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Assessment of Effective Factors in Bacterial Oxidation of Ferrous Iron by Focusing on Sweetening Natural Gas

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ABSTRACT

In this study, the effects of some factors on bacterial growth and ferrous oxidation rates were investigated by Acidithiobacillus ferrooxidan in 250 ml shake flasks. One factor at a time (OFAT) design approach was used for preliminary evaluation of various factors affecting the process, such as pH, initial ferrous and elemental sulfur concentrations, shaker agitation rate, and liquid to flask volume ratio. After that, optimal levels of the last three effective factors in achieving high oxidation rate and cell growth rate were investigated using a full-factorial design. It was obtained that agitation rate and liquid to flask volume, as well as their binary interaction are significant factors in ferrous iron bio-oxidation rate. In contrast, initial high ferrous iron concentration was the only effective factor in the cell growth rate. Maximum bio-oxidation rate of 0.417 g/L was achieved at the media with Fe^{2+} ion concentration of 30 g/l, agitation rate of 200 rpm, and liquid to flask volume ratio of 20 % by full-factorial optimization, which is an about 40 % increase compared to the result obtained in OFAT method. Then, the effect of step-wise adaptation of A. ferrooxidans on high Fe^{2+} concentration was studied, and about 40 % reduction in bacterial lag phase time, and 36 and 86 % increase in bacterial growth rate and bio-oxidation rate were acquired, respectively.

1. Introduction

The oxidation of ferrous iron is a quite useful process which is employed in many industrial processes in the regeneration of ferric iron. Ferric iron has beneficial applications in various industries, such as removal of H_2S from combustible gases, biogases and tail gases, in the pulp and paper industry [1], bioleaching of sulfide minerals, treatment of acid mine drainage, and desulphurization of coal [2].

One of the important applications of ferrous iron oxidation is in natural gas sweetening process. Removal of sulfur components from sour gas, which is mainly in the form of its hydride (H_2S) and oxide (SO_2), is required for reasons of health, safety, and to prevent corrosion [3]. Traditional physicochemical methods, such as dry gas reduction/oxidation (redox) processes, liquid redox processes, and

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liquid adsorption processes, have been used for treatment of sour gas [4]. However, they have high capital costs, high energy intensive, and association with the release of other by-products such as sulfur dioxide [5]. A chemo-biochemical process for desulfurization of gaseous effluents has been developed in two stages. In the first step of the process, sour gas is contacted with an aqueous ferric iron solution in an absorber column. The solution absorbs H_2S and oxidizes it to elemental sulfur with a reduction of ferric iron into ferrous ions (Eq. (1)) after recovering elemental sulfur by a suitable solid-liquid separation method [6]. The second stage involves biologically oxidation of ferrous to ferric ions by appropriate bacterial species, such as *Acidithiobacillus ferrooxidans*, to recycle into the absorber unit (Eq. (2)) [1, 2]:

$$H_2S + Fe_2(SO_4)_3 \rightarrow S\downarrow + 2FeSO_4 + H_2SO_4 \quad (1)$$

$$2FeSO_4 + H_2SO_4 + 0.5O_2 \xrightarrow{bacteria} Fe_2(SO_4)_3 + H_2O \quad (2)$$

The reaction of hydrogen sulfide with ferric sulfate is so rapid [2]; thus, the biochemical reaction acts as a limiting step in natural gas sweetening process. In recent years, some studies [2, 7-9] have been focused on improving the rate of bio-oxidation of Fe^{2+} in a shaken culture. Several factors have been studied in order to obtain better results, such as initial pH, temperature, and initial concentrations of Fe^{2+}, Fe^{3+} and elemental sulfur. However, less attention has been paid to agitation rate and ratio of the flask volume to the liquid volume simultaneously for controlling of the input and output of O_2 and CO_2transfer rates.

A. ferrooxidans is an acidophilic, obligately aerobic, chemoautotrophic organism that has the ability to oxidize ferrous to ferric iron in the presence of atmospheric oxygen and carbon dioxide concentrations [10-12]. Appropriate shaking agitation rate is required to ensure an adequate supply of oxygen and carbon dioxide for a rapid bacterial growth and bio-oxidation. Because of low solubility of oxygen in the medium, agitation rate and consequent mass transfer coefficient should be optimized to make use of high rate of ferrous iron bio-oxidation process [13]. According to Eq. (3), the oxygen transfer coefficient (k_La) in the bacterial shake-flask culture decreases with increasing the liquid volume in flask, whereas the agitation rate increases k_La value [14]:

$$k_L a(h^{-1}) = 0.141\, N^{0.88} \left(\frac{V_L}{V_0}\right)^{-0.80} \quad (3)$$

where N is a shaker agitation rate (min^{-1}), V_L is the liquid volume in flask, and V_0 is the flask volume.

One factor at a time (OFAT) approach is a popular design of an experiment in many researches when performing experiments to find out the setting of main factors [15]. This method consists of doing some trials in several steps, involving the selection of a starting point for amount (level) of a factor in each step and, then, successively varying level of the factor over its range in each trial while the amount of the other factors is held constant [5]. The best level of each factor is obtained in every step and fixed in the remaining steps. This method does not consider the effect of interactions of various factors. OFAT may be desired when quick reaction to data is important during experiments, and also the effects of factor are three or four times the standard deviation [16].

An experimental design refers to a parallel study of several process factors. A combination of several factors in each trial,

instead of one experiment for each factor, reduces the number of required tests greatly, and also a better understanding of the process will be obtained. Some of the important applications of experimental design are as follows: improving the efficiency of the process, reducing development time, the total cost and variability, and also better conforming to the nominal value [17]. A full-factorial design method is a statistical model, which is commonly used to verify 2-4 important factors affecting the process. It is the basis for all classical experimental designs used in screening, optimization, and robustness tests. It can be applied as an alternative approach to understanding complex processes and describing interactions between different process factors [18].

According to our knowledge, limited statistical studies have been done on the factors affecting the ferrous bio-oxidation rate. Khavarpour et al. evaluated the effect of initial ferrous iron concentration, pH, and temperature and agitation rate on iron oxidation rate and ferric despites formation of an indigenous thermophilic bacterium by response surface method on a shake-flask scale [5]. Mousavi et al. investigated the effect of ferric iron concentration, pH, temperature, agitation rate and dilution rate in a packed bed bioreactor on ferrous iron oxidation rate by a thermophilic bacterium using Taguchi methodology [4]. In addition, Rahman et al. evaluated the changes of rate constants of ferrous iron oxidation in a system containing four experimental factors: pH, phosphate and chlorine concentration and dissolved organic matters [19]. Hence, investigating the simultaneous effects of operational factors on ferrous iron oxidation rate and microbial growth is essential.

The bacterial lag phase shows a tendency to become longer with the higher initial ferrous iron concentration [2], which is related to inhibitory effects of high Fe^{2+} concentration. This substrate inhibition can be overcome by adapting the bacteria to high concentration of Fe^{2+}. To the best of the authors' knowledge, there are little data available regarding the use of adapted bacteria to decrease lag phase length of growth of *A. ferrooxidans* in high substrate concentration culture [20].

This article first describes an investigation of the factors that influence biological oxidation rate of ferrous iron with *A. ferrooxidans* in a shaken culture using OFAT method. Initial concentrations of Fe^{2+} and elemental sulfur, initial pH of medium, shaking agitation rate, and liquid to flask volume ratio have been studied. A full-factorial design approach (3^3) was used to find the optimum level of three effective factors (initial Fe^{2+} concentration, liquid to flask volume ratio, and agitation rate) to achieve high ferrous biological oxidation rate. Finally, we compared the biological oxidation rates of ferrous iron using both adapted and non-adapted bacteria in high Fe^{2+} concentration.

2. Materials and method

2.1. Materials

All chemicals used for the experiments were analytical graded and 1,10-phenantroline salt supplied by Orbital Company.

2.2. Microbial strain and growth media

Acidithiobacillus ferrooxidans PTCC 1646 was used throughout this study. The cells were cultured in a mineral medium containing (g/L): KH_2PO_4, 0.4; $(NH_4)_2SO_4$, 0.4; $MgSO_4$, 0.4; $FeSO_4.7H_2O$ in the range of 5-40 according to the different experimental conditions. The initial pH was adjusted by

concentrated H_2SO_4 in relation to the experimental design.

2.3. Experimental design

All experiments were carried out in 250 ml conical flask at 30 °C that was obtained as optimum temperature for the highest specific bacterial growth rate in literatures [21]. The media were sterilized by autoclaving at 121 °C for 15 min and inoculated with 5 % (v/v) of a fresh seed culture. The flasks were incubated on an orbital shaker at an appropriate shaking agitation rate (ranging from 0 to 200 rpm) according to experimental design (Table 1).

Ferrous biological oxidation rate and bacterial growth rate were applied as responses of the trials and calculated during 48 hours by dividing the reduction of ferrous ions and the increase of cell concentrations in time, respectively. Each experiment of ferrous ions bio-oxidation was carried out in duplicate, and the average value of experimental results was reported.

Table 1
Levels of factors in one factor at a time desig.

Step	Factor	Sulfur conc. (g/L)	Initial pH	Shaker agitation rate (rpm)	Ferrous iron conc. (g/L)	Liq. to flask vol. ratio (%)
1	Elemental sulfur conc. (g/L)	0-20	1.6	150	6.7	20
2	Initial pH	0	1.2-2.2	150	6.7	20
3	Liquid to flask vol. ratio (%)	0	1.6	150	6.7	20-40
4	Shaker agitation rate (rpm)	0	1.6	0-200	6.7	20
5	Initial ferrous iron conc. (g/L)	0	1.6	150	4-28	20

2.3.1. One factor at a time design

Primary experiments were carried out according to one factor at a time design approach. Five factors and range of factors levels in the design of experiment are shown in Table 1.

2.3.2. Full-factorial design

Three factors, including shaker agitation rate, high initial ferrous iron concentration, and liquid to flask volume ratio, were examined in three levels using a factorial design. The factors and their real and coded levels of the design of experiment are shown in Table 2. A range of factors' levels in a factorial experimental design was achieved through the results of "one factor at a time" approach. Table 3 presents the design matrix of experiments for a full-factorial design with three factors each at three levels (3^3) including related results of ferrous iron bio-oxidation and cell growth rates. Design-Expert software (version7) was applied to analyse the experimental design's results statistically.

Table 2
Factors and their levels in the full-factorial design of experiments.

Factor	Symbol	Low level (-1)	Middle level (0)	High level (+1)
Shaker agitation rate (rpm)	A	100	150	200
Liquid to flask vol. (%)	B	20	30	40
Initial ferrous iron conc. (g/L)	C	20	25	30

Table 3
Full-factorial design of experiments matrix and corresponding ferrous iron bio-oxidation rate and cell growth rate results.

Run order	Level of factor			Ferrous iron bio-oxid. rate (g/L.h)	Cell growth rate (cells number/L.h)
	A	B	C		
1	-1	-1	-1	0.306	9.95
2	-1	-1	0	0.327	9.532
3	-1	-1	+1	0.293	7.423
4	-1	0	-1	0.263	8.801
5	-1	0	0	0.245	9.219
6	-1	0	+1	0.228	6.598
7	-1	+1	-1	0.296	5.670
8	-1	+1	0	0.205	4.418
9	-1	+1	+1	0.186	3.814
10	0	-1	-1	0.275	15.014
11	0	-1	0	0.359	11.330
12	0	-1	+1	0.271	4.699
13	0	0	-1	0.290	8.804
14	0	0	0	0.250	8.278
15	0	0	+1	0.286	5.119
16	0	+1	-1	0.383	9.119
17	0	+1	0	0.234	4.804
18	0	+1	+1	0.295	3.857
19	+1	-1	-1	0.401	11.889
20	+1	-1	0	0.359	11.251
21	+1	-1	+1	0.417	6.145
22	+1	0	-1	0.321	12.102
23	+1	0	0	0.348	3.592
24	+1	0	+1	0.360	8/805
25	+1	+1	-1	0.347	12.422
26	+1	+1	0	0.315	10.294
27	+1	+1	+1	0.339	9.443

2.4. Adaptation of bacteria to grow in high ferrous sulfate concentration media

Adapted bacteria culture was prepared by the following sequential methods. The concentration of ferrous sulfate increased step by step in a serial sub-culturing process to reach the maximum ferrous sulfate concentration in maximum oxidation rate [22]. The adapted culture from the immediately preceding adaptation was used as the starting point for the next adaptation experiment. Sub-culturing process was carried out in 250 ml shake flasks containing 50 ml medium supplemented with 6.8 to 25 g/L Fe^{2+} (inhibitory concentration of ferrous iron) with a 5 g Fe^{2+}/L/step upwards ramping rate, shaken at 150 rpm and 30 °C.

2.5. Analysis methods

2.5.1. Cell counting

The number of bacteria in the culture was counted visually by a Neubauer chamber (0.1 mm depth and 1/400 mm^2 Area) under an optical microscope (Hund Wetzlar).

2.5.2. Determination of ferrous iron concentration

A colorimetric method was applied to determine the ferrous iron concentration in the samples [23]. The red-orange colored

complex formed by 1,10-phenanthroline and ferrous iron in acidic solution was analyzed using spectrophotometer at 512 nm (Labomed, IncmodelUVS-2800). In this method, 10 μl samples containing ferrous iron are mixed with 2 ml sodium acetate buffer (pH 4) and 4 ml of 3 % 1,10-phenanthroline solution. To measure the total iron concentration, the ferric ion is reduced to the ferrous state by 1 ml of 10 % hydroxylamine solution before colored Fe^{2+} complex is formed. The measurement of absorbance of this solution at 396 nm would give the amount of the total iron. The ferric iron concentration present in the solution was calculated by subtracting the ferrous iron concentration from the total iron concentration.

3. Results and discussion

As described above, the effects of some factors on bacterial growth and ferrous bio-oxidation rates were investigated in two parts: primary evaluation by OFAT method and statistical optimization using a full-factorial design approach. Finally, the optimum result of experimental design was used to reduce the bacterial lag phase time at high concentrations of ferrous iron.

3.1. Results of one factor at a time design

3.1.1. Effect of elemental sulfur concentration

Elemental sulfur concentration effect was examined at the first step of OFAT experiments (Table 1). Based on Eq. (1), H_2S is converted to elemental sulfur using ferric sulfate in the first step of sweetening gas process. The effect of generated sulfur should be considered which is not completely separated in the biochemical process step. The results of bio-oxidation of ferrous sulfate rate in response to sulfur concentration are presented in Fig. 1-A.

It can be seen from the results that the efficiency of the system is highly affected by this factor. According to this plot, the oxidation efficiency decreased with an increase in elemental sulfur concentration ranging from 0 to 20 g/L. In fact, inhibition effects were observed in cultures initially containing elemental sulfur. In other words, the increase in viscosity at high concentrations of elemental sulfur, restricting effective mass transfer inside the bacterial culture, may lead the process to reduce cell activity [2]. Although Mousavi et al. reported the negative role of elemental sulfur in ferrous iron concentration [2], Malhotra et al. showed that the presence of elemental sulfur did not have any impact on the process efficiency [9].

Hence, in order to achieve high performance in the biological gas sweetening process, it ought to be attempted to separate the produced elemental sulfur completely before iron bio-oxidation step. Therefore, the culture medium free of elemental sulfur was considered in the remainder steps of the OFAT experiments.

3.1.2. Effect of initial pH of medium

In the second step of the OFAT experiments (Table 1), the influence of initial pH of the culture medium on biological iron oxidation was investigated, and the results of experiments are shown in Fig. 1-B. *A. ferrooxidans* has been reported to be able to grow at pH ranges from 1.2 to 4 [1]. The maximum bio-oxidation efficiency was observed at pH range of 1.8 to 2.2. The bio-oxidation rate was reduced drastically at pH values less than 1.8 and was constant at pH rather than 1.8. The iron precipitation rate

increased, as medium pH increased. Bacterial oxidation activity at pH over 1.8 is inhibited due to the formation of a layer of ferric precipitates on bacteria, hindering the diffusion of protons through the cell wall [24, 25]. In addition, ferric precipitation limits the solubility of Fe (3+) which is the main oxidant of different industrial processes [22, 24, 26, 27]. At pH value below 1.8, bio-oxidation rate was decreased due to the inhibition of bacterial activity in high acidity medium [21]. On the other hand, minimal deposit formation was achieved at pH 1.6. As a result, with regard to maximal bio-oxidation efficiency and minimal ferric participate formation, pH 1.6 is appropriate for the process.

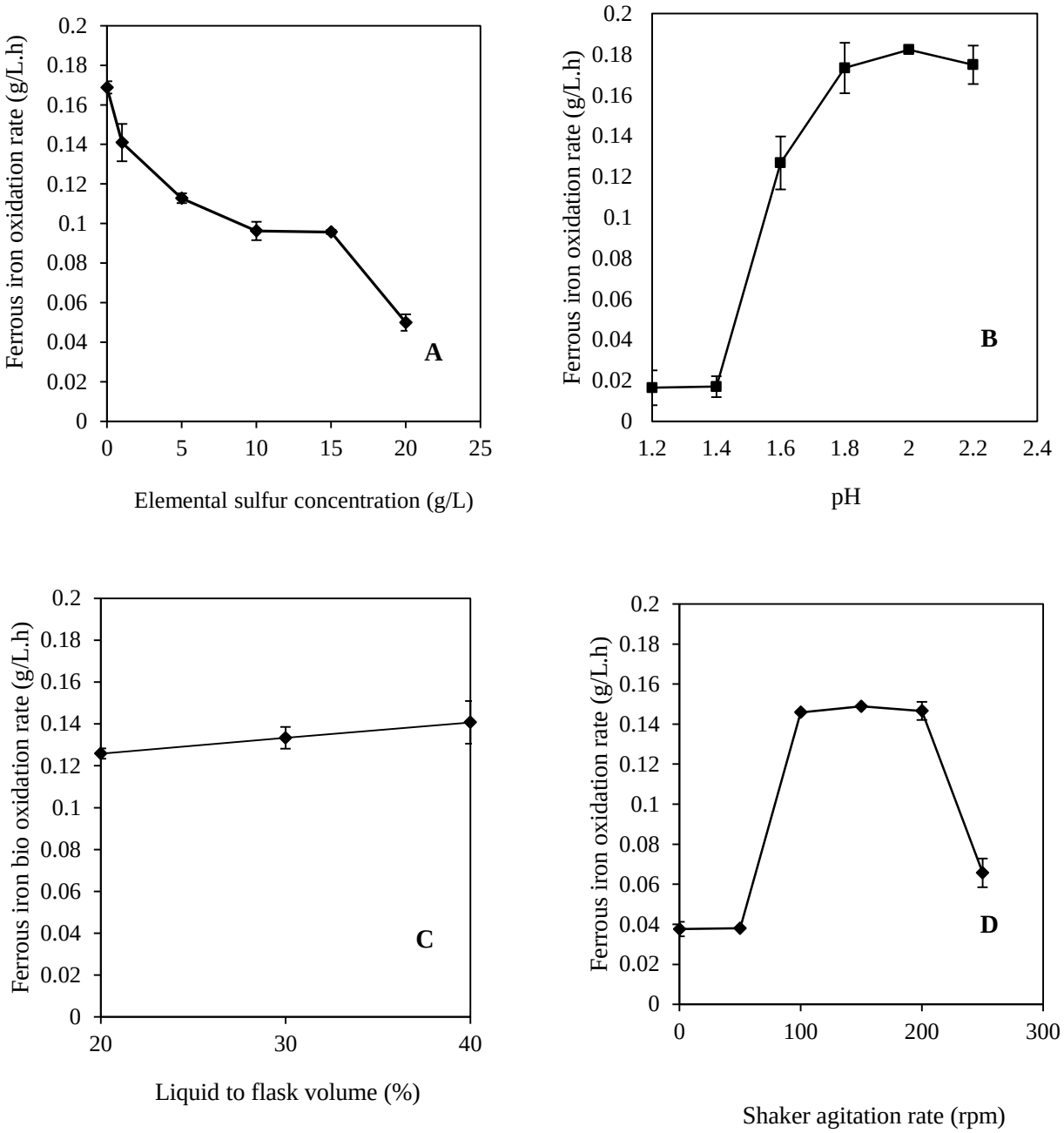

Figure 1. Effect of (A) elemental sulfur concentration, (B) pH, (C) liquid to flask volume, and (D) shaker agitation rate on ferrous iron bio-oxidation rate using OFAT method.

3.1.3. Effect of liquid to flask volume

The bio-oxidation rates at different liquid-to-flask volume percentages (20, 30, and 40 %) at concentration of 6.7 g/L of Fe^{2+} are plotted in Fig. 1-C. It can be seen clearly that liquid-to-flask volume is ineffective. To reduce culture consumption, the volume of 20 % in other steps of OFAT design is considered.

3.1.4. Effect of shaker agitation rate

A. ferrooxidans uses molecular oxygen as an electron acceptor in aerobic conditions, while carbon dioxide is demanded by the bacteria as carbon source. Oxygen and carbon dioxide mass transfer rates increase with an increase in shaker agitation intensity. Moreover, agitation rate enhances the heat transfer rate to a certain level to keep operation temperature within the desired range [28]. On the other hand, agitation rate may limit the bio-oxidation rate if it is beyond a certain level due to cell lysis in strong shear rate and the denaturation of extracellular protein [29]. Therefore, the agitation rate plays an important role in the bio-oxidation process. Fig. 1-D illustrates the results of the bio-oxidation of ferrous iron rate in different agitation rates ranging from 0 to 250 rpm. The average maximal oxidation rate for the agitation rates ranging from 100 to 200 rpm was found to be 0.145 g/L.h. There is a direct relationship between the increase in the agitation rate value and bacterial oxygen consumption. On the one hand, bio-oxidation rate reduces agitation rate values higher than 200 rpm gently due to the oxygen transfer limitation to the bacteria (Eq. 3) [5]. On the other hand, high agitation rate may damage the cells because the ferric iron can be deposited as solid particles form in the medium and the activity of cells would decrease due to the collision produced by solid particles [28]. Therefore, 150 rpm of agitation rate was considered to be the optimum level in the remainder of the tests.

3.1.5. Effect of ferrous iron concentration

A. ferrooxidans use Fe^{2+} as a nutrient and convert to Fe^{3+}. At the second stage of gas sweetening process, ferric ions are oxidizing agents which react with the sulfide ions and are converted to Fe^{2+} ions [26]. Since bacterial growth rate is directly related to the ferrous iron oxidation rate, the effect of variation of the initial Fe^{2+} concentration in the medium is important to predict the growth kinetics. Fig. 2 depicts the effect of initial ferrous iron concentration on both the bio-oxidation rate and bacteria growth rate.

The results of these experiments indicate that, in the range of 4–12 g/L of Fe^{2+}, increasing the initial substrate concentration increases the amount of the oxidation rate. At ferrous concentration above 12 g/L up to 24 g/L, bio-oxidation rate is approximately constant and, at concentration above 24 g/L, bio-oxidation rate decreases. The maximum bio-oxidation rate of 0.28 g/L.h was attained in a culture initially containing 20 g/L of ferrous ions. The results of this study are comparable with those of other studies where maximum bio-oxidation rates of 0.31 and 0.47 g/L.h were obtained, respectively, in the cultures initially containing 20 and 25 g/L.h of ferrous iron [1, 30].

Initial ferrous iron concentration also affects bacterial growth rate. The optimum ferrous iron concentration for the bacterial growth rate was obtained to be 8 g/L. Inhibition effect on bacterial growth rate was observed in the cultures initially containing 20 g/L of ferrous and higher.

The theoretical and experimental data for specific growth rate (μ) of *A. ferrooxidans* versus ferrous initial iron concentration can be observed in Fig. 3. The values obtained for μ suggested that, for non-competitive substrate inhibition, the Andrews equation (Eq. 4) was applied [1]:

$$\mu = \mu_m \frac{[Fe^{2+}]}{([Fe^{2+}]+k_s)(1+\frac{[Fe^{2+}]}{k_I})} \quad (4)$$

where μ is a specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), k_s (g/L) is the saturation constant (i.e., substrate concentration at half μ_m), and k_I (g/L) is the substrate inhibition coefficient. If $k_I >> k_s$, then:

$$\mu = \mu_m \frac{[Fe^{2+}]}{k_s+[Fe^{2+}]+\frac{[Fe^{2+}]^2}{k_I}} \quad (5)$$

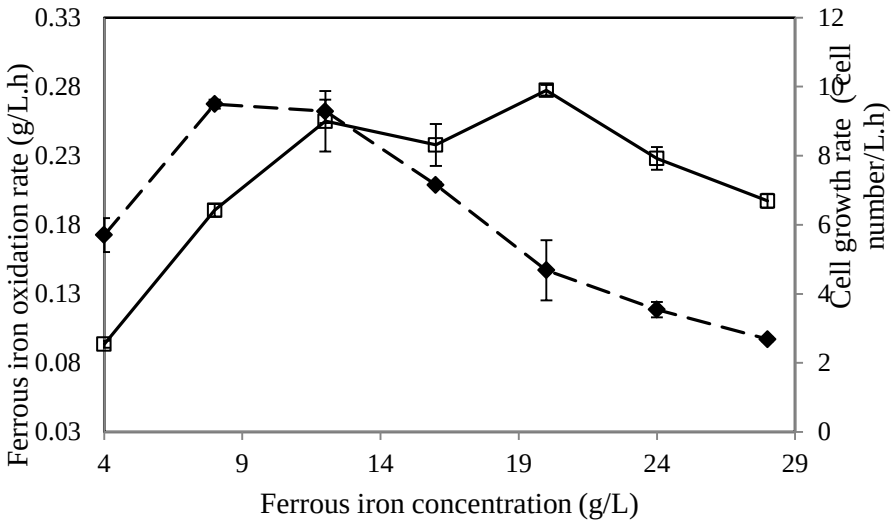

Figure 2. Ferrous iron bio-oxidation rate (□) and bacteria growth rate (♦) vs. initial ferrous iron concentration.

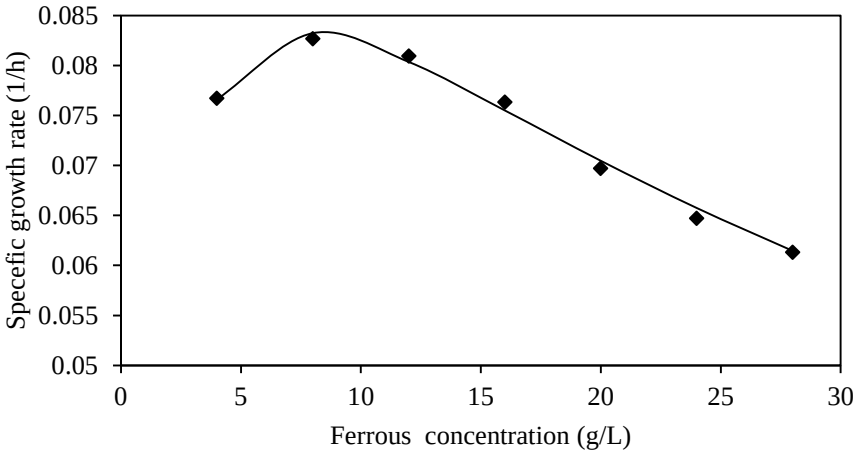

Figure 3. Theoretical (—) and experimental (♦) data of specific bacterial growth rate at different initial concentrations of Fe^{2+}.

Having carried out the required calculations, the following values were obtained: μ_m =0.13 (h^{-1}), k_s=2.36 g/L, and k_I=25.63 g/L. These values are comparable with the constants published by other investigators, especially constants reported by

Kahrizi et al. [1] (Table 4). μ_m indicates maximum specific growth rate, and k_s points to the ability of bacteria to grow in medium with low ferrous iron concentration [31] which fall into the range of the other published surveys and are better than constants reported by Kahrizi et al. [1]. The value of k_I shows the concentration range in which inhibition effect can be observed [31].

Table 4
Comparison of Andrew's equation constant in some researches.

Bacteria	k_I (g/L)	k_s (g/L)	μ_m (h^{-1})	Ref.
An indigenous thermophilic bacterium	9.06	2.85	0.194	[2, 5]
A. ferrooxidans DSM 584	16.5	1.11	0.084	[1]
A. ferrooxidans PTCC 1646	25.6	2.36	0.133	This study

As can be seen in Table 4, this value is higher than the values obtained by other researches. In two other studies, ferrous iron oxidation rate was evaluated in 500 ml shake flask containing 200 ml medium shaking at 150 rpm. While the current study was investigated at medium-to-flask volume equal to 1 to 5 at agitation rate of 150 rpm. According to Equation 3, liquid-to-flask volume is one of the important factors in mass transfer rate. Therefore, higher ferrous iron oxidation rate and higher inhibitory concentration of ferrous iron were obtained due to better mass transfer conditions in the current study.

3.2. Full-factorial design results

Having found the factor level interval and effects of factors on ferrous bio-oxidation rate in one factor at a time approach, a full-factorial design was applied to determine optimal conditions and interactions. Therefore, a full-factorial design was used to find out the effects of the following three factors at three levels (3^3) on ferrous iron bio-oxidation rate and cell growth rate as responses: initial ferrous iron concentration, agitation rate, and liquid-to-flask volume (Table 3). The experimental results were evaluated by the analysis of variance (ANOVA) and probability value (P-value) as the criteria for statistical significance was considered equal to 0.05.

3.2.1. Optimization based on ferrous bio-oxidation rate response

The ANOVA results of factorial design based on ferrous iron oxidation rate as the response are shown in Table 5. The model F-value of 4.30 implies that the model is significant. Moreover, value of “Prob > F” less than 0.05 indicates that model terms are significant. There is only a 0.02 % chance that a "Model F-Value" this large could occur due to noise. As can be seen in the table, agitation rate and liquid-to-flask volume had highly significant effects, whereas ferrous concentration was found to be ineffective in ferrous iron oxidation rate. Moreover, the most efficient interaction effect was observed between agitation rate and liquid-to-flask volume.

3.2.1.1. Effect of high ferrous iron concentration

Due to results obtained by one factor at a time approach, ferrous iron oxidation rate improved by increasing initial ferrous iron concentration up to 12 g/l and was approximately constant through the range of

12 to 20 g/l of Fe^{2+} and, then, decreased gradually (Fig. 2). As described in the last paragraph of section 3.1.5, the inhibitory concentration of ferrous iron was obtained about 25 g/l in the suitable operational conditions. Therefore, the effect of high concentration of ferrous iron with different levels of the other factors was investigated on ferrous iron oxidation rate by a full-factorial design.

According to the iron oxidation metabolism of *Acidithiobacillus ferrooxidans*, ferrous iron is converted to ferric iron by proteins on the cell surface [32, 33]. At ferrous iron concentration higher than 12 g/L, cell surface becomes saturated with the ferrous ions and, consequently, bio-oxidation rate remains constant. At too high concentration of ferrous iron, formation of ferric deposits cannot be controlled by operational conditions such as pH. Therefore, part of the cells' surface is covered by these deposits, and bio-oxidation rate decreases. In addition to one factor at a time approach, initial high ferrous iron concentration in the range of 20-30 g/l of Fe^{2+}has no significant effect on ferrous iron oxidation rate at a full-factorial approach (Fig 4-A).

Table 5
ANOVA calculation based on ferrous iron bio-oxidation rate.

Source	SS[a]	DF[b]	MS[c]	F-value	p-value	Significance
Model	0.20	26	7.676E-003	4.30	0.0002	**
A-shaker agitation rate	0.10	2	0.052	28.86	<0.0001	**
B-liquid to flask volume	0.033	2	0.016	9.17	0.0009	**
C-ferrous concentration	2.422E-003	2	1.211E-003	0.68	0.5159	
AB	0.020	4	5.109E-003	2.86	0.0425	*
AC	6.920E-003	4	1.730E-003	0.97	0.4405	
BC	0.010	4	2.506E-003	1.40	0.2595	
ABC	0.024	8	2.998E-003	1.68	0.1493	
Lack of Fit	0.048	27	1.785E-003			
Pure Error	0.25	53				
Cor Total						

[a] Sum of square of errors; [b] Degree of freedom; [c] Mean square of errors.
[*]Significant (P-value <0.05); [**]Highly significant (P-value <0.01).

3.2.1.2. Effect of liquid-to-flask volume ratio and agitation rate

In one factor at a time approach, it was found that, at low initial ferrous iron concentration, agitation rate and liquid-to-flask volume ranging respectively from 100 to 150 rpm and from 20 to 40 % are ineffective factors in ferrous iron oxidation rate (Fig. 1). However, as shown in Figs. 4-B and 4-C, the effects of these two factors in high initial ferrous iron concentration are highly significant on ferrous iron oxidation rate.

On the other hand, at high ferrous iron concentration, the formation of high concentration of ferric ions and conversion of some parts of these ions to solid deposits could lead to oxidative degradation. Malhotra et al. [9] indicated that the presence of total dissolved solid (TDS) in the media leads to oxidative degradation that may be due to the mass transfer rate decline. Therefore, ferrous iron oxidation rate enhances by decreasing liquid-to-flask volume and increasing agitation rate due to mass transfer improvement.

Savić et al. [13] evaluated ferrous iron oxidation rate at shake flask containing 9 g/l ferrous iron, and concluded that liquid-to-

flask volume is ineffective. This outcome corresponds with our results in one factor at a time approach with low initial ferrous iron concentration. However, to the best of author's knowledge, no investigation has been carried out yet into the simultaneous effect of these two factors in ferrous iron oxidation rate at high initial ferrous iron concentration.

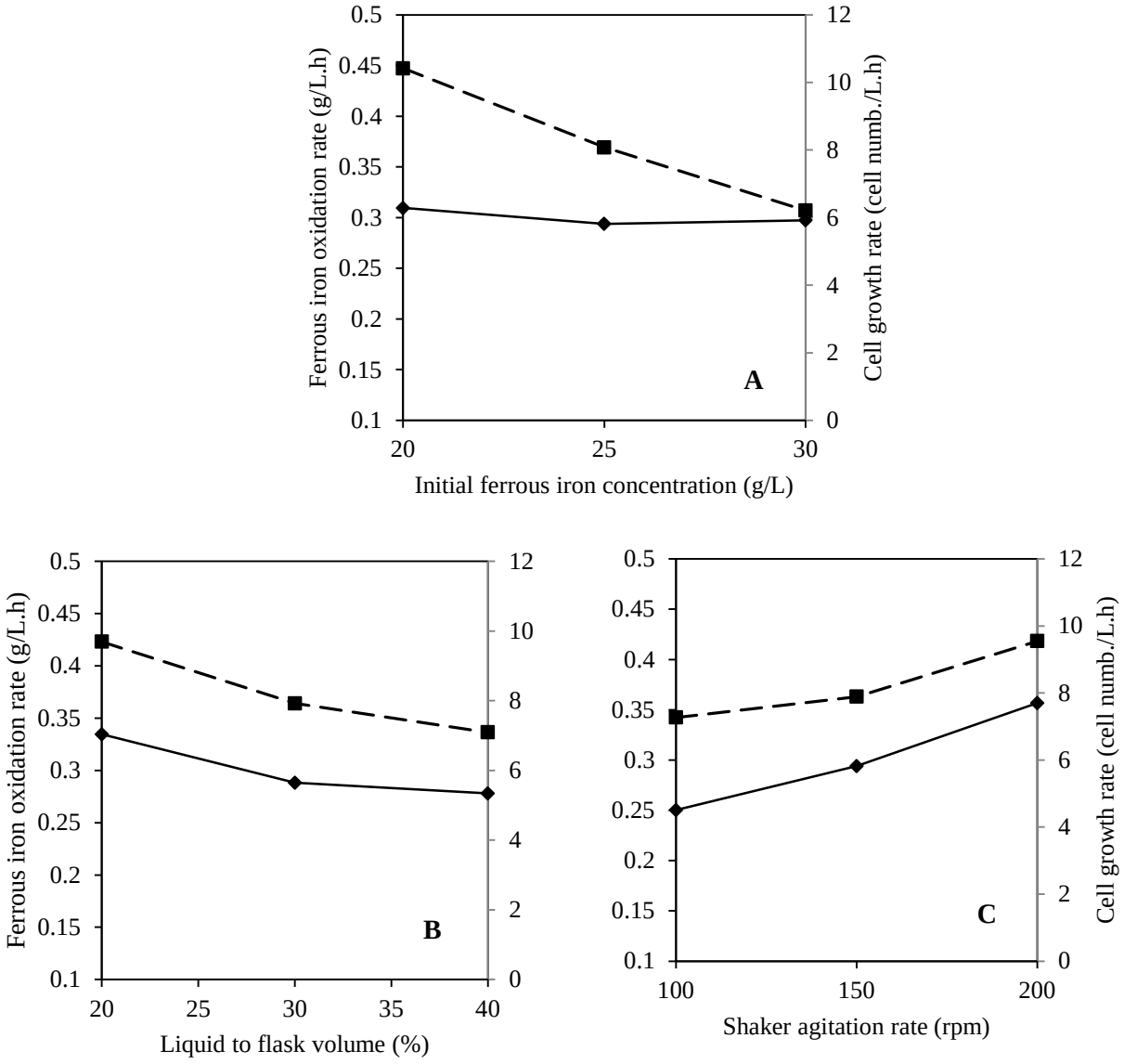

Figure 4. Effect of initial ferrous iron concentration (A), shaker agitation rate (B), and liquid-to-flask volume (C) on ferrous iron bio-oxidation rate (◆) and cell growth rate (■).

3.2.1.3. Effect of binary interaction of agitation rate and liquid-to-flask volume

As is shown in Fig. 5, no interaction can be observed by increasing agitation rate at levels 1 and 2 of liquid-to-flask volume, and ferrous oxidation rate increases by increasing agitation rate. However, at level 3 (40 %), liquid volume interaction, especially with level 2 (30 %), can be observed. A reduction in mass transfer due to high liquid-to-flask volume ratio (40 %) cannot be overcome by increasing agitation rate (up to 150 rpm) at in high initial ferrous iron concentration. Therefore, at level 3, inadequate amount of oxygen and carbon dioxide is available. According to Table 5, other binary and

ternary interactions have no effect on ferrous iron oxidation rate.

As a result, maximum oxidation rate of 0.417 g/L.h was achieved at 200 rpm, 30 g/L initial ferrous iron concentration and liquid-to-flask volume equal to 20 % (Table 3). It is evident that, in this approach, a bio-oxidation rate has improved about 40 %, compared with one factor at a time approach. That is due to the effect of applying factor's interactions on ferrous iron oxidation rate.

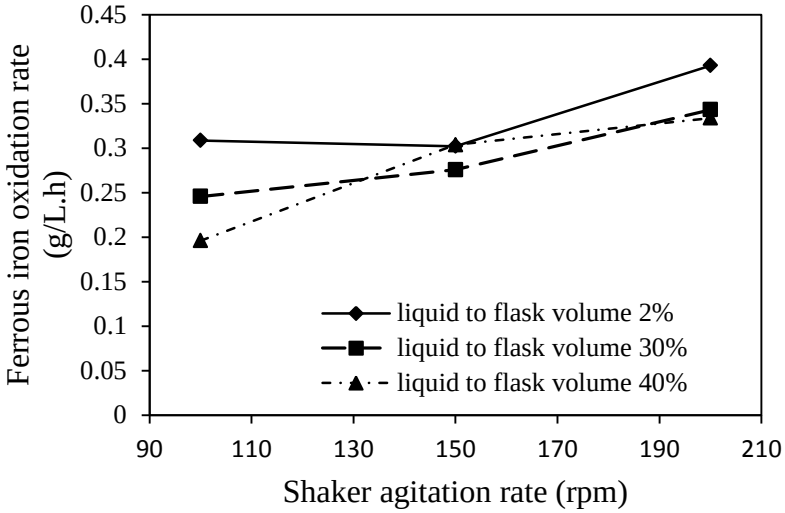

Figure 5. Effect of binary interaction of shaker agitation rate and liquid-to-flask volume on ferrous iron bio-oxidation rate (the other variables were set at average level value).

3.2.2. Optimization based on cell growth rate responses

Cell growth rate improvement is important for biological ferrous oxidation using immobilized microbial cells. ANOVA results of cell growth rate are shown in Table 6. The model F-value of 3.18 implies that the model is significant, and there is only a 4.9 % chance that the "Model F-Value", large-sized, could occur due to noise. In contrast with ferrous iron oxidation rate, just ferrous iron concentration has a significant effect on cell growth rate. The effects of shaker agitation rate, liquid-to-flask volume, and initial ferrous iron concentration on bacterial growth rate are plotted in Figure 4.

Table 6
ANOVA calculation based on cell growth rate.

Source	SS[a]	DF[b]	MS[c]	F-value	p-value	Significance
Model	216.41	18	12.02	3.18	0.0496	*
A-shaker agitation rate	25.00	2	12.50	3.31	0.0897	
B-liquid to flask volume	31.72	2	15.86	4.20	0.0566	
C-ferrous concentration	80.00	2	40.00	10.59	0.0056	**
AB	40.80	4	10.20	2.70	0.1081	
AC	20.51	4	5.13	1.36	0.3295	
BC	18.39	4	4.60	1.22	0.3753	
Residual	30.21	8	3.78			
Cor Total	246.62	26				

[a] Sum of square of errors; [b] Degree of freedom; [c] Mean square of errors.
[*]Significant (P-value <0.05); [**]Highly significant (P-value <0.01).

Because the cell growth rate is limited by high ferrous iron concentration, shaker agitation rate and liquid-to-flask volume cannot affect it significantly. Further ferrous concentration rises, which reduces cell growth rate significantly which corresponds with other researches [1, 2, 34] and the results of OFAT design in the current study (Fig. 2). Binary interactions have no effect on cell growth rate according to ANOVA results of Table 6.

Finally, maximum cell growth rate (14.2 cell number/L.h) was achieved at 150 rpm, 20 g/L initial ferrous iron concentration, and liquid-to-flask volume equal to 20 %.

3.3. Ferrous oxidation with a culture adapted to high ferrous sulfate

In bacterial physiology, lag phase is defined as the phase in which cells adapt to a new environment. Culture history influences the oxidation process [35]. Reducing process timeframe is considered as a critical factor in process industrialization. Lag phase prolongation extends this timeframe and also increases bio-oxidation rate. Therefore, it is necessary to minimize lag phase before any biological process industrialization.

As mentioned before, the reduction of ferrous iron oxidation rate in high ferrous concentration can be for the reason of shock is applied to the bacteria and causes an increase in lag phase. Therefore, if the bacteria are adapted to high concentrations of ferrous iron gradually in a multi-stage adaptation process, this problem may be solved. This matter is important in the cell immobilization process as the first step of the continuous ferrous oxidation in the bioreactor. Fig.6 shows the effect of serial increasing ferrous iron concentration on the prolongation of the lag phase of bacterial growth. As is obvious, increasing initial ferrous iron concentration extends the lag phase substantially. The adaptation of *A. ferrooxidans* to high ferrous iron concentration decreased the bacteria lag time from 24 to 10 hours (Fig. 7). Because of the bacterial lag phase reduction, ferrous iron oxidation rate enhanced significantly (about 86 %), and maximum bacterial concentration was obtained in a shorter period of time in contrast with non-adapted bacteria. The effect of this factor on reducing the lag phase of the bacteria has not been reported yet in any articles.

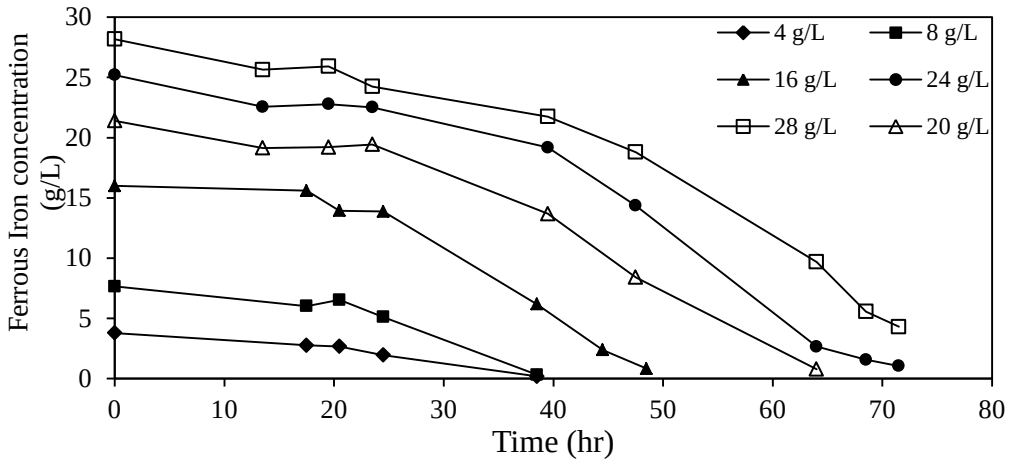

Figure 6. Effect of increasing ferrous iron concentration on the prolongation of the lag phase of bacterial growth.

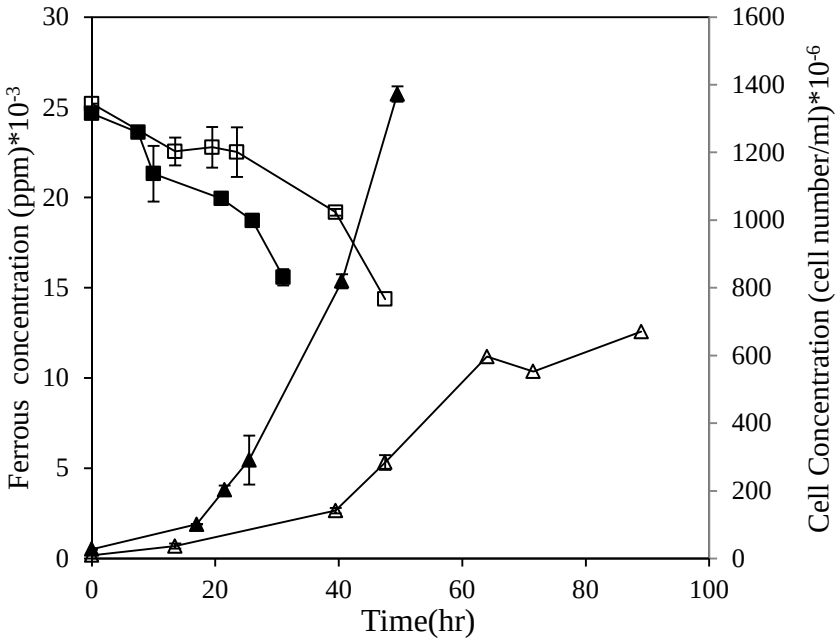

Figure 7. Comparison of bacterial growth rate (▲,△) and ferrous iron oxidation rate (■,□) for adapted (filled symbols) and non-adapted (blank symbols) bacteria in culture containing 25 g/L initial ferrous iron.

4. Conclusions

Bio-oxidation of ferrous iron may be an alternative method for producing ferric sulfate, which is a reagent used for removal of H_2S from natural gas, desulphurization of coal, treatment of acid mine drainage, etc. The ability of a strain of *A. ferrooxidans* in the bio-oxidation of ferrous sulfate and inhibitory effects of ferrous iron concentration on bacterial growth was investigated using two experimental design methods. By using a full-factorial design, it was found that, at high initial ferrous iron concentration, agitation rate and liquid-to-flask volume ratio and their interaction had significant effects on ferrous iron bio-oxidation rate with about 1.5-fold enhancement in comparison to OFAT method. The experimental results showed that maximum growth rate was achieved at low initial ferrous iron. Furthermore, it was obtained that ferrous iron bio-oxidation rate increases with the increase of the initial ferrous iron concentration up to 12 g/L; after that, it has no effect on biological oxidation rate significantly. A 2.4-fold time reduction in the lag phase of bacterial growth and an 86 % ferrous iron oxidation rate increase were achieved by using serial subculturing with linear ramping upwards from 6.8 to 25 g/L of Fe^{2+}.

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