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Response surface methodology (RSM) analysis of organic acid production for Kaolin beneficiation by *Aspergillus niger*

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ABSTRACT

In the present investigation, *Aspergillus niger* isolated from pistachio shell was applied to remove iron impurities from an Iranian kaolin sample. In order to study the effects of initial pH, sucrose and spore concentration on oxalic and citric acid production, and consequently iron dissolution, response surface methodology based on a five-level, three-variable central composite design of experiments was employed. Three models were suggested to predict response values based on the mentioned variables. The most important variables on iron dissolution were initial pH, sucrose and spore concentration, respectively. Also, the highest iron concentration, 311.30 mg/l, was obtained when initial pH was 2, sucrose concentration, 70 g/l, and spore concentration, 35 × 10^7 spore/l, and represented the removal of 67.4% of the total iron contents of the clay.

1. Introduction

Organic acids have been found to have the capacity to remove metals from contaminated soils without seriously deteriorating the soil properties. They are much less toxic, and therefore much easier to dispose. In addition, they do not remain in the soil after treatment like other chemical agents [1].

Because chemically produced organic acids are very expensive and may impose significant costs to the process, some species of fungus like *Aspergillus niger* are applied to generate these substances by fermentation of organic substrates [2]. *A. niger* is the source of three organic acids, gluconate, citrate, and oxalate [3].

Organic acids from fermentations by *A. niger* have been proved to be useful in kaolin bleaching [4,5]. Kaolin belongs to a group of clay minerals called hydrous aluminum silicates with properties such as brightness, white color, very fine particle size, chemical stability, and kiln firing characteristics quite attractive for many applications including cement, bricks, chinaware, paper coating, rubber, pharmaceuticals, paint, plastics, pesticides, and fertilizers. One of the key parameters that govern the quality of kaolin is the pigment brightness achieved by removal of colored impurities such as iron contaminations [6].

The most commonly used organic acids are oxalic and citric. The dissolution mechanism comprises three steps [7]: (1) adsorption of organic ligands on system interface: activation of solid surface, (2) reductive dissolution of active centers: generation of ferrous ions in the solution (induction period), and (3) autocatalytic dissolution of active centers.

In the present research work, effects of initial pH, amount of carbon source, and spore concentration on oxalic and citric acid generation, and iron removal extent were investigated in a leaching process using *A. niger* isolated from pistachio shell, proved to have good ability to remove iron impurities from kaolin in the previous work [5].

2. Materials and methods

2.1. Kaolin sample

Kaolin sample with the particle size of 90% under 5.74 μm (d_{90} = 5.74 μm) was provided by Mehrkhak Company, Tehran, Iran. The presence of 2.19% iron impurities (Table 1) makes the clay unsuitable for using in sanitary ware manufacturing.

2.2. Microorganisms and culture media

The fungus strain originally isolated from pistachio shell on potato dextrose agar (PDA) and Czapek Dox agar (CZ) by streak method. It was later identified as *A. niger* according to the method of Klich [8].

A solid media (malt extract, 30 g/l; meat peptone, 3 and agar, 15 g/l, at pH 5.6) was employed for the growth and maintenance

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Table 1
Chemical composition of the clay sample

Component	Percent
P_2O_5	0.108
MnO	0.001
TiO_2	2.660
MgO	0.500
K_2O	2.720
Na_2O	0.190
CaO	0.130
Fe_2O_3	2.190
Al_2O_3	23.570
SiO_2	56.380

of the microorganism at 30 °C. A synthetic media [4] containing NH_4NO_3, 450 mg/l; KH_2PO_4, 100 mg/l; $MgSO_4 \cdot 7H_2O$, 300 mg/l; $FeSO_4 \cdot 7H_2O$, 0.1 mg/l; $ZnSO_4 \cdot 7H_2O$, 0.25 mg/l, and sucrose at five different levels (Table 2) was employed as culture media.

2.3. Bioleaching of mineral samples

Fungal spores were suspended from a 7-day agar slant in a sterile solution (0.1% Tween80, and 0.9% NaCl) and enumerated by a microscope. Bioleaching experiments were carried out in 500-ml Erlenmeyer flasks containing 100 ml of culture media inoculated at five different concentrations (Table 2), and incubated at 30 °C, and 160 rpm on a rotary shaker. Amount of 3 g of kaolin were added to the culture media in the beginning of the cultivation. All experiments were performed in duplicate, and the average of the results reported, were with 2% deviation.

2.4. Methods of analysis

In order to determine the kaolin composition, and specially its iron contents, XRF analysis was done by ARL 8410 instrument, tube anode: Rh, and 60 kV. Also, to determine kaolin particle size, particle size analysis made by Fritsch Particle Sizer “Analysette 22”. Results showed 90% of the clay particle size was below 5.74 µm.

To register the changes in pH value, dissolved iron, sucrose, and oxalic and citric acid concentrations, sampling was done in determined intervals. Then the solid phase containing kaolin and biomass was separated from leaching medium by centrifuging at 1000 rpm.

The pH value of the liquor was measured by Metrohm pH meter model 744. Dissolved iron concentration was measured by the *o*-phenanthroline method [9]. To quantify the concentration of organic acids exerted to the media, pyridine acetic anhydride method [10] was applied to determine the citric acid concentration, and manganometry method [9] to measure oxalic acid concentration. To determine total sugar contents, the spent media were hydrolyzed, and analyzed by colorimetry using the Nelson and Somogyi method [11,12].

2.5. Design of experiments

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize the response [13]. The central composite design (CCD) is the standard RSM [14], and allows estimating the second degree polynomial of the relationships between the independent variables and the dependant variable and gives information about interaction between variables in relation to the dependant variable [15]. Therefore, a 2^3 CCD with 15 runs, and six replications of the center points was selected to determine the initial pH, sucrose and spore concentration for the maximum production of oxalic and citric acid, and consequently improvement of iron removal from kaolin clay. The experiments were designed by using the Design Expert 7 Trial (State Ease, Inc., Minneapolis, MN, USA), and performed in duplicate.

A CCD consists of l^k factorial points (usually coded ±1 notation), augmented by $2k$ axial points $\{(\pm\alpha, 0, 0, \ldots, 0), (0, \pm\alpha, 0, \ldots, 0), (0, 0, \pm\alpha, \ldots, 0), (0, 0, \ldots, \pm\alpha)\}$ and n_c centre points $(0, 0, 0, \ldots, 0)$. Where k is the number of controllable process variables, l is the number of levels for each process parameter, α equals to $(n_f)^{1/4}$, and n_f is the number of points used in factorial position (2^k) [16]. The levels of the chosen independent variables used in the experiments are given in Table 2. The chosen independent variables used in this experiment were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1, 2, \ldots, k \tag{1}$$

where x_i is the dimensionless value of a variable, X_i the actual value of a variable, X_0 the value of X_i at the center point, and ΔX is the step change [15].

The behavior of the system is explained by the following empirical second-order polynomial:

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

where Y is the predicted response, X_i and X_j are the input variables, β_0 is the intercept term, β_i is the linear effects, β_{ii} is the squared effect, and β_{ij} is the interaction term [13].

The polynomial equations for the three responses were validated by the statistical test called ANOVA (analysis of variance), for determination of the significance of each term in equations and also to estimate the goodness or fit in each case. Response surfaces were drown for the experimental results obtained from the effect of different variables on the iron, oxalic and citric acid concentration in order to determine the individual and cumulative effects of these variables, and the mutual interactions between them.

3. Results and discussion

The coded values of the test variables, namely initial pH (A), sucrose concentration in g/l (B), and spore concentration in spores/l (C) and the experimental results of iron, oxalic and citric acid concentration in each case are presented in Table 3. Using the results listed in Table 3, data analysis is performed for each response variable explained in the following sections.

Table 2
levels and codes of variables for central composite design

Factors	Levels				
	−1.68	−1	0	+1	+1.68
Initial pH	0.98	2	3.5	5	6.02
Spore concentration (spore/l)	0.14×10^7	9×10^7	22×10^7	35×10^7	43.9×10^7
Sucrose (g/l)	49.5	70	100	130	150.5

Table 3
Central composite design matrix of three test variables in coded values, three observed responses and final pH of the cultivated media

Run no.	Coded values			Responses			Final pH of the media
	A	B	C	Oxalic acid (g/l)	Citric acid (g/l)	Iron concentration (mg/l)	
1	−1	−1	−1	7.17	10.83	247.84	1.44
2	+1	−1	−1	8.97	12.18	307.13	1.41
3	−1	+1	−1	4.46	6.68	221.15	1.57
4	+1	+1	−1	5.56	6.28	262.88	1.70
5	−1	−1	+1	9.93	14.70	311.30	1.39
6	+1	−1	+1	10.23	14.18	306.05	1.46
7	−1	+1	+1	8.31	12.22	260.22	1.64
8	+1	+1	+1	9.32	12.19	302.71	1.56
9	−α	0	0	0.44	0.55	50.34	0.95
10	+α	0	0	6.48	8.03	281.90	1.50
11	0	−α	0	8.71	11.01	305.15	1.38
12	0	+α	0	5.17	7.68	223.32	1.51
13	0	0	−α	7.60	8.51	225.97	1.58
14	0	0	+α	11.04	15.19	282.91	1.47
15	0	0	0	9.42	13.34	288.30	1.52
16	0	0	0	10.03	16.14	295.16	1.40
17	0	0	0	9.52	12.30	278.51	1.47
18	0	0	0	9.52	10.28	270.51	1.52
19	0	0	0	10.74	13.15	284.18	1.41
20	0	0	0	9.92	12.76	291.76	1.48

A: initial pH; B: sucrose concentration; C: spore concentration.

3.1. *Statistical analysis*

In the Design Expert software, the response data were analyzed by default. Some raw data might not be fitted and needs the transformation of data. This transformation applies a mathematical function to all the response data to meet the assumptions that makes the ANOVA to be valid. Data transformations were needed for all three responses. Therefore, a power function was applied for these responses. In the next step, the effects for all model terms were calculated, and statistics such as *F*-values, lack of fit, and R^2-values were used for comparing the models, and consequently, a quadratic model was selected. The model terms in the equations are calculated after elimination of some insignificant variables and their interactions which have the lowest *F*-value. The ANOVA results for all responses have been summarized in Table 4. The models adequacy was tested through lack-of-fit *F*-tests. Lack of Fit compares the residual error to the pure error. Lack of fit is not desirable, so a small F value and probability greater than 0.1 are desired. If a model shows lack of fit, it should not be used to predict the response [17]. As indicated in Table 4, the lack-of-fit of all the models (0.156, 0.170, and 0.178) have a probability more than 0.1, and the models are highly significant with very low probability values (<0.0001). Moreover, the models present relatively high determination coefficients (0.790, 0.658, and 0.606). The R^2-value provides a measure of how much variability in the observed response values can be explained by the experimental variables and their interactions. The closer the R^2-value is to 1, the better the model predicts the response [18]. The low values of coefficient of variation (20.56, 29.43, and 26.85) indicate good precision and reliability of the experiments [19]. Adequate precision (14.832, 11.680, and 10.107) measures the signal to noise ratio and a ratio grater than 4 is generally desirable [20]. Also, the predicted sum of squares (PRESS) is a measure of how a particular model fitted each point in the design [21].

Furthermore, Table 5 presented *p*-value of those variables included in each model. Values of “Prob. > *F*” less than 0.05 indicate model terms are significant. Values greater than 0.10 indicate the model terms are not significant.

3.2. *Oxalic acid production*

For iron, oxalic acid is five times more effective than citric acid, and is capable of complexing and reducing iron [22]. In order to dissolve a mole of iron, three equivalents of oxalic acid are necessary [23].

$$3C_2O_4^{2-} + Fe^{3+} \rightarrow [Fe(C_2O_4)_3]^{3-}$$

Taking the produced oxalic acid concentration to the account, effects of the three mentioned variables on oxalic acid generation in the *A. niger* cultured media was investigated. Referring to Table 5, it can be concluded that all three main variables are clearly significant; while the interactions between initial pH and spore concentration (AC), and between sucrose and spore concentration (BC) are not significant. Also, according to the greater *F*-value of the spore concentration, it is the most important variable on the response. The interaction between initial pH and sucrose concentration (AB), and C^2 were removed to improve the model adequacy.

Table 4
Summary of the analysis of variance result for the response models

	Responses		
	Oxalic acid concentration	Citric acid concentration	Iron concentration
R^2	0.790	0.658	0.606
R^2 adjusted	0.744	0.596	0.534
Prob. > *F*	<0.0001	<0.0001	<0.0001
Lack of fit (LOF)	0.156	0.170	0.178
PRESS	875.709	6204.689	1.7 E + 14
Adequate precision	14.832	11.680	10.107
Coefficient of variation	20.56	29.43	26.85

Table 5
F-value and *p*-value for each variable in the polynomial model

Response	Statistics	Factors								
		A	B	C	A^2	B^2	C^2	AB	AC	BC
Oxalic acid concentration	*F*-value	12.12	18.54	28.87	52.09	10.91	–	–	0.29	1.40
	p-Value	0.0015	0.0001	<0.0001	<0.0001	0.0024	–	–	0.5965	0.2460
Citric acid concentration	*F*-value	1.94	10.78	23.67	24.05	3.53	–	–	–	0.90
	p-Value	0.1725	0.0024	<0.0001	<0.0001	0.0691	–	–	–	0.3491
Iron concentration	*F*-value	19.08	14.47	6.91	8.93	–	–	0.24	1.11	–
	p-Value	0.0001	0.0006	0.0129	0.0053	–	–	0.6264	0.3008	–

A: initial pH; B: sucrose concentration; C: spore concentration.

Oxalic acid formation depends on the pH value [24]. There are several different reports on the optimum pH leading to high yields of oxalic acid [24–26]. Some authors consider a neutral pH as the most adequate, while using sucrose as the carbon source, pH of 6 has been reported as optimum [27]. In our experiments, the initial pH ranged from 0.98 to 6.02. Analyzing the final results, it was concluded that the initial pH has the least effect on the oxalic acid production because of its lowest *F*-value (Table 5). The reason is that for the organic acid exertion, the pH of the media in all of the flasks dropped to a level below 2 in the early days of the experiment, and became relatively identical for all of them (Table 3). Also, as low pH inhibits the production of gluconic and oxalic acid [28], the concentration of this acid in the media is not desirable. It is suggested that in order to prevent pH from changing, some buffered solutions be added to the cultured media [26].

Table 3 denotes that the highest oxalic acid concentration was 11.04 g/l which is achieved in Run 14, with the initial pH, sucrose, and spore concentration of 3.5, 100 g/l, and 43.9 × 10^7, respectively. Also, the lowest oxalic acid concentration was 0.44 g/l, which is

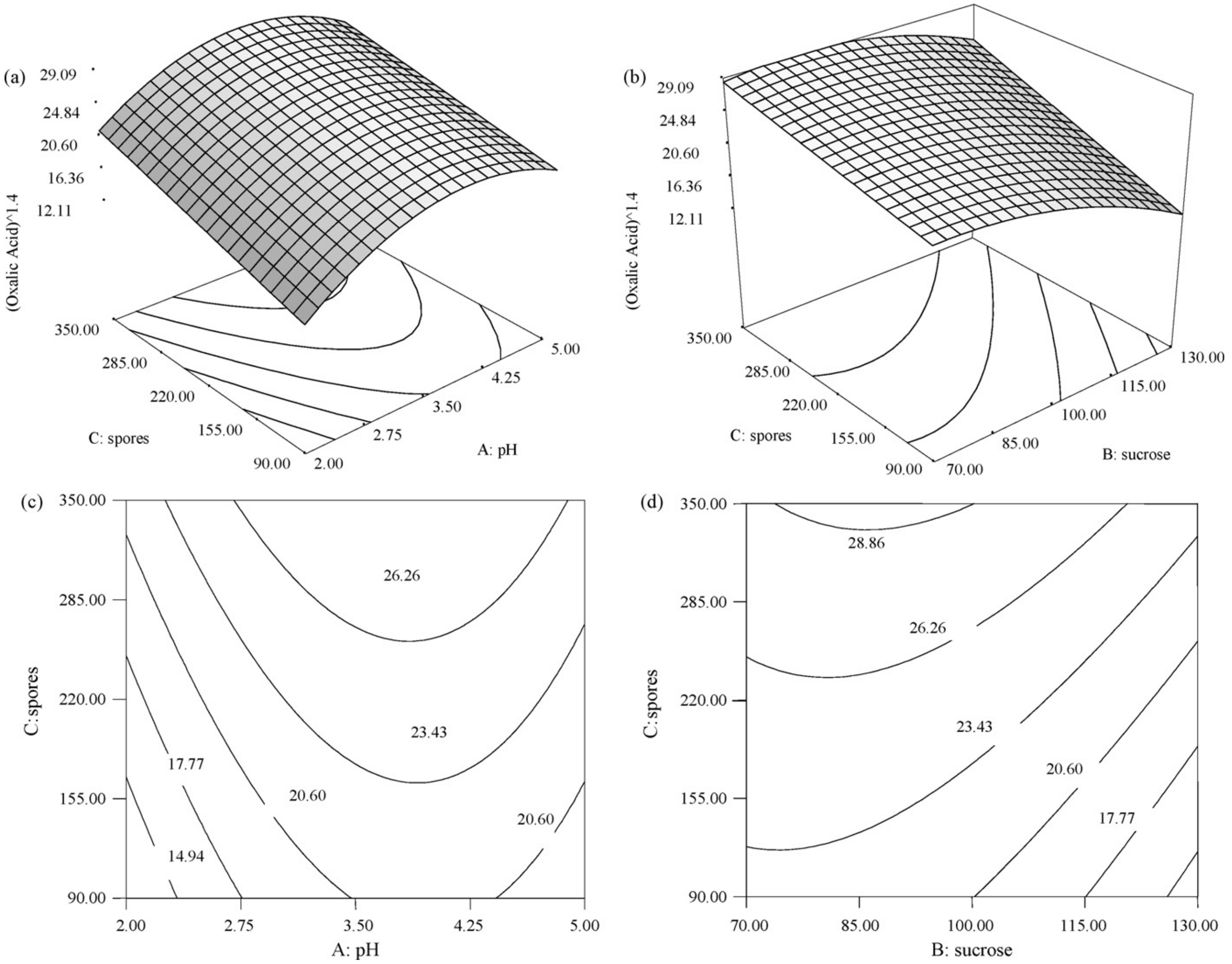

Fig. 1. Response surface plots for effects of initial pH, and spore concentration (a), and sucrose and spore concentration (b) on oxalic acid production, and contour plots of interaction between initial pH, and spore concentration (c), and sucrose and spore concentration (d).

obtained in Run 9, with initial pH, sucrose, and spore concentration of 0.98, 100 g/l, and 22×10^7, respectively.

The regression models made to predict oxalic acid formation by *A. niger* in the presence of kaolin sample for coded and actual variables, are presented in Eq. (3), and Eq. (4), respectively. The Model *F*-value is 17.20; there is only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise.

$$\begin{aligned}(\text{Oxalic acid})^{1.4} &= +24.77 + 2.66\text{A} - 3.29\text{B} + 4.11\text{C} \\ &\quad -5.35\text{A}^2 - 2.45\text{B}^2 - 0.53\text{AC} + 1.18\text{BC}\end{aligned} \tag{3}$$

$$\begin{aligned}(\text{Oxalic acid})^{1.4} &= -29.16905 + 19.01082(\text{pH}) + 0.36737(\text{sucrose}) \\ &\quad + 0.010922(\text{spores}) - 2.37615(\text{pH})^2 \\ &\quad - 2.71866\,E - 003(\text{sucrose})^2 - 2.73998\ E \\ &\quad - 003(\text{pH})(\text{spores}) + 3.02779\,E \\ &\quad - 004(\text{sucrose})(\text{spores})\end{aligned} \tag{4}$$

The three-dimensional and contour plots in Fig. 1 show the effects of initial pH, and spore concentration, and also, sucrose and spore concentration on oxalic acid production while sucrose concentration and pH are fixed at their center values, i.e., 100 and 3.5 g/l, respectively. In Fig. 1a and c, oxalic acid concentration has a maximum value on the pH 3.9. After this point, at a constant value of spore concentration, increasing the initial pH decreases the oxalic acid generation. Moreover, Fig. 1b and d indicate that oxalic acid formation increases by increasing sucrose concentration up to 79.84 g/l, but after that, acid concentration decreases. Also, considering the pH or sucrose concentration constant, increase in spore concentration, causes oxalic acid generation to increase.

3.3. Citric acid production

In terms of tonnage, citric acid is one of the world's major microbial metabolites that is produced on a commercial scale [29]. The most common method for commercial production involves submerged fermentation using the filamentous fungus, *A. niger* growing on media containing sucrose or glucose [30].

Effects of initial pH, sucrose and spore concentration were also investigated on citric acid production. Referring to Table 5, it can be seen that sucrose and spore concentration are the effective variables on the citric acid formation, and the highest effect belongs to spore concentration because of the greater amount of *F*-value it has (23.67), compared to the other variables. Also, it can be concluded from the table that initial pH, and all of the interactions (AB, AC, and BC) are not significant variables. pH of the media is very important for citric acid generation. Its importance is at two different times in the fermentation. Firstly, the spores require a $\text{pH} > 5$ in order to germinate. Secondly, the pH for citric acid production needs to be low ($\text{pH} \leq 2$) [28]. However, as it is mentioned later pH of the media is not controlled during the experiment, therefore the pH was reduced to a level of below 2, and became the same for all of the flasks; consequently, pH did not show its real effect on the response variable. Considering the results of this experiment, it is recommended that the best initial pH to maximize citric acid production is 3.7. More increase in initial pH will lower the citric acid concentration in the media.

The highest citric acid concentration was 16.14 g/l which attained in a condition in which initial pH, sucrose, and spore concentration were 3.5, 100 g/l, and 22×10^7, respectively (Run 16). Also, the lowest acid concentration was 0.55 g/l, and gained when initial pH, sucrose, and spore concentration were 0.98, 100 g/l, and 22×10^7, respectively (Run 9) (Table 3).

The regression models made to predict citric acid formation by *A. niger* for coded and actual variables are presented in Eq. (5), and Eq. (6), respectively. The Model *F*-value is 10.57; there is only a 0.01% chance that a "Model *F*-Value" this large could occur due to noise. In order to improve the model adequacy, the interactions between initial pH and sucrose concentration (AB), and also between pH and spore concentration (AC), and C^2 were removed from the model.

$$\begin{aligned}(\text{Citric acid})^{1.5} &= +47.44 + 2.97\text{A} - 7.00\text{B} + 10.38\text{C} - 10.13\text{A}^2 \\ &\quad - 3.88\text{B}^2 + 2.65\,\text{BC}\end{aligned} \tag{5}$$

$$\begin{aligned}(\text{Citric acid})^{1.5} &= -37.07467 + 33.50490(\text{pH}) + 0.47983(\text{sucrose}) \\ &\quad + 0.011949(\text{spores}) - 4.50319(\text{pH})^2 - 4.31288\,E \\ &\quad - 003(\text{sucrose})^2 + 6.78606\ E \\ &\quad - 004(\text{sucrose})(\text{spores})\end{aligned} \tag{6}$$

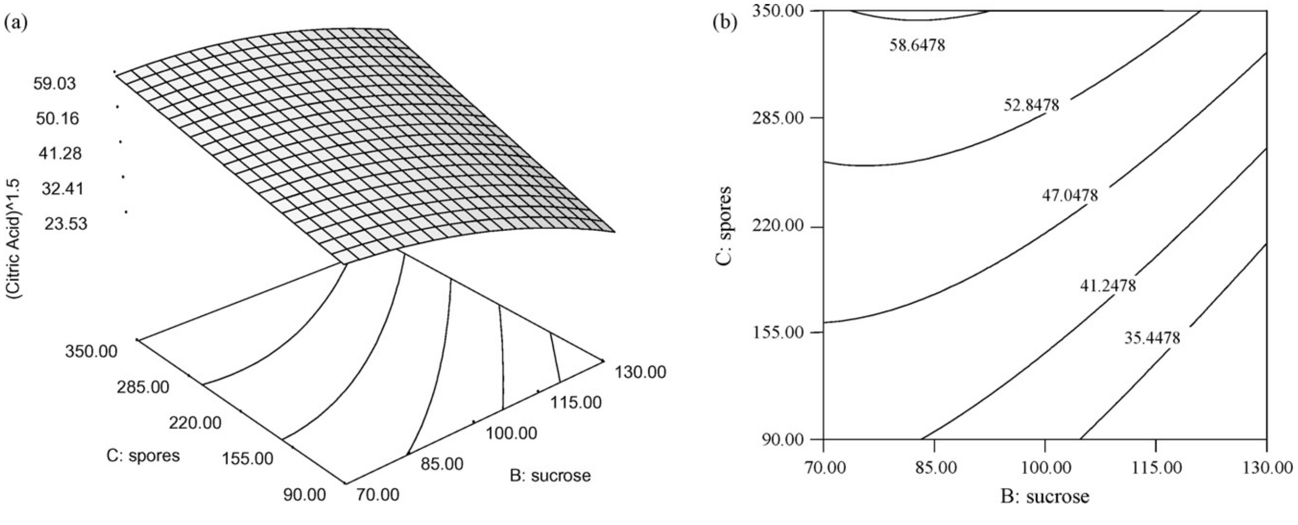

Fig. 2. Response surface plot for effects sucrose and spore concentration on citric acid production (a), and contour plot of the interaction between spore and sucrose concentration (b).

The response surface and contour plots in Fig. 2 show the effects of interaction between sucrose and spore concentration on citric acid formation at the pH value of 3.5. This figure suggests that the best sugar concentration in order to achieve maximum citric acid generation is 73.12 g/l. Further increase in sugar amount caused citric acid exertion to decrease.

Both the type of carbon source and its concentration are critical to citric acid fermentation [28]. Sucrose is preferable to glucose as *A. niger* has a potent extracellular myceliumbound invertase that is active at low pH and rapidly hydrolyzes sucrose [31]. Studies on the effect of sugar concentration on citric acid yield in submerged fermentation showed that highest yields attained at sugar concentration of 10% w/v. No citric acid was produced in media that contained less than 2.5% sugar [32]. A higher sugar concentration leads to greater amount of residual sugar, making the process uneconomical. While a lower concentration of sugar leads to lower yield of citric acid due to the accumulation of oxalic acid in the culture broth [33].

Also, in terms of spore concentration, it can be said that the higher the spore concentration, the greater the citric acid generation.

3.4. *Iron dissolution*

As stated earlier, the main purpose of this research is to optimize the leaching of iron in kaolin by *A. niger*. Investigating the effects of the three mentioned variables on the iron concentration as the response variable, it is concluded that all the main variable can be considered significant (Table 5). However, none of the interactions (AB, AC, BC) are significant. The most important variables are initial pH, sucrose and spore concentration with the *F*-values of 19.08, 14.47, and 6.91, respectively.

It has been agreed by several workers [34,35,36] that the optimum pH for dissolving iron oxide is pH 2.5–3.0. The solution pH governs the distribution of various oxalate ions in the leach system. Below pH 1.5, oxalic acid exists mainly as $H_2C_2O_4$, whereas $HC_2O_4^-$ is the most predominant species at pH 2.5–3.0 [37].

The highest iron concentration was 311.30 mg/l, and represented the removal of 67.4% of the total iron contents of the clay. This concentration achieved in the Run 5 with initial pH, sucrose, and spore concentration of 2, 70 g/l, and 35×10^7, respectively. In contrast, the lowest dissolved iron concentration was 50.34 mg/l, and obtained in Run 9, when initial pH, sucrose, and spore concentration were 0.98, 100 g/l, and 22×10^7, respectively.

To predict the dissolved iron concentration in the kaolin bleaching experiments by *A. niger*, regression models using coded and actual variables are presented in Eq. (7) and Eq. (8), respectively. The sucrose and spore concentration interaction (BC), B^2, and C^2 were removed from the model to improve its

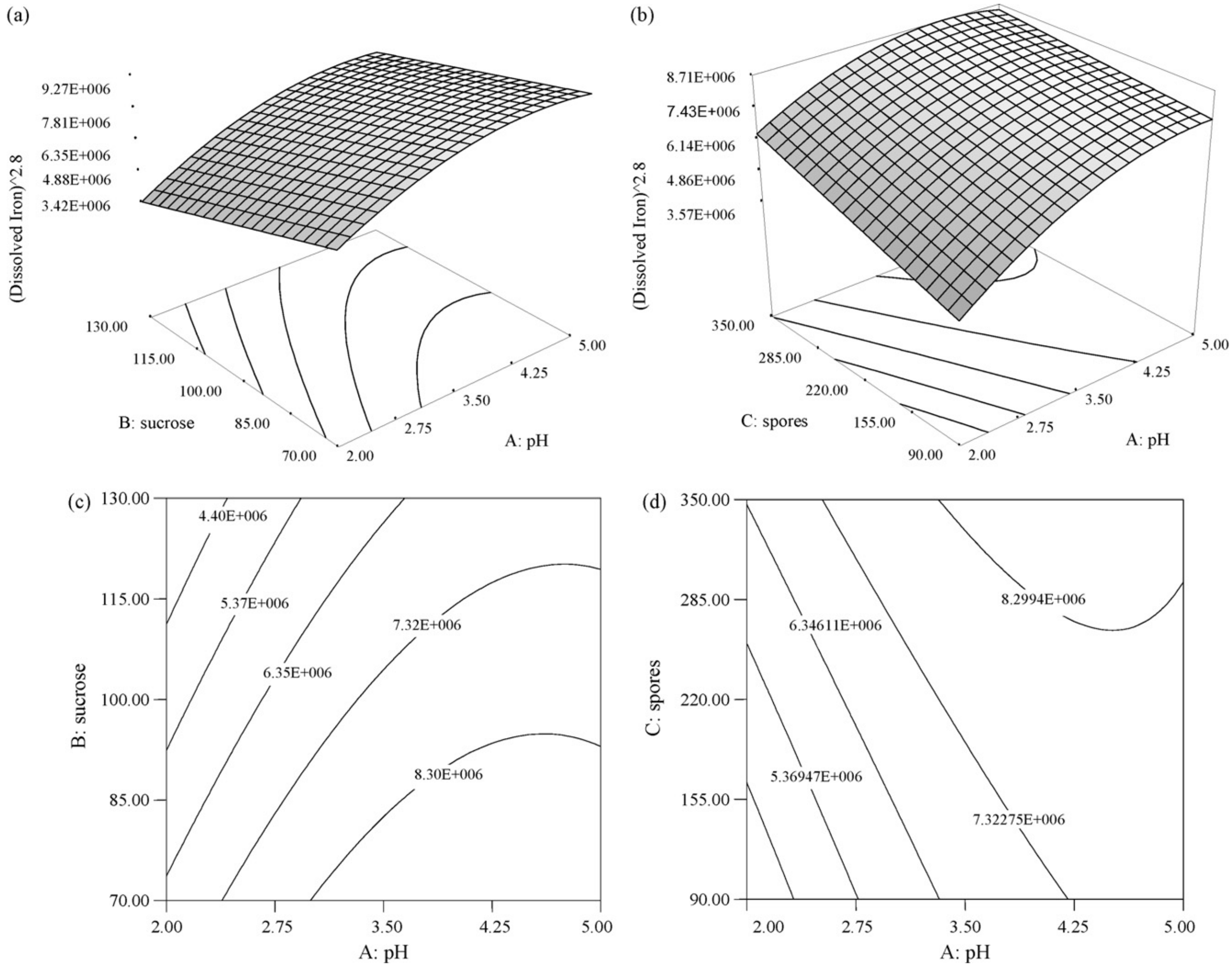

Fig. 3. Response surface plots for effects of initial pH, and sucrose concentration (a), and initial pH and spore concentration (b) on dissolved iron concentration, and contour plots of interactions between initial pH and sucrose concentration (c), and initial pH and spore concentration (d).

adequacy.

$$\begin{aligned}(\text{Dissolved iron})^{2.8} = {} & +7.519\,E+006+1.532\,E+006\,\text{A} \\ & -1.334\,E+006\,\text{B}+9.221\,E+005\,\text{C} \\ & -1.011\,E+006\,\text{A}^2+2.252\,E+005\,\text{AB} \\ & -4.818\,E+005\,\text{AC}\end{aligned} \tag{7}$$

$$\begin{aligned}(\text{Dissolved iron})^{2.8} = {} & +1.17559\,E+006+4.21027\,E+006(\text{pH}) \\ & -61997.48064(\text{sucrose}) \\ & +15740.68755(\text{spores})-4.49385\,E \\ & +005(\text{pH})^2+5005.39246(\text{pH})(\text{sucrose}) \\ & -2470.66089(\text{pH})(\text{spores})\end{aligned} \tag{8}$$

The response surface and contour plots in Fig. 3 shows the effects of pH and sucrose interaction (AB) (Fig. 3a and c), and also, pH and spore interaction (AC) (Fig. 3b and d) is redundant on iron concentration while the other variables, spore and sucrose concentration were set to their central values, 22×10^7 spores/l, and 100 g/l, respectively. It can be deduced from the figure that the dissolved iron concentration increases by increasing the initial pH value until it reaches to 4.6. Also, increase in sugar concentration leads to decrease in iron removal, while increasing the spore concentration results in increasing the iron concentration of the cultured media.

4. Conclusion

The response surface methodology based on a three-variable central composite design was used to determine the effect of initial pH, sucrose and spore concentration on oxalic and citric acid generation, and iron dissolution from kaolin by *A. niger*. Second-order regression models were developed using Design Expert software for predicting the responses in all experimental regions. For each response the coefficients of the postulated model calculated, and the high adequacy of the model was proven by presenting the statistical specifications of them. Statistical analysis showed that initial pH has a positive effect on the responses until the initial pH reaches to 3.9 for oxalic acid, 3.7 for citric acid, and 4.6 for dissolved iron concentration. After these points, the effect of initial pH is negative on oxalic and citric acid production. Also, spore concentration has a positive effect on all of the response variables. Furtheremore, sucrose concentration of the media has a negative effect on iron dissolution, but positive effect on oxalic and citric acid production until it reaches to 79.84 and 73.12 g/l, respectively.

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