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Response Surface Methodology Optimization of Dibenzothiophene Biodesulfurization in Model Oil by Nanomagnet Immobilized *Rhodococcus Erythropolis* R1

Zahra Etemadifar[1], Peyman Derikvand[1], Giti Emtiazi[1] and Mohammad H. Habibi[2]

1. *Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan 81746-73441, Iran*

2. *Department of Chemistry, Faculty of Chemistry, University of Isfahan, Isfahan 81746-73441, Iran*

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Abstract: *Rhodococcus erythropolis* R1 is a capable strain in bioconversion of dibenzothiophene (DBT) to 2-hydroxybiphenyl (2-HBP) in oil model. In order to prevent the contamination of biodesulfurization (BDS) products by free cells, microbial cells were immobilized using different materials such as magnetic Fe_3O_4 nanoparticles (NPs). In this study, magnetic NPs were produced by two different procedures and their characteristics were determined via transmission electron microscopy (TEM) and X-ray diffraction (XRD). Also, binding of NPs on the cell surface was studied and better NPs were used for cells immobilization. Both NPs were crystallized and less than 10nm. The BDS by immobilized cells was carried out in biphasic system, and media conditions were optimized statistically by response surface methodology (RSM). The DBT concentration, temperature and interaction between them had statistically significant effects on 2-HBP production by nanomagnet immobilized cells. The optimum DBT concentration, temperature and pH for 2-HBP production by immobilized *R. erythropolis* R1 were obtained at 6.76mM, 29.63 °C and 6.84 respectively by HPLC analysis.

Key words: Biodesulfurization, biphasic system, nanomagnet particles, *Rhodococcus erythropolis* R1.

1. Introduction

The extensive consumption of sulfur-rich fossil fuels leads to release a number of harmful chemicals such as sulfur oxides, which in turn, causes severe environmental problems including air pollution and acid rain. In fact, a major part of the petroleum sulfur content consists of organic compounds which are hard to separate through conventional methods and are considered as one of the major problems in crude oil refining [1]. For instance, it is reported that some organic components such as dibenzothiophene (DBT) remain in the oil even after desulfurization processes [2]. As a remedy, several effective bioprocesses have been developed based on the ability of a few bacterial strains such as *Rhodoccocus* species which can remove sulfur from organic compounds like DBT and produce 2-hydroxybiphenyl (2-HBP) as the final product without causing oxidative loss of fuel carbon [3]. Although bioprocesses have been shown to be promising in organic desulfurization, there are still certain problems within the system which hindered their large scale application. For example, using the free cells in BDS leads to formation of a two phase oil/water mixture containing the suspended cells which requires cost intensive unit operations e.g. centrifugation at the downstream of the process. In addition, there is a possibility to have cell contaminations at the final products [4].

To address the problem, immobilization methods are frequently used in the industrial processes. Clearly, immobilization has inherent advantages compared to the free cells including enhanced stability of the system,

Corresponding author: Zahra Etemadifar, Dr., professor assistant, research field: microbiology-biodegradation-bioremediation. E-mails: z_etemadifar@yahoo.com and z.etemadifar@sci.ui.ac.ir.

easy separation of cells, minimizing or eliminating the cell contaminations in the products, convenient recovery and re-use of cells which enable their frequent use in the process [5]. Magnetic separation is a promising technology in the support systems for immobilization, since the rapid separation and easy recovery of immobilized cells could be reached in an external magnetic field, and the capital and operation costs could also be reduced [6]. In this study, magnetic Fe_3O_4 nanoparticles (NPs) were prepared in two different procedures, their characteristics were investigated and appropriate NPs were used for immobilization of bacterial cells.

Some factors such as pH, temperature and the concentration of DBT can affect the BDS rate of immobilized cells. Evidently, maximum BDS efficiency can be achieved by setting the parameters at their optimized values. Response surface methodology (RSM) is a statistical method based on the multivariate non-linear model and has some advantages including reduction in the time and number of experiments and improvement the statistical interpretation possibilities [7]. Consequently, the RSM was used in this paper to optimize the important parameters and to increase the BDS efficiency of the immobilized cells in oil/water biphasic system.

2. Materials and Methods

2.1 Chemicals

Ferric chloride ($FeCl_3{\cdot}6H_2O$), ferrous chloride ($FeCl_2{\cdot}4H_2O$) and methanol (HPLC grade) were purchased from Sigma Chemical Co. DBT and n-tetradecane were purchased from Merck. 2-HBP was prepared from Fluka Chemical Co. All other chemicals were analytical grade and commercially available.

2.2 Bacterial Strain and Growth Condition

Rhodococcus erythropolis R1 (NCBI GenBank Accession No. GU570564) was used in desulfurization experiments. This strain, which has a high capability in the conversion of DBT to 2-HBP, was previously isolated from an oil-contaminated soil sample [8]. It was cultured in basal salt medium (BSM) supplemented with 0.3 mM DBT as the sole sulfur source. Cell cultivation was carried out in a 1,000 mL flask containing 200 mL of BSM medium on an orbital shaker incubator (n-biotech,inc) at 180 rpm and 30 °C. The BSM had the following composition: $Na_2HPO_4{\cdot}7H_2O$ 8 $g{\cdot}L^{-1}$, KH_2PO_4 4 $g{\cdot}L^{-1}$, NH_4Cl 2 $g{\cdot}L^{-1}$, $MgCl_2$ 0.2 $g{\cdot}L^{-1}$, $FeCl_3$ 0.001 $g{\cdot}L^{-1}$, $CaCl_2$ 0.001 $g{\cdot}L^{-1}$, DBT 0.3 mM as sulfur source and glucose 15 $g{\cdot}L^{-1}$ as carbon source.

2.3 Preparation of Magnetic Fe_3O_4 Nanoparticles

Magnetic Fe_3O_4 NPs were prepared in two different procedures:

In the procedure 1, magnetic Fe_3O_4 NPs were prepared by Yeh et al. [9] method with a little change. Briefly 25 mL of 0.2 M ferrous chloride was mixed with 100 mL of 0.1 M ferric chloride solution at ambient temperature under nitrogen gas and mechanical stirring and then 3 ml of 2 M HCl solution was slowly added to make the solution slightly acidic. Then 1 g of glycine was added, and afterward, 11 mL 5 M NaOH solution was added dropwise into the mixture to increase its pH to over 10, to provide an alkaline environment for Fe_3O_4 to precipitate. Next, an additional 3 g of glycine was added, and the mixture stirred for 15 min and then sonicated for 30 min. Finally, 5 mL acetone solution was added and agitated. The Fe_3O_4 NPs were separated with a magnetic field and the supernatant discarded by decantation. The precipitate was washed several times and resuspended in deionized water.

In the procedure 2, the oleate-modified Fe_3O_4 NPs were synthesized using the protocol described by Liu et al. [10]. Briefly, 6.76 g of ferric chloride and 2.73 g of ferrous chloride were dissolved in 100 mL deionized water under nitrogen gas with mechanical stirring. The solution temperature was set at 85 °C. Then, 16 mL 25% wt. $NH_3.H_2O$ was added and afterward 4 mL of oleic acid was dripped into the suspension by a syringe. The reaction was kept at 85-90 °C for 30 min. The Fe_3O_4 precipitates were separated using a magnetic

decantation and washed several times with deionized water. Hydrophilic magnetic NPs were obtained by modification of magnetic precipitate with 7.1 M of $NH_3.H_2O$ to pH 8-9 which were mono-disperse in aqueous solution.

2.4 Nanoparticles Characterization

Two different produced NPs were characterized and the better NPs were chosen to be used in immobilization of bacterial cells.

Transmission electron microscopy (TEM) (model EM 280, Philips, Germany) was used for morphology studying of the NPs. In order to preparation of TEM samples, the NPs solutions were sonicated for 5 min to better disperse. A drop of each sample was placed with a carbon-coated copper TEM grid (200-300 mesh) and kept at room temperature to dry and then, imaging was done [11].

Powder X-ray diffraction (XRD) study was used to determination the presence of Fe_3O_4 nano crystals and performed between 20° and 80° with a copper X-ray source on a Bruker instrument (Germany).

In order to study the binding of NPs on the cell surface, immobilization by both produced NPs was done using the procedure described in the next section. Afterward, immobilized cells were harvested by a magnetic field. Remained cells in the supernatant were counted by colony plate count on nutrient agar and considered as not absorbed cells (colony count of non-immobilized cells was done as a positive control).

2.5 Immobilization of Cells by Nanomagnetic Fe_3O_4

A volume of 40 ml of the bacterial cell culture at the late exponential phase (5 $g{\cdot}DCW{\cdot}L^{-1}$) was transferred into 100 mL Erlenmeyer flask and then, 1.5 mL of 30 $g{\cdot}L^{-1}$ magnetic suspension was added and mixed thoroughly [12]. After absorption of the magnetic NPs on the cell surface, a permanent magnet was placed at the side of the vessel. The supernatant was decanted and immobilized cells were washed and suspended in fresh BSM.

2.6 Batch Biodesulfurization of DBT in Model Oil

The biphasic media was consisted of BSM (aqueous phase) and n-tetradecane (organic phase) in a 2:1 ratio and DBT as the sulfur source. The BSM medium as aqueous phase helps the generation of the necessary cofactors in 4S pathway such as FMN and NADH, and aids the cells to survive. The BDS experiments were carried out in 100 mL flasks at 30 °C on an orbital shaker at 180 rpm (n-biotech, inc). The incubation time of DBT utilization and 2-HBP production was 20 h. In order to investigate the effect of nanomagnet immobilization on DBT BDS, an equal amount of immobilized and non-immobilized cells were added to biphasic media separately and their 2-HBP production was measured after 20 h.

2.7 Statistical Design of Experiments

Response Surface Methodology has been generally adopted to optimize the design variables in a timely manner and at lower costs. It can be used to manage the system by a set of factors at different levels and facilitates identifying the influence of individual factors, the relationship between them and finally establishing the performance at the optimum levels obtained by a few selected experimental sets [13]. DBT concentration (X_1), temperature (X_2) and pH (X_3) were regarded as the important factors in BDS activity of immobilized cells. A 3-factor and 3-level Box-Behnken design (BBD) based on RSM methodology was applied to determine the optimum level of variables and to study their relationship. The factors and their levels are shown in Table 1.

All factors at middle (0) level constitute the central points while combination of factors consisting of one at its lowest (-1) level or highest (+1) level. A total of 15 experimental runs of three factors in different combinations were carried out in duplicate and the observed results are shown in Table 2. All experimental design and data analysis were performed using the Design Expert software version 8.0.1.

Table 1 Coded values of experimental variables in immobilized cells.

Independent variables	-1	0	+1
X_1: DBT concentration (mM)	2	6	10
X_2: Temperature (°C)	20	30	40
X_3: pH	5	7	9

Table 2 Response surface Box-Behnken design (BBD) for immobilized cells.

Run	X1	X2	X3	2-HBP (mM)
1	0	+1	+1	0.55
2	+1	0	-1	0.76
3	0	-1	-1	0.66
4	+1	+1	0	0.65
5	+1	-1	0	0.67
6	+1	0	+1	0.73
7	-1	0	+1	0.60
8	0	0	0	0.98
9	-1	-1	0	0.58
10	0	+1	-1	0.59
11	0	0	0	0.92
12	-1	0	-1	0.62
13	0	0	0	0.97
14	0	-1	+1	0.59
15	-1	+1	0	0.43

2.8 Analytical Methods

High-performance liquid chromatography (HPLC) was used to quantitatively assay the DBT (retention time = 5.29 min) and 2-HBP (retention time = 3.16 min) in n-tetradecane phase. HPLC was performed on a KNAUER advanced scientific instruments (Germany) equipped with an MZ-analysentechnic C18 column (5 µ-250 mm) and a UV detector (Smartline 2600) set at 254 nm. The mobile phase was a solution of methanol-water (90:10, v/v) with a flow rate of 1.5 mL·min^{-1}.

3. Results and Discussion

3.1 Nanoparticle Characterization

The Fe_3O_4 NPs were stable in distilled water and the magnetic fluid did not settle after 5 months of storage at room temperature. The obtained TEM images showed that both produced NPs had approximately spherical morphology and were in the range of 5-10 nm (Fig. 1). The large particles cannot well be binding to the cell surface and therefore, smaller NPs are of interest. In addition, the magnetic NPs should be smaller than the critical magnetic domain size (around 50 nm) to be superparamagnetic [14].

The XRD patterns of the two produced NPs are shown in Fig. 2 and indicated the presence of predominantly Fe_3O_4 crystals. The intensity of NPs produced by procedure 1 was obtained 55 (Fig. 2a) and for sample prepared by procedure 2 was 90 (Fig. 2b).

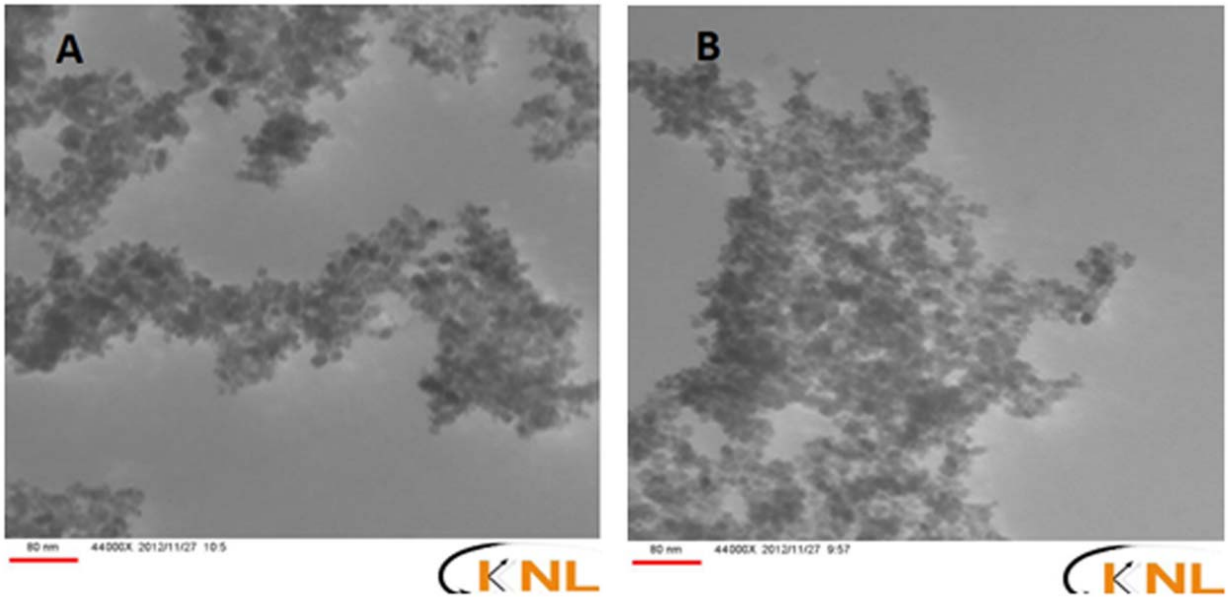
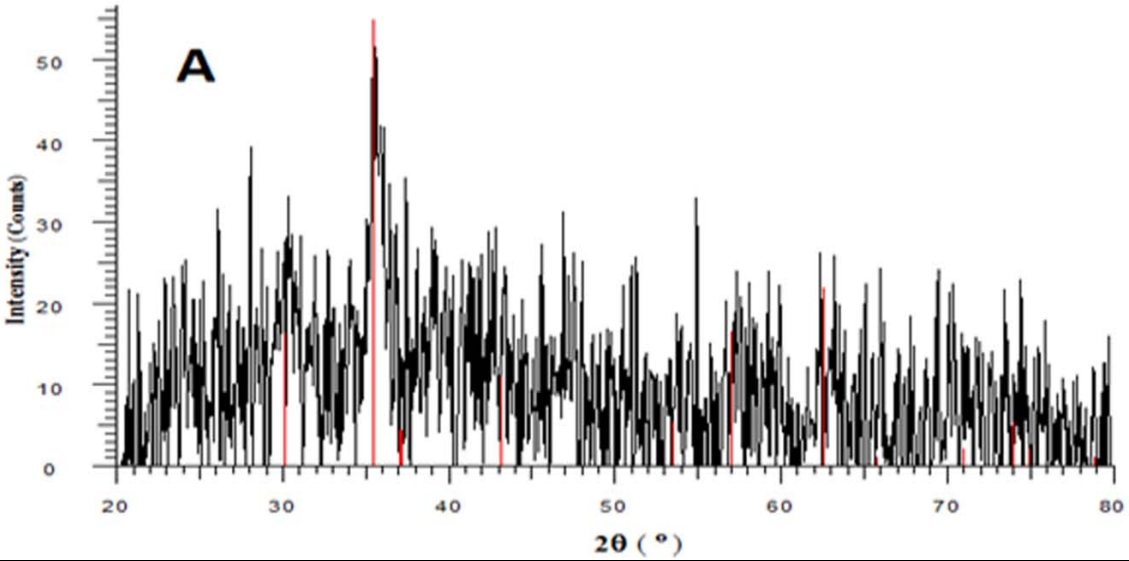
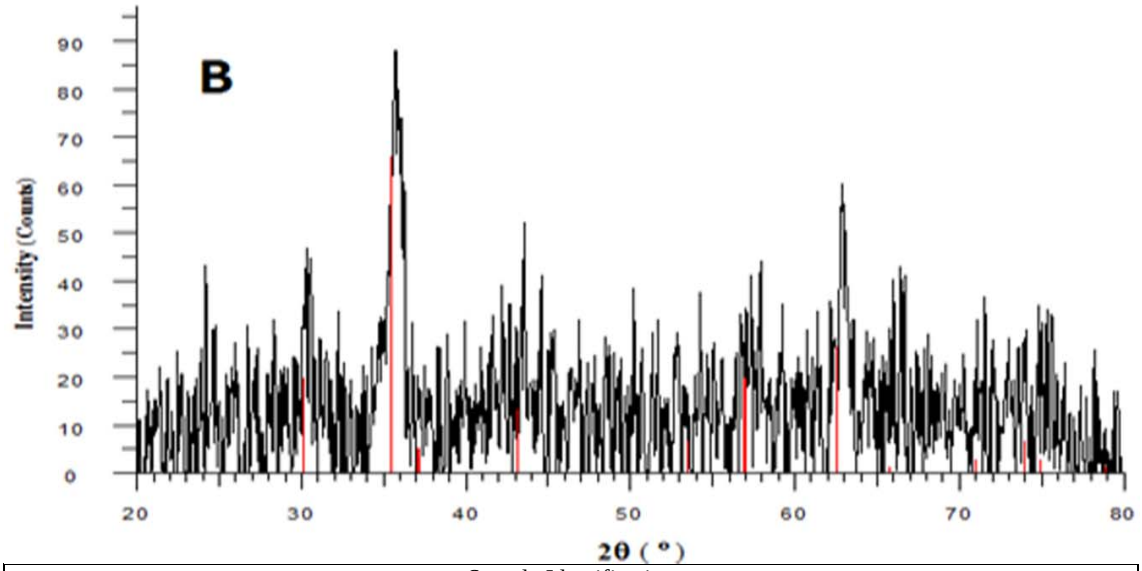


Fig. 1 Transmission electron microscopy image of magnetic Fe_3O_4 nanoparticles. Nanoparticles produced by (A) procedure 1 and (B) by procedure 2.



Sample Identification		
Line color	Compound Name	Formula
Red	Magnetite, Syn	$FeFe_2O_4$



Sample Identification		
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Red	Magnetite, Syn	$FeFe_2O_4$

Fig. 2 The XRD pattern of the two produced nanoparticles. Nanoparticles produced by procedure 1 (A) and by procedure 2 (B).

Colony count analysis showed that in cell immobilization using NPs produced by procedure 1, only 78% of the cells had absorbed NPs while in immobilization using NPs produced by procedure 2, 94% of the cells were decorated by NPs and separated by magnetic field. The high surface energy and larger specific surface area of the Fe_3O_4 NPs make it strongly adsorbed on the surfaces of microbial cells. But, in oleate-modified NPs, the hydrophobic interaction between the cell membrane and the hydrophobic tail of oleate plays another important role in cell adsorption [12]. Therefore, due to better absorption, NPs produced by procedure 2 were used for immobilization of bacterial cells and BDS of DBT in model oil.

3.2 Statistical Analysis

The variables interaction can be simultaneously investigated by response surface model. A quadratic polynomial equation was established to recognize the relationship between 2-HBP production of immobilized cells and variables based on the experimental results of BBD (Table 2). The model of coded units is calculated using:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=1+1}^{3} \beta_{ij} X_i X_j$$

where, Y is the predicted response, X_i is the variable β_0 is constant, β_i is the linear effect, β_{ii} is the quadratic effect, and β_{ij} is the interaction effect.

In this experiment, model of coded units after removing non significant parameters can be expressed as:

$$Y = 1.97 - 0.19X_1 + 0.12X_2 - 0.063X_1X_2 - 0.40X_1^2 - 0.41X_2^2$$

where, Y is the response value (mM), X_1 is DBT concentration (mM), X_2 is temperature (˚C) and X_3 is pH. Positive and negative sign before terms indicates synergistic and antagonistic effect respectively [15].

The equation indicates a quadratic linear relationship between variables and 2-HBP. The effects of factors levels on the BDS efficiency were determined employing analysis of variance (ANOVA) and the statistically significant factors were distinguished for (P value < 0.05). The Model F-value was obtained 62.06 that implied the model was significant and there was only a 0.01% chance that a Model F-value this large could occur due to noise (Table 3). Values of Prob > F (P value) less than 0.05 indicate model terms are significant. In this case X_1, X_2, X_1X_2, X_1^2, X_2^2 and X_3^2 are significant model terms. The Lack of Fit F-value of 0.35 implies the Lack of Fit is not significant relative to the pure error, which indicates the model is good. There is a 79.53% chance that a Lack of Fit F-value this large could occur due to noise. The R-Squared (R^2) is 0.9911 and (A*dj* R^2) is 0.9752 indicate the model is significant. The Pred R-Squared was 0.9377, which was reasonable agreement with the A*dj* R-Squared. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Our ratio was 43.31 that indicate an adequate signal. According to the present model, DBT concentration, temperature and interaction between them were significant but, pH and its interaction with other factors were not statistically significant. This model can be used to navigate the design space.

3.3 Biodesulfurization Analysis

The response surface and its contour plot at the base can represent the regression model developed to investigate the interaction between factors and specify the optimum level of each factor. The interaction of two independent factors can be shown by each response surface with a contour plot while another factor is fixed at the level of zero. The fitted surface and contour plots between DBT concentration and temperature, DBT concentration and pH, temperature and pH are presented in Fig. 3. The highest 2-HBP production was obtained when all factors were at the middle level (Table 2).

Li et al. [12] showed that coated and non-coated *R. erythropolis* LSSE8-1 cells had the same desulfurizing activity but, Ansari et al. [11] reported that decorated *R. erythropolis* IGST8 cells with nanomagnet particles had a 56% higher DBT desulfurization activity in basic

Table 3 Analysis of variance (ANOVA).

Source of variance	df	Mean square	F value	P value
Model	9	0.039	62.06	0.0001 significant
X_1	1	0.042	66.39	0.0005
X_2	1	9.800E-003	15.47	0.0110
X_3	1	3.200E-003	5.05	0.0745
X_1X_2	1	4.225E-003	6.67	0.0493
X_1X_3	1	2.500E-005	0.039	0.8503
X_2X_3	1	2.250E-004	0.36	0.5771
X_1^2	1	0.080	126.12	<0.0001
X_2^2	1	0.19	300.63	<0.0001
X_3^2	1	0.064	101.71	0.0002
Residual	5	6.333E-004		
Lack of Fit	3	3.667E-004	0.35	0.7953 not significant
Pure error	2	1.033E-003		

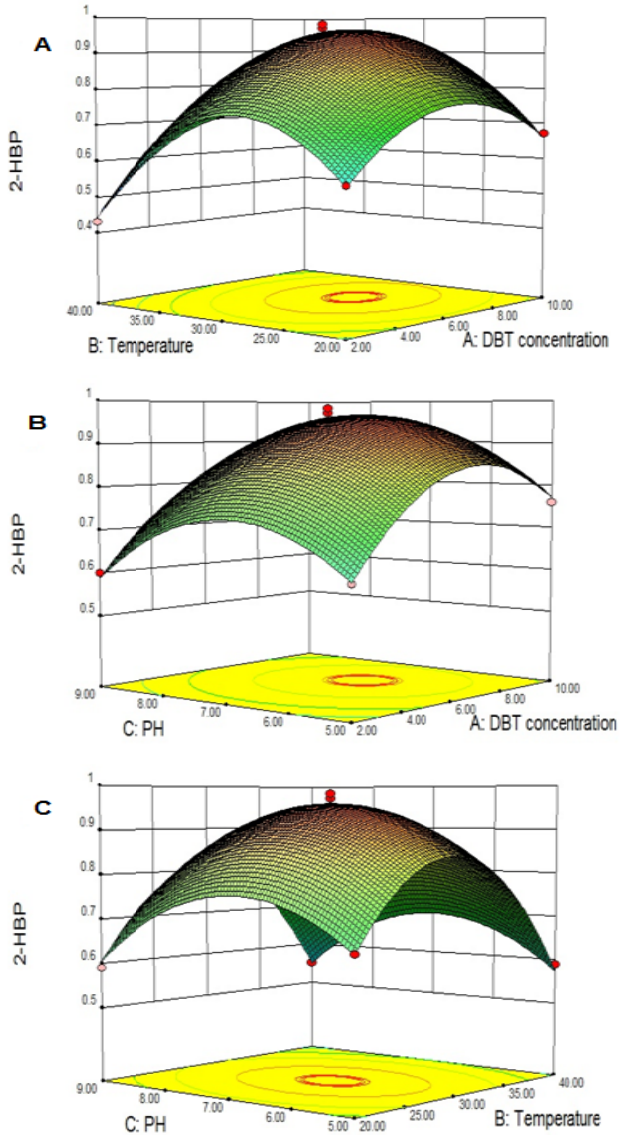

Fig. 3 The response surface and contour plot of 2-HBP production of magnetic Fe_3O_4 nanoparticles immobilized *Rhodococcus* cells. DBT concentration (A), temperature (B), and pH effects (C).

salt medium compared to non-decorated cells. Obtained results in this study showed that biodesulfurization activity of immobilized and free cells in the biphasic system were approximately the same and no significant difference was seen between them.

3.4 Effect of DBT Concentration

In biphasic system, the rate limiting step for BDS is the transfer of DBT from the oil to the cell [16]. *R. erythropolis* is a resistant species to high concentration of DBT and solvents [17] and hydrophobic nature of *Rhodococcus* strains causes the absorption of DBT from oil to the cell surface [18]. Increasing in DBT concentration causes the increasing of DBT availability to cells and leads to enhance in BDS. But at high concentration of DBT, bacterial growth and BDS activity will be inhibited, presumably because of the toxicity of high concentrations of DBT that bacteria cannot tolerate it [11]. In biphasic medium, DBT is dissolved in n-tetradecane (organic phase) that leads to a reduction in its toxic effect on bacteria. Therefore compared to aqueous medium, in oil/water systems, DBT can be used at high concentrations. Fig. 3A shows that the optimum concentration of DBT was 6.76 mM and BDS activity was reduced by increasing DBT concentration up to 10 mM or decreasing it to 2 mM.

3.5 Effect of Temperature

Temperature is a potentially limiting factor like essential chemical elements and organic substrates. In particular, temperature should be studied as an interactive factor, because it affects all chemicals and biochemical processes [19]. *R. erythropolis* R1 is a mesophilic bacterium and its optimum temperature for BDS of DBT in model oil was determined at 29.63 °C. Therefore, unlike HDS or thermophilic bacteria, biodesulfurization by immobilized *R. erythropolis* R1 can be conducted at the ambient temperature which reduces the reaction cost. The surface and contour plot in Fig. 3B indicates that at high or low temperature, 2-HBP production was reduced because at high or low temperatures, the activity of enzymes can be reduced and as previously suggested [20], the first and third enzymes in 4S-pathway (Dsz C and Dsz B) are more sensitive to temperature changes compared to other enzymes and are BDS rate-limiting.

3.6 Effect of pH

The most favorable pH value is known as the optimum pH. The pH is an effective parameter that

controls the bacterial activity. In addition, enzymes are affected by changes in pH that can alter the 3-D shape of enzymes. Changes in pH may not only affect the shape of an enzyme but it may also change in shape or charge properties of the substrate so that the substrate cannot bind to the active site or it cannot undergo catalysis [21]. Fig. 3C shows the surface and contour plots of pH effect on 2-HBP production of immobilized cells. As can be seen, the optimum pH was 6.84 and by a change in pH, 2-HBP production was reduced. Therefore, the reaction can be performed at the ordinary condition.

4. Conclusions

BDS using nanomagnetic Fe_3O_4 particles-immobilized *R. erythropolis* R1 in a biphasic system can be improved by setting significant factors at the optimum level. Also the immobilized cells could be recovered by magnetic power to prevent the oil contamination and use the biocatalyst repeatedly.

Acknowledgments

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