

Sulfur Removal from Dibenzothiophene by Newly Isolated *Paenibacillus validus* Strain PD2 and Process Optimization in Aqueous and Biphasic (Model-Oil) Systems

PEYMAN DERIKVAND¹, ZAHRA ETEMADIFAR^{1*} and HOSSEIN SABER²

¹Department of Biology, Faculty of Sciences, University of Isfahan

²Department of Biotechnology, Faculty of Advanced Sciences and Technology, University of Isfahan, Iran

Submitted 6 August 2014, revised 20 December 2014, accepted 20 December 2014

Abstract

Dibenzothiophene (DBT) is an organic sulfur compound which remains in oil after hydrodesulfurization (HDS) process and can be removed by biodesulfurization (BDS). A new strain of *Paenibacillus validus* (strain PD2) was isolated from oil contaminated soils that is able to desulfurize DBT. HPLC analysis and Gibb's assay showed that this strain was capable to convert DBT to 2-Hydroxybiphenyl (2-HBP) as final product. The presence of *dszC* gene confirmed that DBT desulfurization occurred through the 4S pathway. Maximum growth and the highest induction in *dsz* operon obtained in the presence of dimethyl sulfoxide (DMSO) as sole sulfur source. DBT concentration, temperature and pH were optimized statistically for growing and resting cells by using Response Surface Methodology (RSM). All parameters in growing cells had a significant effect on 2-HBP production during BDS of DBT by *P. validus* PD2, although in resting cells temperature in range of 20–40°C was not a significant factor. Maximum BDS for growing cells was obtained at 0.41 mM DBT concentration, pH 6.92 and temperature 31.23°C. For resting cells, optimum pH, temperature and DBT concentration were 6.62, 27.73°C and 7.86 mM respectively. The results of this study showed that high concentrations of DBT could be desulfurized by *P. validus* strain PD2 in model-oil. Thus, the isolated strain could be introduced as a proper candidate for biodesulfurization of organic sulfur in the oil industry.

Key words: *Paenibacillus validus*, 2-hydroxybiphenyl (2-HBP), dibenzothiophene (DBT), Response Surface Methodology (RSM)

Introduction

Sulfur is the third most common element in the crude oil composition after carbon and hydrogen which causes severe environmental pollutions. For example, burning the petroleum based fuels containing sulfur components will release SO_x gases to the atmosphere leading to acid rain and corrosion problems. Consequently, deep desulfurization of the crude oil is compulsory to prevent the production of such pollutants (Derikvand *et al.*, 2014).

Hydrodesulfurization (HDS) process which has been usually employed in oil refineries, works at high temperatures and pressures (more than 300°C and 100 atm) (Ma *et al.*, 2002) and is effective in removing a large amount of inorganic and a part of organic sulfur from the crude oil. However, the major part of organic sulfur content is resistant to HDS and remains in the oil composition after this treatment (Borgne and Quintero, 2003). As a remedy, biodesulfurization (BDS) process has been suggested to remove the recalcitrant organic

sulfur compounds like dibenzothiophene (DBT) which is often used as a model of heterocyclic sulfuric compounds in biodesulfurization studies. In fact, BDS has several advantages which make it a potentially alternative or at least a complementary process to the HDS. For instance, BDS is more specific and cost effective and takes place at ambient temperature and pressure (Monticello, 2000). In addition, the combustion value of the oil would not be affected in the BDS process because the effective bacterial strains (such as *Rhodococcus* and *Gordonia*) can remove the sulfur through the 4S pathway, which attacks the C-S bounds selectively (Gou *et al.*, 2002).

BDS through 4S pathway has been studied using different bacterial species such as *Gordonia* (Rhee *et al.*, 1998), *Bacillus* (Kirimura *et al.*, 2001), *Lysinibacillus* (Bahuguna *et al.*, 2011) and *Rhodococcus* (Derikvand *et al.*, 2014). Generally, there are four enzymes involved in the 4S pathway: first, DBT monooxygenase (*DszC*) oxidizes DBT to DBT-sulfone (DBT-O₂). In the second step, flavomonooxygenase (*DszA*) catalyzes

* Corresponding author: Zahra Etemadifar, Department of Biology, Faculty of Sciences, University of Isfahan, Hezarjarib Isfahan, Iran; e-mail: z.etemadifar@sci.ui.ac.ir, z_etemadifar@yahoo.com

transformation of DBTO₂ to 2-2'-hydroxyphenyl benzene sulfinate (HPBS) and in the final step, HPBS is desulfinated by DszB to produce 2-hydroxybiphenyl (2-HBP) as the final product (Yan *et al.*, 2000). Three catabolic genes, *dszA*, B, C, are clustered in the *dsz* operon. The fourth enzyme (DszD) prepares FMNH₂ required for oxygenase reactions and is a chromosomal gene (Derikvand *et al.*, 2013). Clearly, performance and activity of the enzymes in the 4S pathway is dependent on their operating conditions and a high BDS efficiency could be achieved in the optimum level of the effective parameters. Although BDS process has been studied for physiology of microorganisms and their gene modifications, metabolic pathways and kinetics of desulfurization in both model and diesel oils, the significance of operational factors and their optimization through statistical methods are rare. Response Surface Methodology (RSM) is one of the well known statistical methods which is utilized to find a relationship between a few effective variables and one or more responses in a system. In this approach, analysis of variance (ANOVA) and regression techniques have been employed to estimate a low degree polynomial model for optimization of the levels of significant explanatory variables in a limited number of experiments (Khurana *et al.*, 2007).

In the present study, *Paenibacillus validus* PD2 which was isolated from an oil contaminated soil sample was employed to investigate the effects of important operational conditions such as initial DBT concentration, temperature and pH on the BDS efficiency. Box-Behnken RSM was used to determine the optimum value of these factors in BDS process for both growing and resting cells conditions in aqueous and biphasic (aqueous / model-oil) systems, respectively. Finally, the BDS efficiency was determined in the obtained optimum conditions.

Experimental

Materials and Methods

Chemicals. All chemicals were analytical grade and commercially available.

Enrichment, isolation and identification of DBT desulfurizing bacteria. Double deionized water was used to prepare sulfur free basal salt medium (BSM). The BSM contains the following composition (g/l): Na₂HPO₄·7H₂O 8.0, KH₂PO₄ 4.0, NH₄Cl 2.0, MgCl₂ 0.2, FeCl₃ 0.001 and CaCl₂ 0.001. More than 100 oil contaminated soil samples were collected from various areas in Iran. One gram of each soil sample was suspended in 50 ml of BSM supplemented with 10 g/l glucose and 0.3 mM DBT as the sulfur source in a 250 ml flask. After incubation for 3 days at 30°C and 180 rpm on a rotary

shaker, 5 ml of supernatant was inoculated in 45 ml of fresh BSM supplemented with 0.3 mM DBT and 10 g/l glucose. Subculturing was repeated with 1% v/v inoculums five times. Afterwards, streak culture was done onto the same medium with 9 g/l agarose. Finally, a single colony was isolated and its 2-HBP production was detected by Gibb's assay. One of the Gibb's positive isolated strains named PD2, was genetically identified by partial 16S rDNA gene sequencing using DG74 and RW01 general primers.

Detection of *dszC* gene from *dsz* operon. Briefly, chromosomal DNA of *P. validus* was extracted (by DNA extraction kit, Fermentas) and separated on 1% agarose gel with 1X TAE buffer (Promega, Germany) at 80 V for 40 min. The extracted chromosomal DNA was amplified by PCR with applying specific *dszC* primers as forward 5'-GACACTGTCTCACCTGAAA-3' and reverse 5'-CTCAGGAGGTGAAGCCG-3' primers. The amplified gene separated on 1% agarose gel with 1X TAE buffer (promega, Germany) at 80 V for 40 min. The amplified gene was purified using gel extraction kit (Promega Germany). The quantity and quality of the purified gene was assessed by optical density at 260 nm and 280 nm and by electrophoresis in 1% agarose gel (Promega, Madison, WI, USA). The correct orientation and nucleotide sequence of the gene was verified by double-strand sequencing.

Characterization of growth and biodesulfurization. Growth rate, DBT consumption and 2-HBP production by isolated *P. validus* PD2 were measured by turbidimetry (600 nm), UV spectrophotometry (323.8 nm) and Gibb's assay (610 nm wavelength) respectively for 4 days incubation period. The culture media was BSM supplemented with 0.3 mM DBT and 15 g/l glucose incubated at 30°C on a rotary shaker.

Biodesulfurization in the liquid media by growing cells. For growing cells, the strain was inoculated in BSM supplemented with different amounts of DBT and 10 g/l glucose and 2-HBP production was measured after 48 h incubation time.

Biodesulfurization in the biphasic media by resting cells. The biphasic media consist of BSM (aqueous phase), n-tetradecane (organic phase) and different amount of DBT as a sulfur source, were prepared to monitor the BDS activity by the resting cells. The ratio of aqueous to oil phase volume was 2:1. 2-HBP production was determined after 20 h.

Effect of different sulfur sources on growth and BDS rate. Several sulfur sources such as DBT, thiophene, dimethyl sulfoxide (DMSO) and MgSO₄ were added to 50 ml BSM in concentrations equal to 0.3 mM, 1 µl/ml, 200 mg/l and 200 mg/l respectively. The carbon source was 10 g/l glucose. 0.5 ml of cell suspension with OD₆₀₀ = 1 was inoculated to each medium and growth rate and 2-HBP production were determined after 30 h.

Table I
Coded values of experimental variables in growing and resting cells of PD2 strain

Independent variables	Growing cells			Resting cells		
	-1	0	+1	-1	0	+1
X ₁ : DBT concentration (mM)	0.2	0.5	0.8	2	6	10
X ₂ : Temperature (°C)	25	30	35	20	30	40
X ₃ : pH	6	7.5	9	5	7	9

Table II
Response surface Box-Behnken design (BBD) and the rate of produced 2-hydroxybiphenyl (2-HBP) in aqueous system by growing cells and biphasic system by resting cells.

Run	A*			B**			2-HBP (mM) Growing cells	2-HBP (mM) Resting cells
	X ₁	X ₂	X ₃	X ₁	X ₂	X ₃		
1	0	1	-1	0	1	1	0.151	0.41
2	1	1	0	1	0	-1	0.112	0.66
3	0	-1	1	0	-1	-1	0.111	0.51
4	1	0	-1	1	1	0	0.113	0.55
5	0	-1	-1	1	-1	0	0.118	0.51
6	0	0	0	1	0	1	0.198	0.59
7	1	0	1	-1	0	1	0.097	0.44
8	-1	-1	0	0	0	-1	0.121	0.88
9	0	1	1	-1	-1	0	0.119	0.45
10	-1	0	1	0	1	-1	0.133	0.49
11	1	-1	0	0	0	0	0.085	0.84
12	-1	0	-1	-1	0	-1	0.158	0.51
13	0	0	0	0	0	0	0.192	0.89
14	0	0	0	0	-1	1	0.201	0.42
15	-1	1	0	-1	1	0	0.146	0.34

*A: growing cell; **B: resting cell

Experimental design for biodesulfurization by growing and resting cells. DBT concentration (X₁), temperature (X₂) and pH (X₃) were considered as the important factors in biodesulfurization activity of growing and resting cells. A 3-factor and 3-level Box-Behnken design (BBD) was used to determine the optimum level of the important factors and to study their relationship to the BDS efficiency. Factors and their levels are shown in Table I. For resting cells design, we increased the range between high and low levels of factors (Table I) to better illustrate the impact of variables. All factors at middle (0) level constitute the central points while combination of factors consisting of one at its lowest level (-1) or highest level (+1). A 0.05 significance level was assumed to perform ANOVA for the model coefficients. A total of 15 experimental runs of three factors in different combinations were carried out in duplicates (Table II). All experimental design and data analysis were performed using Design Expert software package version 8.0.1.

Analytical methods. Cell density was measured by spectrophotometry at 600 nm (OD₆₀₀) (spectronic 21D Milton Roy). In the growing cells system, BDS activity was monitored using the Gibb's reagent (2,6-dichloroquinone-4-chloroimide). To measure the DBT consumption in the growing cells samples, their pH was reduced to 2 before mixing with equal volume of ethyl acetate. After centrifuging at 3000 rpm for 5 min., dissolved DBT in the organic phase was measured by UV spectrophotometry at 328 nm. In the resting cells system, HPLC was used to determine the amounts of 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-analysentechnik C18 column (5 μ–250 mm) and a UV detector (Smart-line 2600) set at 254 nm. The mobile phase was methanol-water (90:10, v/v) with a flow rate of 1.5 ml/min.

For all of the analytical methods standard curves of DBT and 2-HBP were prepared.

Results and Discussion

Identification of the strain PD2. The numerous bacterial colonies isolated from diesel contaminated soils were checked for 2-HBP production from DBT as sole sulfur source. The Gibb's analysis showed that a strain designated as PD2 was able to produce 2-HBP as a final product. Since 2-HBP production indicates selective cleavage of carbon-sulfur bond, further characterization and optimization was performed on this strain. Gram- and acid-fast staining, colony morphology and biochemical activities revealed the PD2 strain as Gram-positive, spore-forming and non-acid fast bacillus. Partial nucleotide sequence analysis of 16S rRNA gene implied 99% homology with *P. validus* SB3263 (NCBI Gene Bank Accession No. GU191921). Therefore, PD2 was identified as a novel strain *P. validus* PD2 and deposited in NCBI (NCBI Gene Bank Accession No. KC161368).

By now, several aerobic DBT-desulfurizing bacteria have been reported to date that are able to desulfurize DBT *via* 4S pathway. In particular, *Rhodococcus erythropolis* IGTS8, the first strain found able to desulfurize DBT through 4S pathway, has been widely used for BDS and the genes related to this pathway have been studied (Denome *et al.*, 1993). However, according to our knowledge, no studies have ever been reported using *P. validus* cells for DBT biodesulfurization *via* 4S pathway. Therefore, strain PD2 is the first *P. validus* strain reported to be capable of DBT desulfurization, without carbon skeleton cleavage.

Characterization of growth and biodesulfurization. The growth curve of *P. validus* PD2 has been shown in Fig. 1. The growth was continued for 96 h, simultaneously with the consumption of DBT and the production of 2-HBP. As shown in Fig. 1, desulfurization of DBT to 2-HBP in the induced cells of PD2 was detected after 8 hours, afterwards, the concentration of 2-HBP in the medium increased up to 0.27 mM at 50 hours (initiation of stationary phase). The production of 2-HBP is less than DBT consumption which as previously suggested by Caro *et al.* (Caro *et al.*, 2007), this is due to accumulation of 2-HBP and other 4S pathway compounds inside and on the surface of the cells.

Detection of *dszC* gene in *P. validus* PD2. The 1300 bp PCR product of amplified *dszC* gene by specific primers (Fig. 2) purified from the gel and sequenced by double-strand sequencing. The length of sequenced fragment was 1153 bp. Sequenced DNA was blasted in NCBI GenBank. The blast result showed that *dszC* in *P. validus* PD2 had 98% similarity with *dszC* gene in *R. erythropolis* FMF. This sequence was submitted in NCBI by accession number KF056797. The presence of *dszC* gene confirmed that DBT desulfurization takes place through the 4S pathway which produced 2-HBP

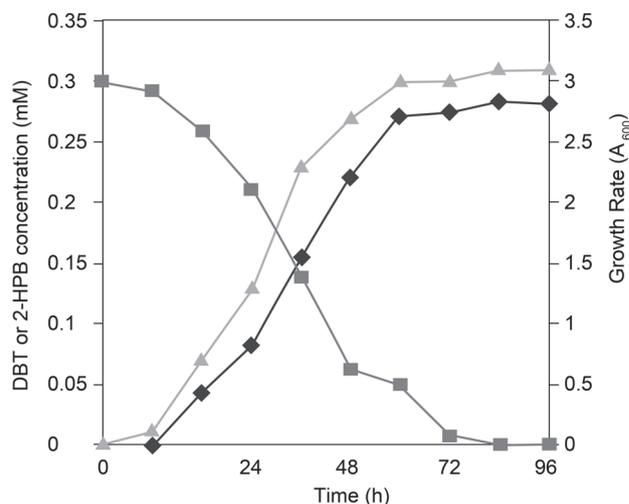


Fig. 1 Growth of *Paenibacillus validus* PD2, DBT utilization and 2-HBP production during cultivation at 30°C and 180 rpm shaking. The medium contained 0.3 mM DBT as sole sulfur source and glucose as carbon source. Symbols: ■, DBT concentration (mM); ♦, 2-HBP concentration (mM); ▲, growth (OD_{600nm}).

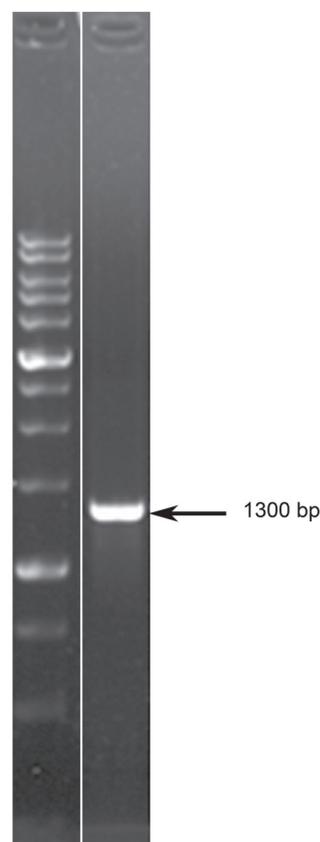


Fig. 2. PCR amplification of 1300 bp fragment of *dszC* gene from isolated *Paenibacillus validus* PD2. M: GeneRuler 1 kb DNA ladder (Fermentas).

as final product without cleavage of carbon ring; therefore, the strain PD2 could be used for BDS of oil without decrease in octane rate.

Effect of different sulfur sources on growth and biodesulfurization rate. *P. validus* PD2 was cultured in

Table III

Growth rate of isolated strain PD2 in the presence of different sulfur sources and 2-HBP production from DBT after cultivating of induced cells in 0.3 mM DBT.

Sulfur source	Growth (A_{600})	2-HBP (mM)
DBT	2.39	0.21
Thiophene	0.83	0.11
DMSO	3.94	0.25
Sulfate	3.39	0.09

the presence of different sulfur compounds. As shown in Table III, a higher cell density was obtained when DMSO was used as the sole sulfur source, because of more easily metabolization of DMSO than others sulfur sources (Bustos-Jaimes *et al.*, 2003). Moreover, the growth rate can be inhibited by production of 2-HBP when DBT is used as the sulfur source (Setti *et al.*, 2003). In fact, induction of *dsz* operon by DMSO in *Rhodococcus* sp. 1AWQ has been shown to be similar to DBT (Ma *et al.*, 2006) while Mohebal *et al.* (2008) showed that 2-HBP production of resting cells grown on DMSO was higher than that of DBT. The results indicated that the cell density in the presence of $MgSO_4$ was more than DBT and thiophene. This occurs due to the lower water solubility of DBT and thiophene. Although the *dsz* operon can be repressed by $MgSO_4$ as a sole sulfur source during the growth (Li *et al.*, 1996), PD2 grown on sulfate showed low BDS activity that could be explained by re-expression of *dsz* operon. Finally, the maximum BDS activity of the PD2 strain resting cells was achieved when they were grown on DMSO.

Statistical analysis. Growing cells: A quadratic polynomial equation was estimated to describe the rela-

tionship between 2-HBP production of growing cells and variables based on the experimental results of BBD (Table II). All of the main and interactive effects can be simultaneously investigated by response surface model. The model of coded units is calculated using follow:

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

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 the growing cells system, the empirical model of coded variables can be expressed as the equation 1 after removing non significant parameters:

$$\text{Equation 1: } Y = 1.97 - 0.19X_1 + 0.12X_2 - 0.100X_3 - 0.063X_2X_3 - 0.40X_1^2 - 0.41X_2^2 - 0.32X_3^2$$

Where Y is the response value (mM), X_1 is the initial DBT concentration (mM), X_2 is temperature ($^{\circ}C$) and X_3 is pH. Positive and negative sign before each term indicates synergistic and antagonistic effects respectively (Tan *et al.*, 2010).

The ANOVA results for 2-HBP production by growing cells have been shown in Table IV. The Model F-value was 142.23 that imply its significance and there is only a 0.01% chance that a Model F-value this large could occur due to the noise factors. Also values of Prob > F (P value) less than 0.05 indicate model terms are significant. In this case, X_1 , X_2 , X_3 , X_2X_3 , X_1^2 , X_2^2 and X_3^2 were significant model terms (P values < 0.05). The proposed model was proved to be adequate through calculation of its Lack of Fit equal to 0.49 and adjusted determination factor ($Adj R^2$) equal to 0.9891. This

Table IV
Analysis of variance (ANOVA)

Source of variance	df	Growing cells			Resting cells		
		Mean square	F value	P value	Mean square	F value	P value
Model	9	0.21	142.23	<0.0001	0.048	130.21	<0.0001
X_1	1	0.29	195.88	<0.0001	0.041	111.27	0.0001
X_2	1	0.11	74.30	0.0003	1.250E-003	3.42	0.1235
X_3	1	0.080	54.98	0.0007	0.012	32.91	0.0023
X_1X_2	1	1.000E-004	0.069	0.8037	5.625E-003	15.41	0.0111
X_1X_3	1	2.025E-003	1.39	0.2912	0.000	0.000	1.0000
X_2X_3	1	0.016	10.74	0.0220	2.500E-005	0.068	0.8040
X_1^2	1	0.60	411.12	<0.0001	0.092	250.94	<0.0001
X_2^2	1	0.61	421.40	<0.0001	0.23	632.24	<0.0001
X_3^2	1	0.37	251.80	<0.0001	0.098	267.12	<0.0001
Residual	5	1.455E-003			3.650E-004		
Lack of fit	3	1.025E-003	0.49	0.7252	1.417E-004	0.20	0.8876
Pure error	2	2.100E-003			7.000E-004		

Growing cells: $R^2=0$

means that the Lack of Fit is not significant relative to the pure error and the model predictions were well fitted the experimental results. According to the present model, all of the main factors were significant in the interval of variation and temperature has significant interactions with pH.

Resting cells: The relationship between the 2-HBP production of resting cells and the studied variables after removing non significant parameters stated as equation 2.

$$\text{Equation 2: } Y = 0.87 + 0.071X_1 - 0.039X_3 - 0.038X_1X_2 - 0.16X_1^2 - 0.16X_3^2$$

In equation 2, all of the coded variables are defined similar to equation 1 in the previous section. The ANOVA results for 2-HBP production by resting cells have been shown in Table IV. P-value of the model was obtained equal to <0.0001 , which warrants the model significance. In this case, X_1 , X_3 , X_1X_2 , X_1^2 and X_3^2 were significant model terms. The Lack of Fit value of 0.2 indicated the Lack of Fit was not significant relative to the pure error and the adjusted coefficient of determination ($AdjR^2 = 0.9881$) confirmed the significance of the model. DBT initial concentration, pH and also the interactions between DBT concentration and temperature were significant in the BDS by resting cells (Table IV).

RSM analysis. The response surface and its contour plots 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 and Fig. 4 for growing and resting cells systems, respectively.

Effects of DBT initial concentration: Results indicated that an increase in the initial DBT concentration would make DBT more available to the cells and led to an enhancement in BDS. However, at higher initial concentrations of DBT, bacterial growth and BDS activity would be inhibited, presumably because of the toxicity of DBT at high concentrations beyond the tolerating level of the bacteria (Ansari *et al.*, 2007). Such inhibitory effect of DBT has been reported formerly (Ohshiro *et al.*, 1995). It is known that only water soluble compounds can play an inhibitory role on the cell activity. DBT has an extremely low solubility in water and expected to precipitate in aqueous medium or fully dissolved in the organic phase of biphasic medium and thus, the initial concentration of DBT expected to have an insignificant effect on the cell or enzyme activity. However, most bacterial species with DBT biodesulfurization activity secrete some biosurfactants to solubilize and enhance the bioavailability of DBT (Kim *et al.*, 2004). Also, recombinant *Pseudomonas* strains with high production of biosurfactant have been developed, which could enhance biodesulfurization activity (Gallado *et al.*, 1997). Therefore, by secretion of biosurfactants inhibitory effect of DBT would not be limited. Fig. 3B shows that the optimum concentration of DBT in growing medium for PD2 strain was 0.41 mM and BDS activity was reduced by both the increasing initial DBT concentration up to 0.8 mM or decreasing it to 0.2 mM due to the limit in the growth rate. Also in biphasic medium and resting cells system, the optimum concentration was determined at 7.86 mM and as can be seen in Fig. 4B, the BDS activity was reduced by going away from the optimal point. For the resting cells, the optimum point for the initial DBT concentration was far more than the growing cells. In fact, in the biphasic medium, DBT can be dissolved in n-tetradecane (organic phase) which reduces its toxic effect on bacteria.

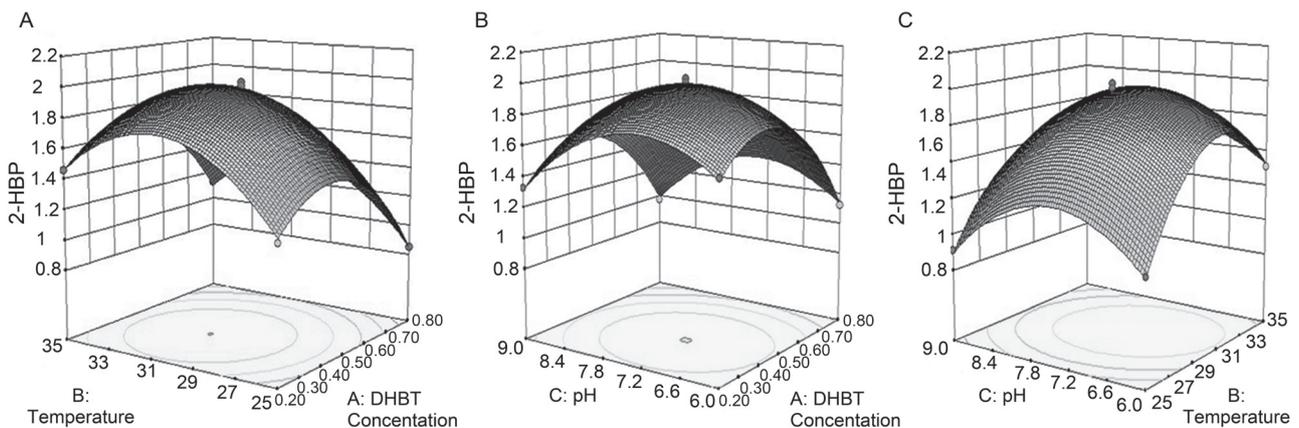


Fig. 3. The response surface and contour plot of 2-HBP production (mM) by growing cells of *Paenibacillus validus* PD2 in aquatic system. A: optimum temperature (°C), B: DBT concentration (mM), C: effect of pH on 2-HBP production.

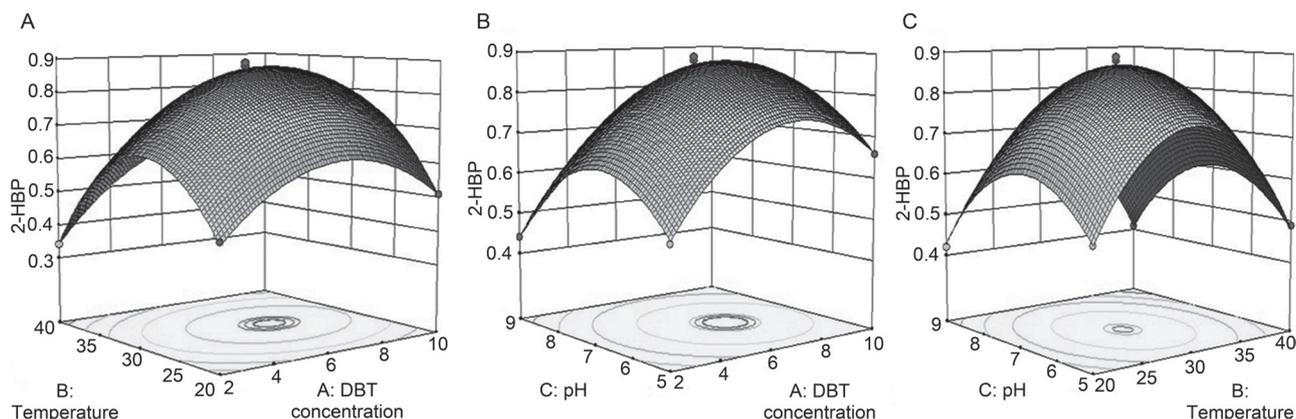


Fig. 4. The response surface and contour plot of 2-HBP production (mM) by resting cells of *Paenibacillus validus* PD2 in biphasic system (tetradecane-water). A: optimum temperature ($^{\circ}\text{C}$), B: DBT concentration (mM), C: pH effect on 2-HBP production.

Effects 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 (Ratkowsky *et al.*, 1982). *P. validus* PD2 is a mesophilic bacteria and its optimum temperature for BDS of DBT in growing medium was determined at 31.23°C . The surface and contour plot in Fig. 3A indicates that in high and low temperatures, 2-HBP production was low because of the limiting influence of temperature on the growth rate and enzyme activity. In resting cells, the optimum temperature for the 2-HBP production was 27.73°C (Fig. 4A). Although the growth rate limiting effect was absent in resting cells, the activity of enzymes can be reduced at the higher or lower temperatures. It has been indicated that in biodesulfurization by *Mycobacterium phlei* WU-F1, degradation of DBT to DBTO_2 stops at high temperatures while degradation of DBTO_2 and other intermediate compounds maintain at this condition. It can be concluded that the activity of the first enzyme oxidizing DBT to DBTO_2 is sensitive to temperature changes (Furuya *et al.*, 2001). In fact, Furuya *et al.* suggested that the first and third enzymes in 4S pathway (DszC and DszB) are more sensitive to temperature changes in comparison with the other enzymes and are considered as the BDS rate-limiting enzymes.

Effects of pH: The pH, like other factors in culture medium such as temperature and carbon source, is an effective parameter that controls the bacterial growth rate (Madigan *et al.*, 2012). In addition, enzymes are affected by the pH variations in the system because of the dependence of the 3-D shape of enzymes on the pH. The impact of pH variation is not devoted to only the shape of enzymes. However, it may also affect the shape or electrical charge properties of the substrate in such a way that the substrate cannot bind to the enzyme active site or it cannot undergo catalysis (Berg *et al.*, 2007). Previous studies showed that the highest DBT

biodesulfurization by the bacterial strains was obtained at pH near to neutral (Kirimura *et al.*, 2001; Etemadifar *et al.*, 2008; Ardakani *et al.*, 2010). Also, it has been indicated that the purified 4S pathway enzymes such as flavin reductase are active only at the pH 6–8 (Matsubara *et al.*, 2001). Figure 3C and Fig. 4C shows the surface and contour plots of pH for 2-HBP production in growing and resting cells systems, respectively. The optimum pH in growing condition was 6.92 and as shown in Fig. 3C, by a change in pH, 2-HBP production was reduced. In resting cells BDS of model oil, the optimum pH was equal to 6.62 (Fig. 4C).

In conclusion, this study demonstrated that the newly isolated strain *P. validus* PD2 can catalyze the conversion of DBT to 2-HBP in aqueous and biphasic systems *via* the 4S pathway. The response surface methodology showed that the optimum DBT concentration in aqueous and biphasic system was 0.41 and 7.86 mM respectively. Also in both media, the optimum temperature was mesophilic and the optimum pH was near the neutral. Therefore *P. validus* PD2 can be efficiently used for oil BDS under ordinary conditions and high activity can be obtained by setting the effective parameters at the optimum levels.

Acknowledgments

We appreciate the financial support for this work from the University of Isfahan .

Literature

- Ansari F., P. Prayuenyong and I.E. Tothill. 2007. Biodesulfurization of dibenzothiophene by *Shewanella putrefaciens*. *J. Biol. Phys. Chem.* 7: 75–78.
- Ardakani M.R., A. Aminsefat, B. Rasekh, F. Yazdian, B. Zargar, M. Zarei and H. Najafzadeh. 2010. Biodesulfurization of dibenzothiophene by a newly isolated *Stenotrophomonas maltophilia* strain Kho1. *WASJ*. 10: 272–2778.

- Bahuguna A., M.K. Lily, A. Munjal, R.N. Singh and K. Dangwal.** 2011. Desulfurization of dibenzothiophene (DBT) by a novel strain *Lysinibacillus sphaericus* DMT-7 isolated from diesel contaminated soil. *J. Env. Sci.* 23: 975–982.
- Berg J.M., J. Tymoczko and L. Stryer.** 2007. *Biochemistry*. Freeman and company, New York.
- Borgne S.L. and R. Quintero.** 2003. Biotechnological processes for the refining of petroleum. *Fuel process technol.* 81: 155–169.
- Bustos-Jaimes I., G. Amador, G. Castorena and S. Le Borgne.** 2003. Genotypic characterization of sulfur-oxidative desulfurizing bacterial strains isolated from Mexican refineries. *Oil Gas Sci. Technol.* 58: 521–526.
- Caro A., K. Boltes, P. Letón and E. García-Calvo.** 2007. Dibenzothiophene biodesulfurization in resting cell conditions by aerobic bacteria. *Biochem. Eng. J.* 35: 191–197.
- Denome S.A., E.S. Olson and K.D. Young.** 1993. Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus* sp. strain IGTS8. *Appl. Environ. Microbiol.* 59: 2837–2843.
- Derikvand P., Z. Etemadifar and D. Biria.** 2014. Taguchi optimization of dibenzothiophene biodesulfurization by *Rhodococcus erythropolis* R1 immobilized cells in a biphasic system. *Int. Biodeterior. Biodegrad.* 86: 343–38.
- Derikvand P., Z. Etemadifar and H. Saber.** 2013. Optimization of nicotinamide and riboflavin in the biodesulfurization of dibenzothiophene using response surface methodology. *BJM.* p-ISSN, 2322–5173. 4: 35–40.
- Etemadifar Z., G. Emtiazi and N. Christofi.** 2008. Enhanced desulfurization activity in protoplast transformed *Rhodococcus erythropolis*. *Am. Eurasian J. Agric. Environ. Sci.* 3: 285–291.
- Furuya T., K. Kirimura, K. Kino and S. Usami.** 2001. Thermophilic biodesulfurization of dibenzothiophene and its derivatives by *Mycobacterium Phlei* WU-F1. *FEMS Microb. Lett.* 24: 129–133.
- Gallado M.E., A. Fernandez, V.D. Lorenzo, J.L. Garcia and E. Diaz.** 1997. Designing recombinant *Pseudomonas* strains to enhance biodesulfurization. *J. Bacteriol.* 176: 6707–6714.
- Gou Z., H. Liu, M. Luo, J. Li S Xing and J. Chen.** 2002. Isolation and identification of nondestructive desulfurization bacterium. *Sci. China Series B: Chem.* 45: 521–531.
- Khurana S., M. Kapoor, S. Gupta and R. Kuhad.** 2007. Statistical optimization of alkaline xylanase production from *Streptomyces violaceoruber* under submerged fermentation using response surface methodology. *Indian J. Microbiol.* 47: 144–152.
- Kim Y.J., J.H. Chang, K. Cho, H.W. Ryu and Y.K. Chang.** 2004. A physiological study on growth and dibenzothiophene (DBT) Desulfurization characteristics of *Gordonia* sp. CYKS1. *Korean J. Chem. Eng.* 21: 436–441.
- Kirimura K., T. Furuya, Y. Nishii, Y. Ishii, K. Kino and S. Usami.** 2001. Biodesulfurization of dibenzothiophene and its derivatives through the selective cleavage of carbon-sulfur bonds by a moderately thermophilic bacterium *Bacillus subtilis* WU-S2B. *J. Biosci. Bioeng.* 91: 262–266.
- Li M.Z., C.H. Squires, D.J. Monticello and J.D. Childs.** 1996. Genetic analysis of the dsz promoter and associated regulatory regions of *Rhodococcus erythropolis* IGTS8. *J. Bacteriol.* 178: 6409–6418.
- Ma C.Q., J.H. Feng, Y.Y. Zeng, X.F. Cai, B.P. Sun, Z.B. Zhang, H.D. Blankespoor and P. Xu.** 2006. Methods for the preparation of a biodesulfurization biocatalyst using *Rhodococcus* sp. *Chemosphere.* 65: 165–169.
- Ma X., L. Sun and C. Song.** 2002. A new approach to deep desulfurization of gasoline, diesel fuel and jet fuel by selective adsorption for ultra-clean fuels and for fuel cell applications. *Catal. today.* 77: 107–116.
- Madigan M.T., J.M. Martinko, D.A. and D.P. Clark.** 2012. *Brock Biology of microorganisms*. Benjamin Cummings (eds.), San Francisco.
- Matsubara T., T. Ohshiro, Y. Nishina and Y. Izumi.** 2001. Purification, characterization, and over expression of flavin reductase involved in dibenzothiophene desulfurization by *Rhodococcus erythropolis* D-1. *Appl. Environ. Microbiol.* 67: 1179–1184.
- Mohebbali G., A.S. Ball, A. Kaytash and B. Rasekh.** 2008. Dimethyl sulfoxide (DMSO) as the sulfur source for the production of desulfurizing resting cells of *Gordonia alkanivorans* RIP190A. *Microbiology* 154: 878–885.
- Monticello, D.J.** 2000. Biodesulfurization and the upgrading of petroleum distillates. *Curr. Opin. Biotech.* 11: 540–546.
- Ohshiro T., Y. Kobayashi, Y. Hine and Y. Izumi.** 1995. Involvement of flavin coenzyme in dibenzothiophene degrading enzyme system from *Rhodococcus erythropolis* D-1. *Biosci. Biotech. Biochem.* 59: 1349–1354.
- Ratkowsky D., J. Olley, T. McMeekin and A. Ball.** 1982. Relationship between temperature and growth rate of bacterial cultures. *J. Bacteriol.* 149: 1–5.
- Rhee S.K., J.H. Chang, Y.K. Chang and H.N. Chang.** 1998. Desulfurization of dibenzothiophene and diesel oils by a newly isolated *Gordonia* strain, CYKS1. *Appl. Environ. Microbiol.* 64: 2327–2331.
- Setti L., S. Bonoli, E. Badiali and S. Giuliani.** 2003. Inverse phase transfer biocatalysis for a biodesulfurization process of middle distillates. *BECTH MOCK YH-TA CEP.* 44: 80–83.
- Tan K.T., K.T. Lee and A.R. Mohamed.** 2010. A glycerol-free process to produce biodiesel by supercritical methyl acetate technology: An optimization study via Response Surface Methodology. *Bioresour. technol.* 101: 965–969.
- Yan H., M. Kishimoto, T. Omasa, Y. Katakura, K.I. Suga, K. Okumura and O. Yoshikawa.** 2000. Increase in desulfurization activity of *Rhodococcus erythropolis* KA2-5-1 using ethanol feeding. *J. Biosci. Bioeng.* 89: 361–366.