 rpm, while the other variable was set at the middle value. It was observed that with increase in the agitation rate value from 80 to 140 rpm, the amount of jarosite decreased and this depletion was more significant in higher pH. This decrease in amount of jarosite was as a result of higher oxidation of ferrous to ferric ions and consequently, lower Fe^{3+} precipitates were formed. Nevertheless, agitation rate values higher than 140 rpm increased the jarosite. The obtained P-value of 0.0004 indicated significant interaction between agitation rate and media initial pH on jarosite formation.

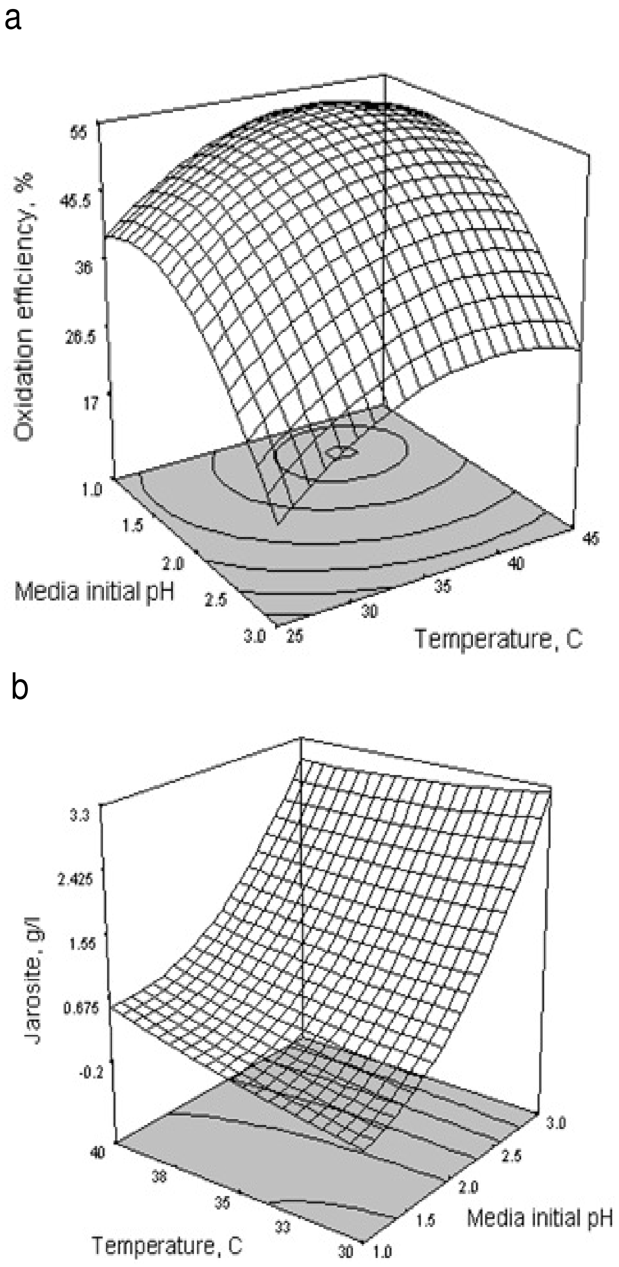

Figure 4. A 3D-response surface plot, of the effect of media initial pH and temperature on (a) oxidation efficiency (b) jarosite formation. The other variables were set at zero levels.

Effect of media temperature and pH on oxidation efficiency and jarosite formation

Figure 4a depicts the interaction between temperature and media initial pH on the oxidation efficiency. The lowest temperature and the highest pH caused minimum oxidation efficiency, which was not desirable. It was observed that, the optimum temperature for the maximum oxidation efficiency of 54.5% was achieved at 39.4°C. A decrease in pH ranging from 3 to 1 and an increase in temperature from 25 to 45°C resulted in maximum efficiency and the pH and temperature optimum values were 1.43 and 39.4°C, respectively. An increase in the biooxidation of ferrous to ferric iron caused by increasing the temperature can be explained by the increase in the cell density in the medium. However, temperature in the range of 39.4 to 45°C had no significant effect on the oxidation efficiency. The high P-value of 0.8054 indicated that the interaction of temperature and initial pH on oxidation efficiency was not significant. The effect of temperature and media initial pH on the amount of jarosite formation is shown in Figure 4b. According to the plot, the minimum amount of jaosite (zero), which is desirable, was achieved at the lowest value of pH and temperature corresponding to 1 and 25°C, respectively. These optimum values indicate that at low pH and temperature, ferric ions existed in the form of ion and the precipitates decreased. The low P-value of <0.0001 emphasized significant interaction between temperature and media initial pH on the amount of jarosite formation.

Effect of agitation rate and Fe^{2+} ion concentration on oxidation efficiency and jarosite formation

Experiments were performed to study the influence of agitation rate and Fe^{2+} ion concentrations, in the range of 80 to 200 rpm and 2 to 10 g/l, respectively, on the oxidation efficiency of ferrous iron (Figure 5a). The estimated P-value (0.2292) for the interaction of these two factors was high, which indicated a low interaction between these two parameters. The biooxidation efficiency of ferrous to ferric ion first increased with increasing agitation rate and reached to a maximum value. A decrease appeared to start with a further increase in the agitation rate. The changes in oxidation efficiency can be explained by taking into consideration the consumption of oxygen by bacteria, which reached to its saturation value at the optimal agitation rate. At the stated conditions, the highest oxidation efficiency was 53.48% with optimum agitation rate value and Fe^{2+} ion concentration of 137 rpm and 8.58 g/l, respectively. The ferric ion concentration ranging from 8.58 to 10 g/l showed no major change on the oxidation of ferrous to ferric ion. When the agitation rate shifted from 80 to 200 rpm, the optimum Fe^{2+} ion concentration was obtained as the value of 2 g/l.

Figure 5b, shows the effect of agitation rate and Fe^{2+} ion concentrations on the amount of jarosite formation. It was observed that by decreasing the Fe^{2+} ion concentrations, the jarosite formation decreased. The minimum value of 0.5 g/l was achieved at the lowest value of Fe^{2+} ion concentrations, while the optimum agitation rate was 154 rpm. An increase in Fe^{2+} ion concentration led to an increase in the oxidation of ferrous to ferric ion, conesquently more ferric ions wer precipitated and caused increase in the jarosite formation. The high P-value of 0.2632 for the effect of Fe^{2+} ion concentration and

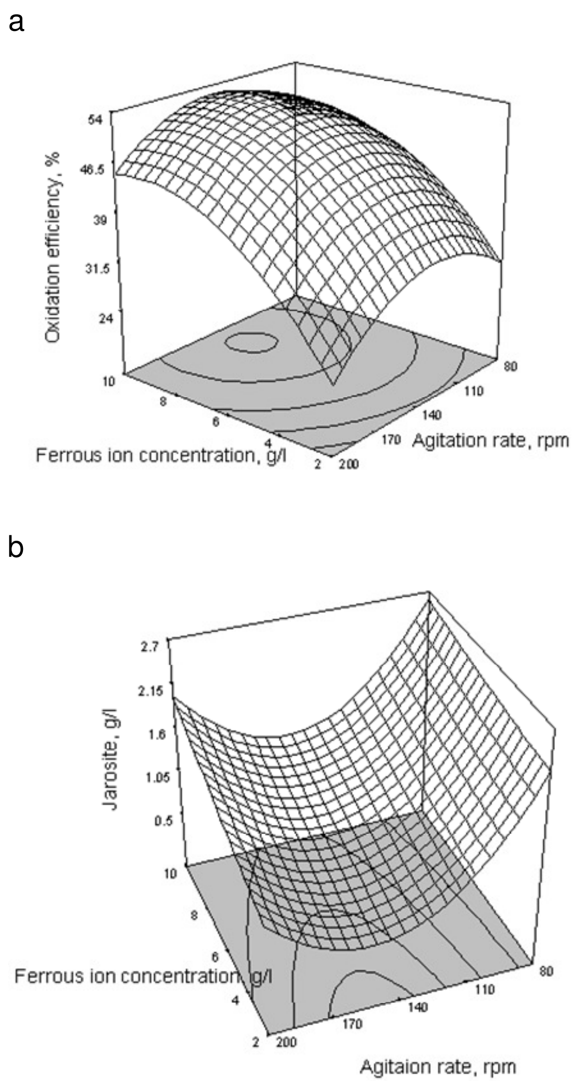

Figure 5. A 3D-response surface plot of the effect of Fe^{2+} ion concentration and agitation rate on (a) oxidation efficiency and (b) jarosite formation. The other variables were set at zero levels.

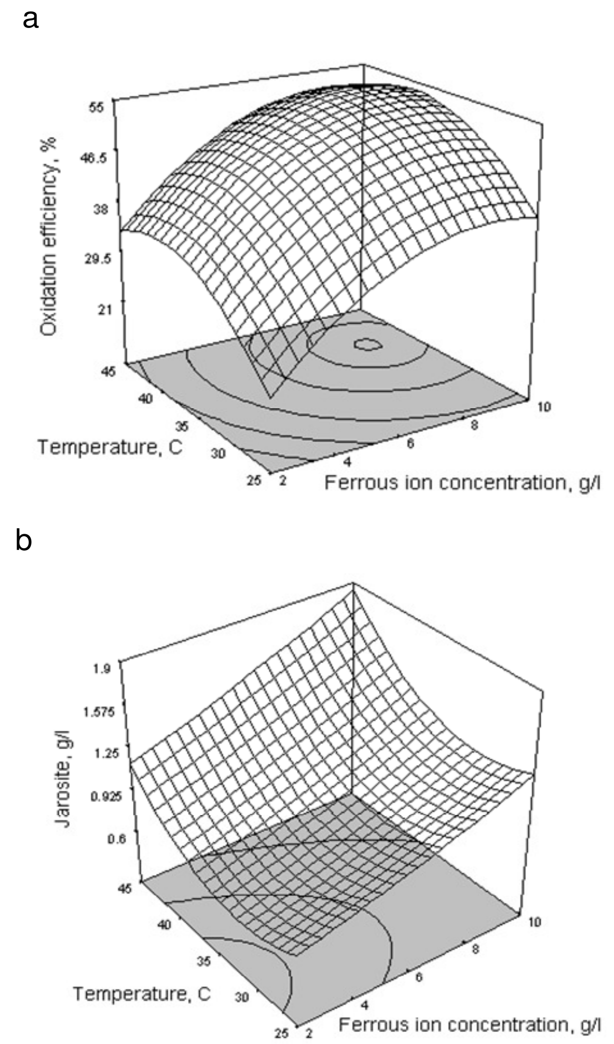

Figure 6. A 3D-response surface plot of the effect of Fe^{2+} ion concentration and temperature on (a) oxidation efficiency and (b) jarosite formation. The other variables were set at zero levels.

agitation rate showed no significant interaction between these factors.

Effect of temperature and Fe^{2+} ion concentration on oxidation efficiency and jarosite formation

Figure 6a, demonstrates the interaction between Fe^{2+} ion concentration and temperature on the oxidation of ferrous to ferric ions. The obtained P-value (0.8054) was high, which depicted the low interaction of the two factors. It was found that an increase in the temperature value resulted in an increase in the oxidation efficiency due to more cell concentration in the high temperature. The maximal oxidation efficiency of 54.52% was achieved at the optimum temperature and Fe^{2+} ion concentration values of 39°C and 8.58 g/l, respectively.

The effect of temperature and Fe^{2+} ion concentration on jarosite formation is illustrated in Figure 6b. According to the presented plot, the jarosite formation increased with increase in Fe^{2+} ion concentration ranging from 2 to 10 g/l as well as with temperature ranging from 25 to 45°C. It was also observed that the amount of jarosite first decreased with an increase in temperature and reached to a minimum value at 31.77°C. Then, an increase appeared to start with a further increase in temperature. The changes in jarosite at temperatures higher than 31.77°C most probably can be explained due to biomass aggregation and ferric ion precipitation. The minimal jarosite amount of 0.6 g/l was obtained at optimal temperature value of 31.77°C, while the Fe^{2+} ion concentration was at its minimum value. The high P-value of 0.6908 indicated low interaction between temperature

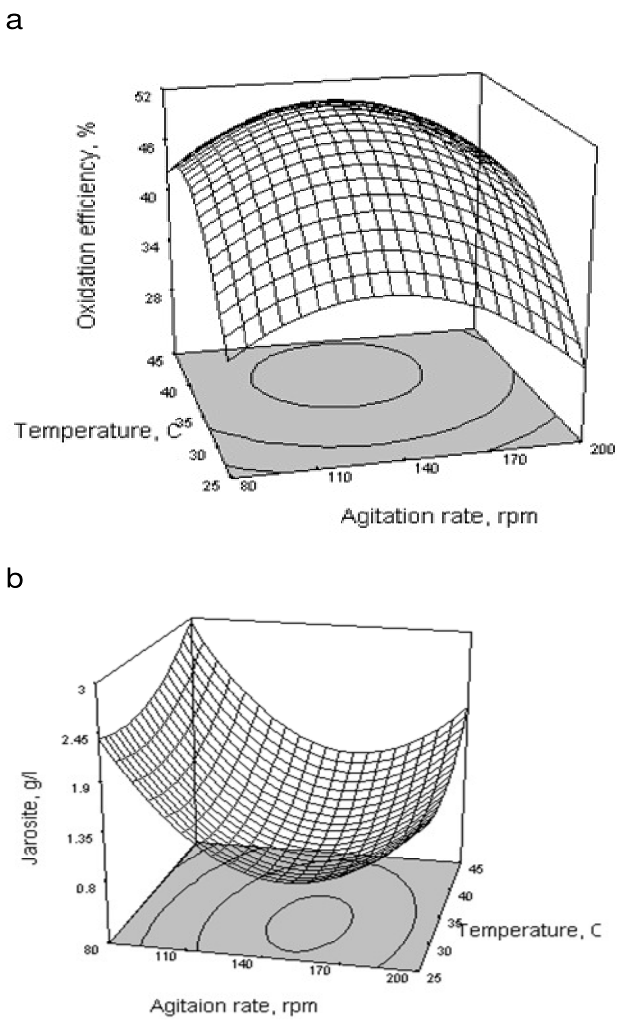

Figure 7. A 3D-response surface plot of the effect of agitation rate and temperature on (a) oxidation efficiency and (b) jarosite formation. The other variables were set at zero levels.

and Fe^{2+} ion concentration on jarosite formation.

Effect of temperature and agitation rate on oxidation efficiency and jarosite formation

Figure 7a, presents the effect of temperature and agitation rate on oxidation efficiency. From this plot, the oxidation efficiency increased with increase in agitation rate ranging from 80 to 135.28 rpm, as well as with temperature ranging from 25 to 45°C. However, agitation rate values greater than 135.28 rpm reduced the oxidation efficiency. The inhibition of the bacterial oxidation of ferrous to ferric ions at agitation rate higher than 135.28 rpm was partially linked to the oxygen consumption limitation. The oxidation efficiency variation may have occurred due to consumption of oxygen by the bacteria, which reached to its saturation value at the optimal agitation rate. In the other hand, oxidation efficiency was increased with increase in the temperature ranging from 25 to 38.95°C. The maximal oxidation efficiency was found to be 51.66% for the optimum agitation rate and temperature values of 135.28 rpm and 38.95°C, respectively. The high P-value of 0.4636 showed an insignificant interaction between the agitation rate and the temperature on biooxidation efficiency. Extensive studies were conducted to examine the relationship between the temperature and agitation rate on jarosite formation (Figure 7b). The jarosite concentration first decreased with an increase in agitation rate from 80 to 153 rpm. However, an increase appeared in the jarosite amount with increase in the agitation rate higher than 153 rpm. This increase was caused by more ferric precipitation which is partially linked to the inhibition of the bacterial oxidation of ferrous to ferric ions at agitation higher than 153 rpm. The minimal jarosite amount of 0.8 g/l was obtained at the optimal agitation rate and temperature of 153 rpm and 31.51°C, respectively. The estimated P-value of 0.6524 indicated an insignificant interaction between the two factors.

Optimization via the desirability function

In numerical optimization, the desired goal for each factor and response were selected. The possible goals were maximization and minimization of target, within range, (for responses only) and setting to an exact value (factors only). A minimum and maximum level must be set for each parameter. The desired goals were combined into an overall desirability function. Desirability is an objective function that ranges from zero outside of the limits, to one at the goal. The numerical optimization finds a point that maximizes the desirability function. For several responses and factors, all goals are combined into one desirability function. The desirability value is fully depended on how closely the lower and upper limits are set relative to the real optimum. The mathematical presentation of the overall desirability (D) was defined as the geometric mean of all transformed responses, according to the Equation 6 (Myers et al., 2009):

$$D = (d_1 \times d_2 \times \ldots \times d_n)^{\frac{1}{n}} = \left(\prod_{i=1}^{n} d_i\right)^{\frac{1}{n}} \qquad (6)$$

Where, n is the number of responses in the measure. A multiple response method was applied for the optimization of any combination of six goals namely media initial pH, Fe^{2+} ion concentration, agitation rate, temperature, biooxidation efficiency and jarosite formation. The numerical optimization found a point that maximized the desirability function. All four factors were selected to be in a range. A maximum biooxidation efficiency and minimum level of jarosite formation were set for maximum desirability.

Figure 8a, shows contour plot desirability of the

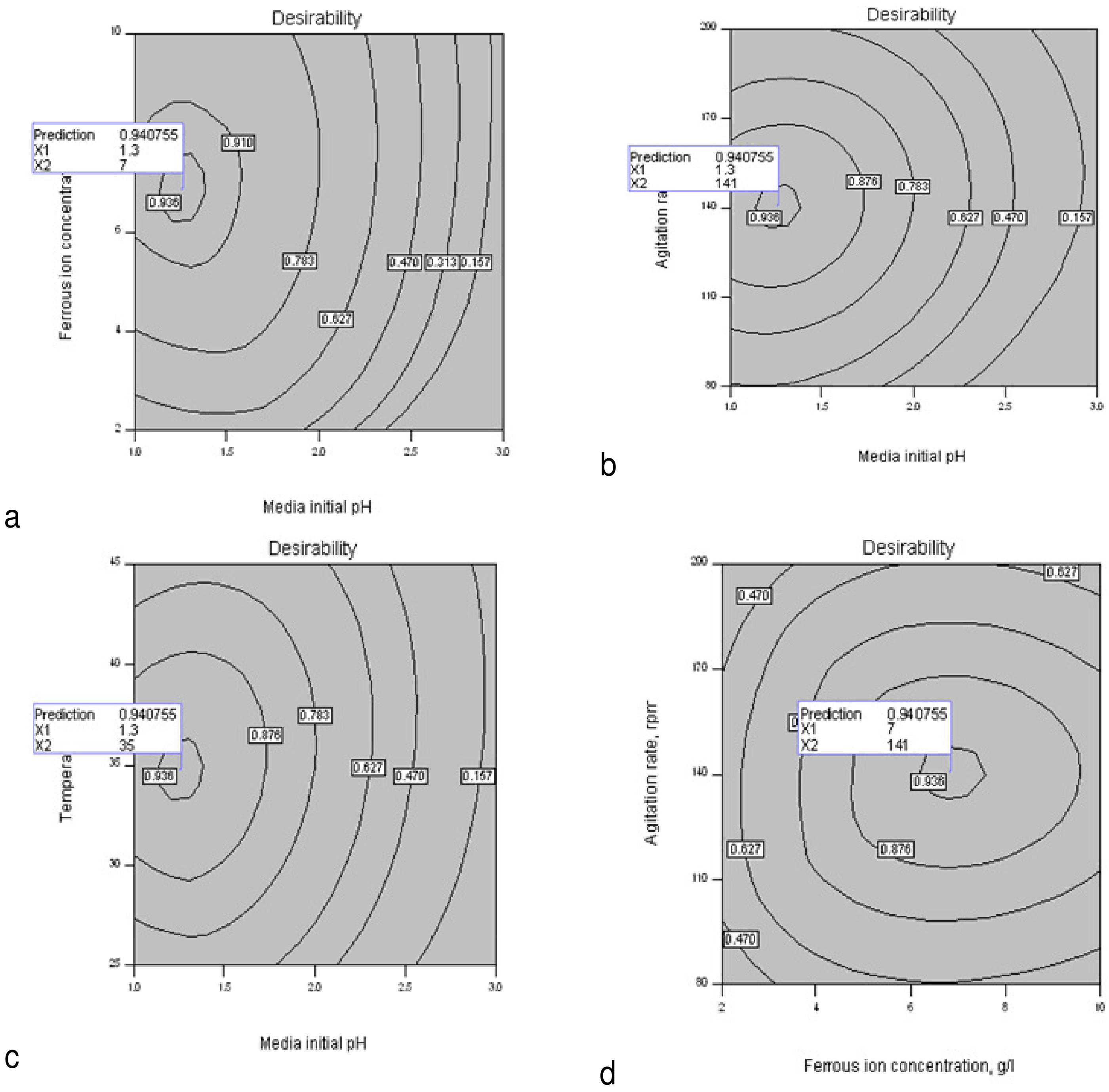

Figure 8. Desirability fitted surface for numerical optimization of the respective goals within the range of four factors of maximum oxidation efficiencyand minimum amount of jarosite; (a), effect of media initial pH and Fe^{2+} ion concentration; (b) effect of media initial pH and agitation rate; (c) effect of media initial pH and temperature; (d), effect of agitation rate and Fe^{2+} ion concentration; (e), effect of temperature and Fe^{2+} ion concentration; (f), effect of temperature and agitation rate.

combined effects of Fe^{2+} ion concentration and media initial pH for optimal objects (maximal oxidation efficiency and minimal jarosite formation), while the actual value of agitation rate and temperature were set at 141 rpm and 35°C. Figure 8b shows the combined effects of agitation rate and media initial pH for optimal goals, while the actual value of Fe^{2+} ion concentration and temperature were set at 7 g/l and 35°C, respectively. The combined effects of temperature and pH for optimal oxidation efficiency and minimum jarosite was depicted in Figure 8c, while the actual value of agitation rate and Fe^{2+} ion concentration were set at 141 rpm and 7 g/l.

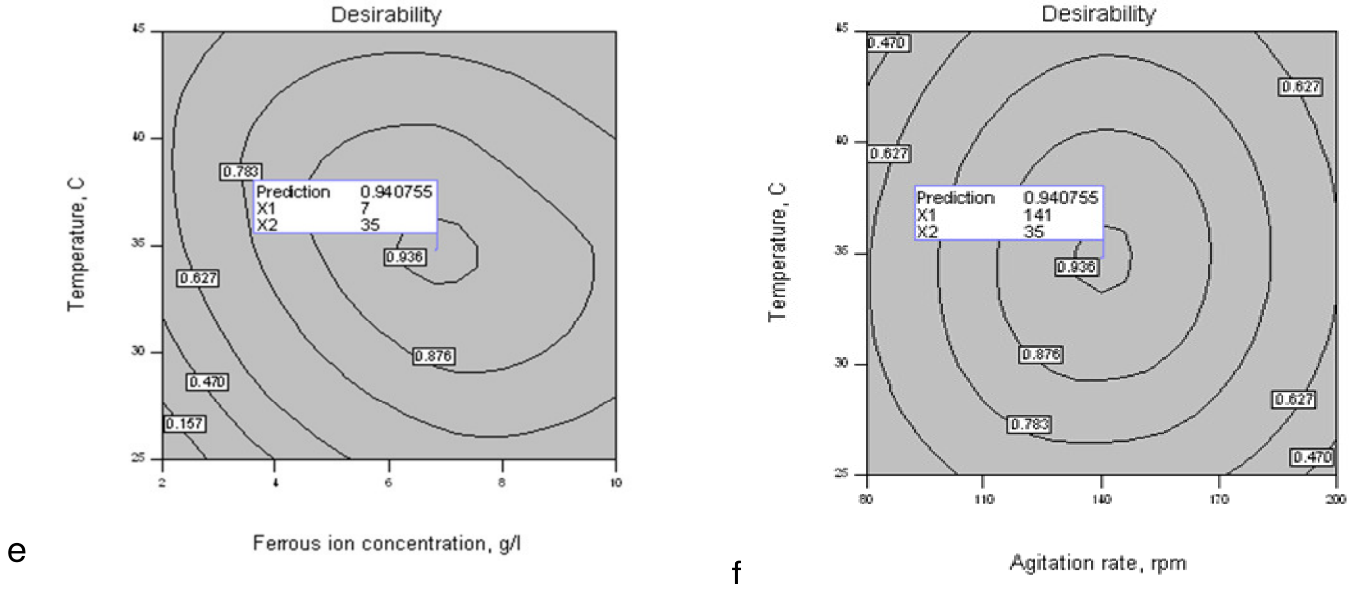

Figure 8. Continue

Furthermore, the combined effect of agitation rate and Fe^{2+} ion concentration for optimal objects is illustrated in Figure 8d, while the actual value of pH and temperature were set at 1.3 and 35°C. Figure 8e shows the combined effect of temperature and Fe^{2+} ion concentration for optimum responses, while the actual value of agitation rate and pH were set at 141 rpm and 1.3, respectively. Finally, the combined effect of temperature and agitation rate is presented in Figure 8f, while the actual value of Fe^{2+} ion concentration and pH were set at 7 g/l and 1.3. Thus, the predicted value and desirability were given by design expert software. The optimal condition was obtained by 6 counter plots. The best local maximum oxidation efficiency and minimum jarosite formation obtained were at media initial pH of 1.3, Fe^{2+} ion concentration of 7 g/l, agitation rate of 141 rpm and temperature of 35°C. At these optimal conditions and at a desirability value of 0.941, the oxidation efficiency and jarosite formation obtained were 54.67% and 0.34 g/l, respectively. The achieved desirability value of 0.941 showed that the estimated function may stand for the experimental model and the desired conditions. Under optimum conditions, an experiment was conducted and the oxidation efficiency of 53% and jarosite amount of 0.32 g/l was achieved, which was equivalent to the predicted values given by the regression model.

Conclusions

The main objective of the study was to employ central composite design coupled with response surface methodology. Optimization was applied for the oxidation of ferrous to ferric irons along with minimal jarosite precipitation due to its suitability to fit the quadratic model which typically works well for the optimization. The model demonstrated that, all the four factors had influence on the objective function. The experimental results and regression model depicted that the maximum biooxidation efficiency of 55% and minimal jarosite of 0.34 g/l was achieved with RSM under design-expert software at media initial pH of 1.3, Fe^{2+} ion concentration of 7 g/l, agitation rate of 141 rpm and temperature of 35°C. The fitted model was also checked by the multiple correlation coefficients (R^2). In this case, the values of R-squared for oxidation efficiency and jarosite formation were R^2= 0.9911 and 0.9927, respectively and revealed that the regression was statistically significant and only 0.89 and 0.73% of the total variations (for oxidation efficiency and jarosite formation, respectively), was not explained by the model. The experiment carried out under optimal condition resulted in oxidation efficiency and jarosite amount of 52% and 0.32 g/l, respectively. These values were similar to the predicted value obtained by the model. The isolated organism was identified as iron-oxidizing bacteria in a batch culture. This mixed culture can obtain the required energy for growth via oxidation of various inorganic substances. The most dominant substance in the media was ferrous sulfate. The results also showed that the CCD with RSM was practical for optimizing the selected parameters for the biooxidation of ferrous ions to ferric ions.

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