

# BIODESULFURIZATION OF SIMULATED LIGHT FUEL OIL BY A NATIVE ISOLATED BACTERIA *BACILLUS CEREUS* HN

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## ABSTRACT

In the present paper, the biodesulfurization of simulated light fuel oil (i.e., dibenzothiophene (DBT) in dodecane) and untreated kerosene with a high total sulfur content has been studied by a native isolated bacterium named *Bacillus cereus* HN. The influences of various parameters such as the reaction temperature (T), biocatalyst cell density, oil phase fraction (OFP), and initial DBT concentration on the fractional conversion of the model sulfur compound were investigated. The experimental data obtained were used to determine the reaction rate constant of the model sulfur compound and the corresponding activation energy. Furthermore, the biodesulfurization of untreated kerosene with a total sulfur content of 2333 ppmw produced by an Iranian refinery company (Isfahan refinery) was investigated to examine the capability of this new microorganism. It was realized that about 33% of the total sulfur content of untreated kerosene could be removed after 72 hrs. The results of the response surface methodology (RSM) showed that a quadratic correlation could be proposed for the influences of biocatalyst cell density, OFP, and initial DBT concentration on the desulfurization of DBT.

**Keywords:** Biodesulfurization, DBT, RSM, *Bacillus cereus* HN, Simulated Light Fuel Oil

## INTRODUCTION

The combustion of fossil fuels releases a huge amount of  $SO_x$  into the atmosphere, which has adverse effects on the environment. Because of the transportation of  $SO_x$  by air streams, international cooperation is required to control emissions [1, 2]. To have a less polluted world, a number of regulations have been enacted to restrict the sulfur content of fossil fuel oils, primarily those intended for transportation [3]. In the past decades, the allowed total sulfur content of transportation fuel oils has been reduced from 2000-5000 ppm to less than 15 ppm [4].

The sulfur-containing compounds of crude oil have many different structures such as elemental sulfur, hydrogen sulfide, sulfides, mercaptans, and heterocycles. Among these groups, heterocycles such as dibenzothiophene (DBT) or its derivatives are quite important, because they have higher boiling points (larger than 200 °C), which makes it difficult to remove them from the atmospheric tower outlet streams of oil refineries [5, 6]. In addition, up to 70% of sulfur in heavy fuel oils is in the form of dibenzothiophene (DBT) and its alkylated derivatives [7]. The conventional method

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### Article history

Received: June 25, 2013  
Received in revised form: September 14, 2013  
Accepted: January 06, 2014  
Available online: March 10, 2014

for removing sulfur-containing compounds of light fuel oils is hydrodesulfurization (HDS), which has poor performance in removing some heterocyclic compounds such as thiophenes, benzothiophenes, dibenzothiophene, and their derivatives by using conventional catalysts. However, new developed catalysts can overcome the poor performance of HDS.

Biological desulfurization (BDS), which attracted research efforts in the oil industries [8, 9], is a process for the desulfurization of fossil fuel oils by means of enzyme-catalyzed reactions [10]. In this process, a biocatalyst in an aqueous solution is contacted with a fuel oil (organic phase) to be treated and the desulfurization reactions occur at the interface of the aqueous and organic phases. DBT and its derivatives were widely used for the desulfurization of simulated light fuel oils. The specific oxidative desulfurization of DBT is a pathway in which carbon-sulfur bond is targeted to produce 2-hydroxybiphenyl as the final product [11]. BDS is normally carried out at mild operating temperature and atmospheric pressure and biocatalyst provides a highly selective desulfurization. Moreover, in this process, greenhouse emissions, capital, and operating costs are lower than those of HDS [12]. Considering these advantages, a number of studies on this process regarding various types of efficient microorganisms have been reported [13–16]. In addition, numerous investigators have studied the influences of biocatalyst cell density, oil phase fraction, and initial DBT concentration as the most important parameters of BDS process on the desulfurization of light fuel oils [17–19].

The major objective of the present work was to investigate the important parameters of BDS by a native strain *Bacillus cereus* HN, isolated from west Paydar oil wells in the south of Iran. Moreover, a response surface methodology was applied to determine the appropriate conditions of the process with maximum desulfurization.

*Journal of Petroleum Science and Technology* 2014, 4(1), 31-40  
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Furthermore, the kinetic rate expression of the BDS reaction for DBT was evaluated.

## EXPERIMENTALS

### Materials and Methods

#### Chemicals

Normal dodecane, DBT, nutrient agar, nutrient broth, and materials for preparing the media including glutamic acid, glycerol,  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{FeCl}_3$ , and  $\text{NH}_4\text{Cl}$  were obtained from Merck Co. (Germany). Nutrient broth (NB) is a microbiological growth medium commonly used for the routine cultivation of non-fastidious bacteria. It is also used as a pre-culture medium for increasing the number of the cells before transferring them to the producing medium. The composition of the medium is 0.5% peptone, 0.3% beef extract/yeast extract, and 0.5% NaCl. All the other chemicals used in the present work were of analytical grade. Deionized distilled water was also used to prepare all media and solutions.

#### Microorganism

##### Isolation of DBT Desulfurizing Microorganisms

Oil samples were collected from west Paydar oil wells in the south of Iran, under aseptic conditions by sterile sampling bottles. Then, 1 ml of oil sample was fed into a 250 ml flask containing 100 ml nutrient broth medium and incubated at two different temperatures, namely 30 and 40 °C, while stirring at 170 rpm for one week. Then, the bacterial strains were purified and more than twenty types of colonies were purified.

##### Identification of Selective Strain

16S ribotyping was carried out to identify the bacterium. The sequence analysis was performed at Ribosomal Database Project (RDP) I & II and the basic local alignment search tool <http://jpst.ripi.ir>

(BLAST-n) analysis was conducted at National Center for Biotechnology Information (NCBI) server. The alignment of the sequence was carried out using CLUSTALW program at European Bioinformatics site. These sequence data have been submitted to the GenBank databases under accession number of JF705198.

### **Media**

The medium containing 4 gr. of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 4 gr. of  $\text{K}_2\text{HPO}_4$ , 20 gr. of glutamic acid, 0.67 gr. of  $\text{NH}_4\text{Cl}$ , 0.0245 gr. of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.001 gr. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001 gr. of  $\text{FeCl}_3$ , and 20 ml of glycerol as the carbon source was dissolved in 1000 ml of deionized distilled water. The pH of the medium was then adjusted to 7 using 1 N sodium hydroxide solution.

### **Analytical Methods**

Cell concentration was determined using optical density value obtained by a Camspec spectrophotometer (Model M501) at 600 nm. DBT concentration and the metabolites in the reaction mixtures were detected and measured by a gas chromatograph (GC, type GC-7890A, Agilent, USA) equipped with a mass spectroscopy detector and a capillary column (30 m  $\times$  0.32 mm  $\times$  0.25  $\mu\text{m}$ , Agilent, USA). The injector temperature was set at 250  $^\circ\text{C}$  while the temperature program used was 40  $^\circ\text{C}$  for 1 min, followed by a ramp of 10  $^\circ\text{C}/\text{min}$  to 120  $^\circ\text{C}$ , and a second ramp of 12  $^\circ\text{C}/\text{min}$  to 232  $^\circ\text{C}$  for 1 min. Helium was used as the mobile phase at a flow rate of 1.3 ml/min. All the experimental data reported in the present work are the mean values of the experimental data obtained from two or three experiments with a mean standard deviation of 5%. Moreover, the total sulfur content of reaction mixtures and kerosene was determined by a total sulfur analyzer (ED-XRF).

### **Determination of Appropriate Conditions of BDS Process Using Response Surface Methodology**

Response surface methodology (RSM) is a collection of useful mathematical and statistical techniques for modeling problems using quantitative data obtained from a number of appropriate experiments. The desired response is influenced by several variables and the response is optimized using an objective function [20]. In this regard, three important parameters of BDS process, including biocatalyst cell density (A; cell/ml), oil phase fraction (B), and the initial DBT concentration (C, ppmw) were chosen. Then, a central composite design was applied to the optimization problem. To have design points, which had no decimal, the value of parameter  $\alpha$  in the RSM was chosen to be 2. This experimental design included twenty experiments for evaluating the model coefficients from which eight points of factorial design, six axial points, and a center point with six replications were obtained. This replication at the center point allowed estimating experimental error for a better fitting. The results of the proposed set of experiments are summarized in Table 1.

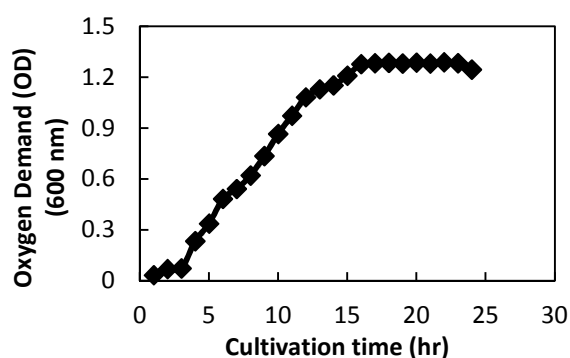
## **RESULTS AND DISCUSSION**

### **Identification of Strain HN**

In the present study, 17 morphologically distinct microbial colonies were isolated. One of the isolates, initially named HN, had the maximum desulfurization activity (65-70% of DBT) and was finally chosen for further studies. The 16S ribotyping and BLAST-n analysis of the 762 base pairs were performed at the National Center for Biotechnology Information (NCBI) server, which confirmed the identity of organism, i.e. a strain of *Bacillus cereus* called *Bacillus cereus* HN. *Bacillus cereus* belongs to the group of *Bacillaceae* organisms, which are rod shaped, spore forming, gram-positive, and motile. Figure 1 shows the growth of HN strain used in the present study.

**Table 1: Experimental runs proposed by the RSM method**

Run	(A): Cell Density (cell/ml) $\times 10^7$	(B): Oil phase fraction	(C): Initial DBT concentration (ppmw)	Desulfurization (%)
1	2	0.3	500	55.9
2	3	0.5	750	56.1
3	1	0.5	750	37.6
4	3	0.5	750	47.2
5	4	0.7	1000	56.4
6	3	0.5	750	69.9
7	2	0.3	1000	58.3
8	3	0.5	750	53.3
9	3	0.5	750	27.8
10	3	0.5	250	31.2
11	3	0.5	750	73.6
12	2	0.7	1000	56.6
13	4	0.3	1000	42.9
14	5	0.5	750	62.3
15	3	0.5	1250	57.8
16	3	0.9	750	56.1
17	2	0.7	500	52.8
18	4	0.3	500	58.9
19	4	0.7	500	54.3
20	3	0.1	750	60.2



**Figure 1: Growth of *Bacillus cereus* HN during cultivation at 30 °C; Experimental conditions: atmospheric pressure and incubator stirring at 180 rpm.**

### Effect of Parameters

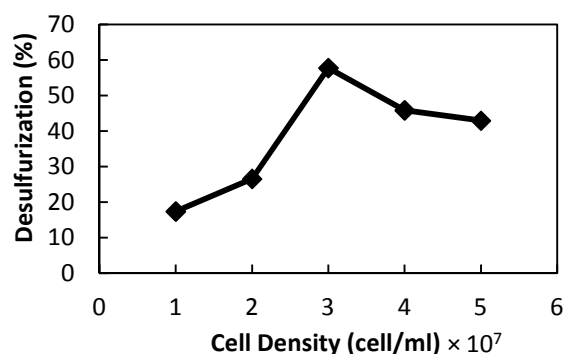
In the RSM, effective variables and their proper range have to be known. Therefore, three basic

parameters such as biocatalyst concentration, oil phase fraction, and initial DBT concentration were chosen [19, 21]. The influences of these parameters on the desulfurization of DBT were systematically examined one at a time. All the assays were carried out in 100 ml Erlenmeyer flasks. Dodecane and DBT were used as the oil solvent and model sulfur compound respectively. The BDS were carried out by incubating at 30 °C and 180 rpm for 48 hrs.

### Effect of Biocatalyst Cell Density

One of the most important parameters in the BDS process is biocatalyst cell density. A high cell density can reduce operating costs. Thus it is important to determine the maximum biocatalyst cell density [22]. To examine this effect, a number of experimental runs were carried out

in 100 ml Erlenmeyer flasks with oil phase fraction and DBT initial concentration of 0.5 and 500 ppm respectively. In these experiments, cell density varied from  $1 \times 10^7$  to  $5 \times 10^7$  (cell/ml) with  $1 \times 10^7$  increments. The obtained experimental results are presented in Figure 2. As can be seen, higher desulfurization can be obtained at higher cell density. This could be due to the increased number of cells, and thus more biocatalyst being available for DBT molecules. However, at a cell density larger than  $3 \times 10^7$  (cell/ml), the percentage of desulfurization falls. This can be explained by the mass-transfer limitations and inability of some cells to be in contact with the organic phase. In fact, the rate at which DBT can be converted by cells is higher than the mass transfer rate of DBT from the organic phase into the cells. Therefore, the mass transfer of DBT from organic phase into the cells controls the overall process. In this regard, the existence of an optimum value of the biocatalyst loading has been reported in the literature [23, 24].

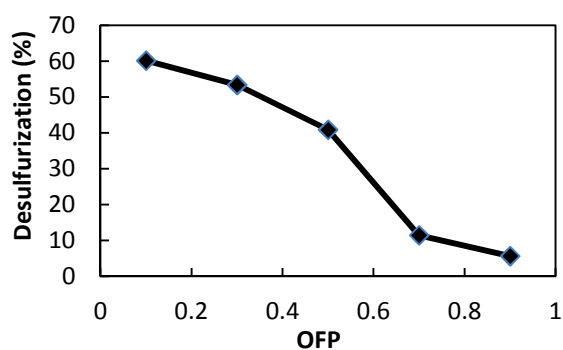


**Figure 2:** Effect of cell density on the percentage of desulfurization; Experimental conditions:  $C_{\text{DBT}}=500$  ppmw, OFP=0.5, atmospheric pressure,  $T=30$  °C, and incubator stirring at 180 rpm.

### Effect of Oil Phase Fraction (OFP)

BDS is typically known as a two-phase aqueous-oil process due to the water requirement for biocatalysts. Therefore, the required amount of water is a key parameter; to this end, the influence of the oil phase fraction on the de-

sulfurization fraction was carefully studied. In the present work, the fraction of oil phase varied from 0.1 to 0.9 while the two other parameters were kept constant (i.e.,  $C_{\text{DBT}} = 500$  ppm and cell density =  $3 \times 10^7$  cell/ml). The experimental results obtained are presented in Figure 3. As can be inferred from this figure, the desulfurization of DBT decreases as the volume percentage of organic solvent increases, which is fully in agreement with other studies reported in the literature [17, 18, 25]. This can be due to the high hydrophobicity of DBT. It should be noted that the transport of DBT molecules into the oil-water interface is essential for the desulfurization reactions.

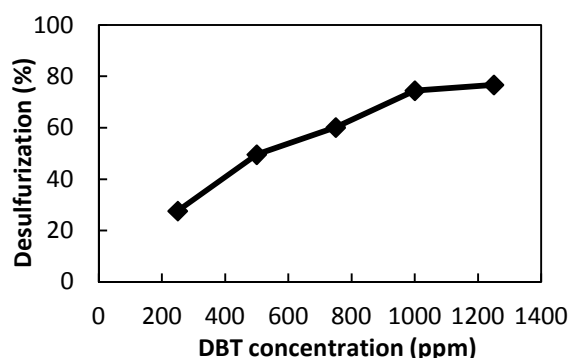


**Figure 3:** Effect of oil phase fraction (OFP) on the percentage of desulfurization; Experimental conditions:  $C_{\text{DBT}}=500$  ppmw, atmospheric pressure,  $T=30$  °C, cell density =  $3 \times 10^7$  (cell/ml), and incubator stirring at 180 rpm.

### Effect of Initial DBT Concentration

The effects of initial substrate concentration on the percentage of desulfurization are shown in Figure 4. Different DBT concentrations from 250 to 1250 ppmw with a 250 ppm increment were prepared, while the biocatalyst concentration and the oil phase fraction were set to be  $3 \times 10^7$  (cell/ml) and 0.1 respectively. Figure 4 clearly shows that with an increase in the DBT concentration increases the percentage of desulfurization due to the enhancement in the DBT diffusion to the oil-water interface. It is worth noting that this behavior has been previously reported by other researchers using various

types of microorganisms [18, 21].



**Figure 4:** Effect of initial DBT concentration on the percentage of desulfurization; Experimental conditions: OFP=0.1, atmospheric pressure,  $T=30$  °C, cell density= $3 \times 10^7$  (cell/ml), and incubator stirring at 180 rpm.

### Correlation of Experimental Data

The experimental results were analyzed by software Design Expert® 8.0.1 package. Evaluating the significant model, the most effective parameters and their possible interactions were obtained by using the technique of analysis of variance (ANOVA) as shown in Table 2.

A quadratic model could be suitable and, therefore, it was chosen for the subsequent analysis of the experimental data. As can be seen in Table 2, the obtained experimental results indicate that predictability of the model is at a confidence level of 91%. Therefore, response function predictions were in reasonable agreement with the experimental data with an  $R^2$  value of 0.912, which clearly showed that less than 9% of the total variations could not be explained by this model. Moreover, as the Fisher

F-test (F value) was found to be 25.77 with a very low probability value (P value  $<0.0001$ ), the quadratic regression model could be valid. This indicated that only 0.01% of the model predictions were due to the noise. The predicted  $R^2$  value of 0.7753 is in reasonable agreement with the adjusted value of 0.8670. In addition, the value of adequate precision of response, which was 21.185 (larger than 4), indicated that the model could be appropriate for describing the present BDS process. The final correlation obtained for the present percentage of desulfurization can be expressed by:

$$\text{Desulfurization}(\%) = 56.48 + 1.57A - 4.74B + 4.99C - 6.34A^2 + 2.56B^2 \quad (1)$$

where,  $A$ ,  $B$ , and  $C$  are the cell density, oil phase fraction, and the initial DBT concentration respectively.

Figure 5 shows the parity plot of the experimental and predicted values of the percentage of desulfurization. It is seen that the predicted and experimental values are distributed close to the diagonal line showing a reasonable agreement between the predicted and experimental values of the percentage of desulfurization. Considering Equation 1, the significant terms are three linear effects of all the parameters and the nonlinear effects of the biocatalyst cell density and the oil phase fraction. Furthermore, there are no interactions between the parameters because all the possible effects of any two parameters are not significant.

**Table 2: Results of ANOVA**

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-value prob> F
Model	225.24	5	445.05	25.77	<0.0001
Residual	241.83	14	17.27		
Lack of fit	202.2	9	22.47	2.83	0.132
Pure error	39.63	5	7.93		
$R^2 = 0.9120$ , $\text{Adj-}R^2 = 0.8670$ , $\text{Pre-}R^2 = 0.7753$ , Adequate precision= 21.185					

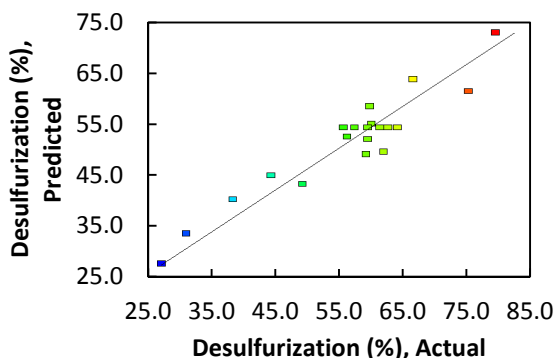


Figure 5: Predicted versus actual values of the percentage of desulfurization; Experimental conditions: atmospheric pressure,  $T=30\text{ }^{\circ}\text{C}$ , and incubator stirring at 180 rpm.

### Optimization of Desulfurization

The optimization of the present BDS process for obtaining the percentage of the maximum biodesulfurization was performed. To this end, the optimum conditions of the BDS were determined as follows: biocatalyst cell density= $3.6\times 10^7$  (cell/ml), oil phase fraction= 0.2, and initial DBT concentration= 1086 ppmw; these conditions provided a percentage of desulfurization of 0.791. Moreover, the optimum conditions predicted by RSM were also verified by carrying out three additional experiments. This amount of oil phase fraction at the optimum conditions provides a great advantage. It should be noted that few microorganisms were able to provide a high percentage of desulfurization of DBT at oil phase fractions larger than 0.1. This finding can be very important, because one of the essential problems of industrial BDS process is the large volume of water.

### Kinetic Rate Expression

The biocatalyst which was in the stationary phase was used to evaluate the kinetic rate expression. In fact, biocatalyst concentration remained almost unchanged during the reaction time. The parameters of process were set at optimum conditions estimated by RSM. Then, the effects of operating temperature on the desulfurization were examined. These experi-

mental results are shown in Figure 6.

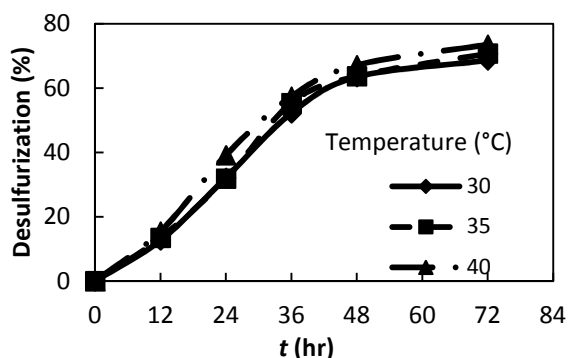


Figure 6: Effect of temperature on the percentage of desulfurization; Experimental conditions:  $C_{DBT}=500$  ppmw, atmospheric pressure,  $T=30\text{ }^{\circ}\text{C}$ , OFP=0.1, cell density= $3\times 10^7$  (cell/ml), and incubator stirring at 180 rpm.

The general  $n$ th-order power-law reaction rate expression was chosen to correlate the experimental data. It was found that the best value of  $n$  for this process could be 1. Therefore, the rate expression for the BDS of DBT can be expressed as:

$$-\frac{dC_{DBT}}{dt} = kC_{DBT} \quad (2)$$

$$k = 2.715 \exp\left(\frac{-12.79}{RT}\right) \quad (3)$$

where,  $C_{DBT}$ ,  $t$ , and  $k$  are DBT concentration (ppmw), time (hr), and reaction rate constant ( $\text{hr}^{-1}$ ) respectively.  $R$  and  $T$  represent the universal gas constant (kJ/mol.K) and temperature (K) respectively. The comparison of DBT degradation experimental results and those predicted by the first-order reaction rate expression is shown in Figure 7. The mean absolute deviation of the model predictions from the experimental data is 5%. Moreover, evaluating the correlation between temperature and the percentage of desulfurization shows a great advantage. This microorganism is able to desulfurize DBT over the temperature range of 30 to 40  $^{\circ}\text{C}$ ; additionally, an increase in the reaction temperature increases the degradation of DBT as the model-sulfur compound. Thus the sulfur-containing

streams do not need an extra cooling to temperature lower than 40 °C. Therefore, this thermophilic bacterium can be quite promising.

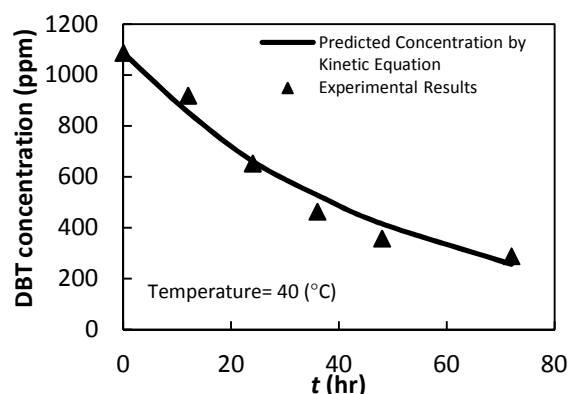


Figure 7: Comparison of the DBT degradation experimental data with those predicted by first-order reaction rate expression; Experimental conditions:  $C_{DBT}=1086$  ppmw, atmospheric pressure,  $T=40$  °C, cell density= $3 \times 10^7$  (cell/ml), OFP=0.1, and incubator stirring at 180 rpm.

### Desulfurization of Kerosene

Finally, the biodesulfurization of kerosene, which has some similarity with the simulated light fuel oil, was carried out to evaluate the capability of this microorganism to degrade real oil samples. The biocatalyst cell density and oil phase fraction were chosen according to the optimum conditions obtained by RSM, while the initial total sulfur content of kerosene was 2333 ppmw. The properties of kerosene produced by Isfahan refinery used in the present study are summarized in Table 3. As can be noticed, the range of boiling point of the kerosene used is 150-272 °C. The main sulfur compounds of fuel oil in the kerosene boiling range are benzothiophene and its alkylated derivatives. The GC-MS analysis confirmed the presence of benzothiophene and its alkylated derivatives such as 5-methyl benzothiophene and 3-methyl benzothiophene in the present kerosene. Besides, some other sulfur compounds such as 1-dodecanethiol were also observed in the kerosene. After 72 hr, the total sulfur content of kerosene was reduced to 1557 ppm. Considering the high

*Journal of Petroleum Science and Technology* 2014, 4(1), 31-40  
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initial total sulfur content of kerosene, it seems that this microorganism could be promising for the desulfurization of DBT; however, it probably does not possess the ability to desulfurize other types of sulfur-containing compounds.

Table 3: Properties of kerosene feedstock

Property	Value
Density (kg/m <sup>3</sup> )	795
Viscosity at 20 °C (mPa.s)	1.46
Aromatic content (Vol.%)	18
Total acid number (mg KOH/g)	0.018
Initial boiling point (I.B.P.), °C	173
5%	179
10%	183
30%	195
50%	209
70%	225
90%	245
95%	254
Final boiling point (F.B.P.), °C	269

### CONCLUSIONS

In the present work, an experimental investigation was conducted on the biodesulfurization of a model solution of DBT as well as untreated kerosene with a high total sulfur content by using a native isolated bacteria *Bacillus cereus* HN. The effects of various operating parameters on the desulfurization of DBT were examined. It was found out that:

- 1- The removal of DBT from the model solution (DBT in dodecane) can be carried out by this type of microorganism. The yield of BDS increases with an increase in the cell density up to  $3 \times 10^7$ , a decrease in the OFP, and an increase in the initial DBT concentration.
- 2- DBT can be desulfurized from the model solution with an appropriate amount of biocatalyst cell density and OFP at relatively low temperature and atmospheric pres-

<http://jpst.ripi.ir>



sure. Moreover, biodesulfurization of DBT obeys the pseudo-first-order rate expression in terms of sulfur concentration.

- 3- Untreated kerosene with a high total sulfur content in comparison with DBT could also be slightly desulfurized.
- 4- The results of the RSM showed that a quadratic correlation could be proposed for the influences of biocatalyst cell density, OFP, and the initial DBT concentration on the desulfurization of DBT.

## ACKNOWLEDGEMENT

The authors are very grateful for the financial support provided by Sharif University of Technology (Tehran, Iran).

## NOMENCLATURE

BDS	Biological desulfurization
BLAST-n	Basic local alignment search tool
DBT	Dibenzothiophene
F.B.P.	Final boiling point
GC	Gas chromatography
HDS	Hydrodesulfurization
I.B.P.	Initial boiling point
NCBI	National center for biotechnology information
OD	Oxygen demand
OFP	Oil phase fraction
RSM	Response surface methodology
RDP	Ribosomal database project

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