**Short Communication**

# Isolation of a dibenzothiophene desulfurizing bacterium from soil of Tabriz oil refinery

**Firoozeh Khadem Haghighat[1,2] , Fereshteh Eftekhar[2], Mahnaz Mazaheri[1]**

*[1] Biotechnology Center of Iranian Research Organization for Science and Technology, No.71, Forsat St., Enghelab Ave., Tehran, Iran.*
*[2] Biology Department, Faculty of Science, Shahid Beheshti University, Evin, Tehran, Iran.*

***Abstract***
*A dibenzothiophene (DBT) degrading bacterium, which utilizes DBT as the sole source of sulfur, was isolated from soil contaminated with crud oil collected from oil refinery of Tabriz (Iran). A convenient spectrophotometric assay (Gibbs' assay) was used to determine the quantity of desulfurized product (Hydroxybiphenyl). This isolate did not grow on DBT, dibenzothiophene sulfone ($DBTO_2$), or 2-Hydroxybiphenyl (HBP) as sole carbon sources. Biodesulfurization activity was observed only in growing cultures and depressed by free sulfate. The desulfurization trait was expressed at increasing levels during the exponential phase of growth and then declined in stationary-phase cells. This gram-positive, non-spore-forming, partially acid fast, polymorphic bacterium with the ability to desulfurize DBT or $DBTO_2$ was identified as Rhodococcus sp. and designated strain FMF.*
***Keywords****:Isolation, Biodesulfurization, dibenzothiophene, Rhodococcus sp.*

Combustion of sulfur-containing fossil fuels result in acidic deposition, which pose a multitude of threats to the environment (Monticello, 1985; Bos et al., 1990; Foght et al., 1990; Kilbane, 1990; Finnerty, 1992; Monticello, 1993; For reviews). Physical and chemical methods to prevent the release of sulfur compounds as atmospheric pollutants from coal and crude oil, are currently being employed, but are expensive. Recently, microbial degradation of organic sulfur compounds has attracted attention for its potential application of desulfurization of coal and petroleum. Microbial desulfurization may provide an improved technology for the pre-combution removal of organic sulfur from fossil fuels.

The biodesulfurization process using microorganisms improved technology for the pre-combustion removal and/or enzymes has the advantage of being carried out more safely under mild conditions. Moreover, bioprocessing in general is strictly specific for substrates. Such high substrate specificity of biological reactions may be more convenient for treatment of petroleum fractions containing a limited number of organic sulfur compounds (Izumi *et al.*, 1994; Kayser *et al.*, 1993; Konishi *et al.*, 1997). Coal and crude oil contain many of the same organic sulfur structures, which include thiophenes, disulfides, sulfones, mercaptans (thiols), and sulfoxides. The compound dibenzothiophene (DBT) has received most attention in recent biodesulfurization studies because it is an accepted model compound representative of thiophenic structures found in coal and petroleum (Kayser *et al.*, 1993). Apart from being a heterocyclic sulfur substructure in coal and crude oil, DBT belongs to a group of recalcitrant polycyclic aromatic sulfur heterocycles (PASHs) of environmental concern (Kayser, 1993 and Van Afferden *et al.*, 1994).

The bacterial strains, *Rhodococcus rhodochrous* IGTS8 (Kilbane, 1989; Kilbane *et al.*, 1990),

*Correspondence to:* **Mahnaz Mazaheri, Ph.D**
*Tel/ Fax: +98 21 8838350,*
*E-mail: mxmazaheriassadi@yahoo.com*

*Rhodococcus erythropolis* D-1 (Izumi *et al.*, 1994; Ohshiro *et al.*, 1994, 1995), *Rhodococcus erythropolis* H-2 (Ohshiro *et al.*, 1996), *Corynebacterium* SY-1 (Omori *et al.*, 1992) and *Agrobacterium* sp. (Constanty *et al.*, 1990) can utilize DBT as the sole source of sulfur and convert it to hydroxybiphenyl (HBP). The genes involved in DBT degradation by *R. rhodochrous* IGTS8 have been identified (Denome *et al.*, 1993, 1994).

We have isolated a DBT-degrading bacterium, which utilizes DBT as the sole source of sulfur. This report describes the desulfurization of DBT and DBT-sulfone by measuring HBP formation. The organism was tentatively identified as *Rhodococcus sp*., strain FMF.

Basal salt medium (BSM) consisting of: $K_2HPO_4$ (4 gr); $Na_2HPO_4$ (4 gr); $NH_4Cl$ (2 gr); $MgCl_2$ $6H_2O$ (0.2 gr); $CaCl_2$ $2H_2O$ (0.001 gr) and $FeCl_3$ $6H_2O$ (0.001 gr) per liter of distilled, deionized water pH 7.0 was used for isolation and growth of the microorganisms under sulfur deficient conditions (Kilbane *et al.*, 1990). Glycerol (20 mM) was used as the carbon source and was omitted when other test compounds were used instead. Soil samples and subsequently isolated strains were inoculated in BSM supplemented with 0.1 mM DBT or $DBTO_2$ or 0.2 mM of $MgSO_4$. The sulfur sources were added to the medium from sterile stock solutions before inoculation (10 mM DBT or $DBTO_2$ in ethanol; 50 mM $MgSO_4$ in deionized water). Media were designated as DBT, $DBTO_2$, or $MgSO_4$ medium, respectively (Wang *et al.*, 1994). BSM solidified with 15 gr of agar per liter was used for isolating bacterial colonies. All cultures were incubated at 30°C and liquid cultures were shaken at 200 rpm.

Cell suspensions were inoculated (1: 200) in DBT or $DBTO_2$ liquid media for sulfur bioavailability assay (Kilbane, 1989, 1990; Krawiec, 1990). Positive and negative controls consisted of BSM with or without $MgSO_4$. After two days of incubation, subcultures were made in fresh media (1: 500) and shaken for four more days (Wang *et al.*, 1994). Cultures with significant growth in DBT or $DBTO_2$ media as compared to the negative controls were centrifuged, the supernatants were used for Gibbs assay and the pellets were streaked on solid media for colony isolation. Individual colonies were then inoculated in DBT and $DBTO_2$ media and incubated at 30°C with shaking (200 rpm). Samples were taken during a period of 2 to 4 days and tested with Gibbs assay. BSM without glycerol was used to determine if positive strains could use organosulfur compounds as sole carbon sources. The ability to inhibit desulfurization activity was assessed by using 20 mM $MgSO_4$ (Kayser *et al.*, 1993).

Soil samples were collected from oil fields and oil refinery regions of Iran. A half gram of each soil sample was added to 10 ml of $MgSO_4$ liquid medium and incubated for 24 h with shaking. The suspension was centrifuged at 500×g for about 2 min to remove soil particles. The supernatant was then centrifuged at 6000×g for 6 min and the cell pellets were washed and resuspended in 0.5 ml of saline (Wang *et al.*, 1994).

Identification of the isolate(s) was based on colony morphology, microscopic observation of the cell cycle, gram stain, acid–fast stain, catalase test, oxygen requirement, motility, and casein hydrolization according to standards for microbial identification in Bergy's manual of systematic bacteriology. The ability to grow on different carbon, carbon and nitrogen sources, and in the presence of some inhibitors were also tested for further identification according to Bergy's manual of systematic bacteriology.

Desulfurization activity was monitored in growing cells using the Gibbs assay (Kayser *et al.*, 1993), a convenient spectrophotometric assay, based on the chromogenic reaction of 2, 6-dichloroquinon-4-chloroimide (Gibbs reagent) with aromatic hydroxyl groups to determine the quantity of desulfurized product (Hydroxybiphenyl). For detecting HBP in growing cultures, aliquots of cultures were removed at various time intervals, centrifuged for 6 min at 6000×g and the supernatant was subjected to Gibbs assay. To monitor the desulfurization activity of resting cells (Resting-cell reaction) cells were harvested after 4 days incubation washed twice with 0.1 mM potassium phosphate buffer (pH 7.0) and stored at -20°C until use. The cells were suspended in BSM and cell density was adjusted to 1 at $OD_{600}$. DBT (20 mM) was then added and the cells were incubated at 30°C for 60 min with occasional shaking. This suspension was subjected to Gibbs assay.

For Gibbs assay, 10 ml of the test solution was placed in a 20 ml test tube, the pH was adjusted to 8.0 with 10% (w/v) $Na_2CO_3$ and then 50 µl of Gibbs reagent (10 mg/ml, ethanol solution ) was added. The solution was incubated at room temperature for 30 min to develop full color. The solution was then centrifuged and the absorbance of the supernatant was determined

at 610 nm (UNICAM 8620 spectrophotometer). The values were reported as p.p.m based on HBP standard curves.

Working on seven soil samples showed that only the soil from tabriz refinery is positive on Gibbs assay when grown in DBT or $DBTO_2$ media, representing the possibility of the existence of desulfurized product (HBP). The mixed culture from this soil was selected for further studies. Mixed culture consisted of two distinct colony types; one Gram-positive bacterium and a gram-negative bacillus. Each of these colonies was grown in pure cultures and tested individually by Gibbs assay. Only the Gram-positive isolate could produce the desulfurized product and a similar amount of desulfurized product was produced compared to the mixed culture. Hence it was concluded that the gram-negative bacilli were unable to desulfurize the organic compounds.

The isolate capable to desulfurize DBT or $DBTO_2$ was a non–endospore forming, partially acid fast, non–motile bacterium which formed coccoid and short rods. In liquid cultures, cells existed as singles, pairs (V-shape), or in groups of 3 or more. Cells from young colonies were sometimes branched. The colonies were creamy to pale orange-pink, round, regular, entire, opaque, convex, smooth, and butyrous. The results show that the isolate belongs to the genus *Rhodococcus* and is perhaps a *R.erythropolis*. Hence, the isolate was designated "*Rhodococcus sp.*, strain FMF".

Expression of the desulfurization trait by the isolate in relation to bacterial growth cycle was studied. The bacterium was grown with DBT or $DBTO_2$ as sole sources of sulfur in two separate experiments and accumulation of HBP from DBT or $DBTO_2$ was monitored throughout the growth cycle using the Gibbs assay (Fig. 1). In both DBT and $DBTO_2$ media production of desulfurized product was initiated from mid-exponential phase and the concentration of the product peaked at the onset of stationary phase and remained constant up to 240 h (duration of the experiments). This fact may indicate that HBP (the desulfurized product) is the end metabolite of DBT and $DBTO_2$ degradation by this strain. It was also observed that formation of HBP started earlier in $DBTO_2$ medium (4h.) when compared with that in DBT (48 h.). It has been proposed in the "4S" pathway that the conversion of DBT to HBP requires the production of $DBTO_2$ as an intermediate (Isbester, 1986; Kraweic, 1990). This may suggest that $DBTO_2$ is indeed one of the intermediates produced during the metabolism of DBT by the strain FMF. This bacterium could not grow on DBT, DBT sulfone and Hydroxybiphenyl as the sole sources of carbon (growth was negligible). As it was expected, desulfurization was repressed in DBT or $DBTO_2$ media con-

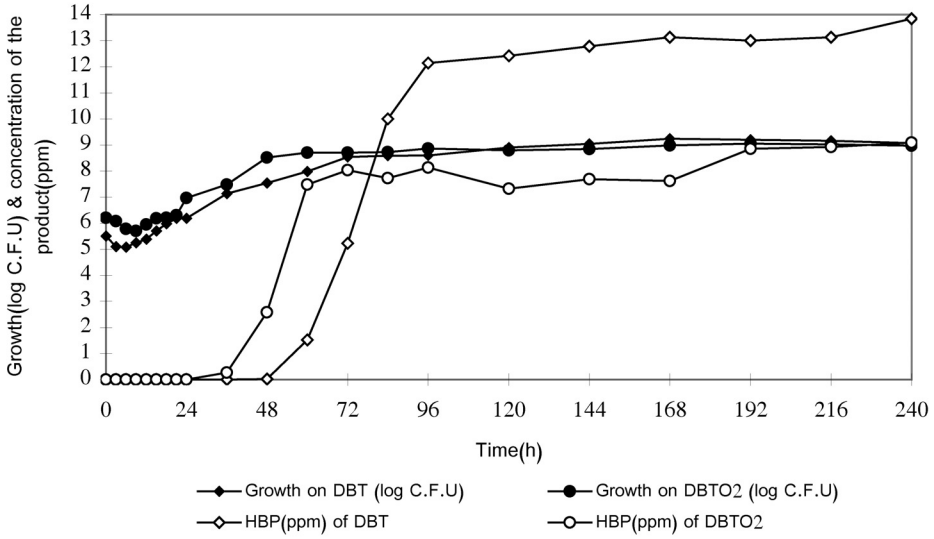


**Figure 1. Growth of *Rhodococcus sp*. Strain FMF on DBT and $DBTO_2$ as sole sourse of sulfur and formation of HPD during growth.**

taining 20 mM $MgSO_4$ (Kayser, 1993; Purdy, 1993). These results indicate and suggest that the strain FMF can selectively desulfurize organo sulfur compounds (DBT & $DBTO_2$) without destroying the carbon skeleton via a pathway similar to that of other recently isolated gram-positive organisms ("4S" pathway; Constanty *et al.*, 1990; Izumi *et al.*, 1994; Kilbane 1989; Omori *et al.*, 1992; Ohshiro *et al.*, 1994) and not carbon destructive pathway (*Kodama et al.*, 1970; Monticello 1985). However the data obtained in this study are insufficient to either confirm or deny this suggestion since the study of desulfurization pathways was not the chief objective of this project. Finally, the results showed that desulfurization of DBT and $DBTO_2$ occurred only in growing cultures and resting cell suspensions did not desulfurize any of the substrates. In conclusion a bacterial strain with stable DBT desulfurization phenotype was isolated. The strain may have potential use for large-scale desulfurization of fossil fuels especially crude oil. The isolate is presumptively identified as *Rhodococcus sp.* strain FMF. Further investigation of the mechanism of desulfurization and genetic and enzymatic aspects of the metabolic pathways used by this organism need to be carried out.

## References


Bos P, Kuenen J G. (1990) Microbial treatment of coal, pp. 343-377. In H.L. Ehrlish and C. L. Brierley (ed.) *Microbial mineral recovery.* Mc G raw-Hill book co. New York.

Constanty M, J Giralt , A. Bordons. (1990) Desulfurization of dibenzothiophene by bacteria. *World J. Microbiol. Biotechnol*. 10: 510-6.

Denome S A, E S Olson, K D Young. (1993) Identification and cloning of genes involved in specific desulfurization of dibenzothiophene by *Rhodococcus sp*. strain IGTS8. *Appl. Environ. Microbiol*. 59: 2837-43.

Denome S A, C Oldfield, L J Nash, K D Young. (1994) Characterization of desulfurization genes from *Rhodococcus sp*. strain IGTS8. *J. Bacteriol*. 176: 6707-16.

Finnerty W R. (1992) Fossil resource biotechnology. Challenges and prospects. *Curr. Opin. Biotechnol*. 3: 277-82.

Foght J M, P M Fedorak, M R Gray, DW S Westlake. (1990) Microbial desulfurization of petroleum, pp: 79-407. *In* H. L. Ehrlich and C. L. Brierley (ed.) *Microbial mineral recovery*. Mc Graw-Hill book co. NewYork.

Isbester J. (1986) Biological removal of organic sulfur from coal. *In Proceeding of biological treatment of coals workshop*, pp: 18-25, Sheladia associates, Rockville, MD.

Izumi Y, T Ohshiro, H Hine, M. Shimao. (1994) Selective desulfurization of dibenzothiophene by *Rhodococcus erythropolis* D-1. *Appl. Environ. Microbiol*. 61(1): 223-6.

Kayser K J, B A Bielaga-Jonse, K Jackowski, O Oduson, Kilbane II J J. (1993) Utilization of organo sulfur compounds by axenic and mixed cultures of *Rhodococcus rhodochrous IGTS8*. *J. Gen. Microb*. 139: 2123-9.

Kilbane II J J. (1989) Desulfurization of coal: The microbial solution. *Trends Biotechnol*.; 7: 97-101.

Kilbane II J J. (1990) Sulfur-specific microbial metabolism of organic compounds. *Resour. Conserv. Recyc*. 3: 69-79.

Kilbane II J J, B A Bielaga. (1990) Toward sulfur-free fuels. *CHEMTEC*. 20: 747-51.

Kodama K, S Nakatani, K Umehara, K Shimizu, Y Minoda, K Yamada. (1970) Microbial conversion of petro-sulfur compounds, III. Isolation and identification of products from dibenzothiophene. *Agric. Biol. Chem*. 34: 1320-4.

Konishi J, Y Ishii, T Onaka, K Okumura, M Suzuki. (1997) Thermophilicarbon-sulfur-bond-targeted biode-sulfurization. *Appl. Environ. Microbiol.* 63 (8): 3164-9.

Krawiec S. (1990). Bacterial desulfurization of thiophenes : screening techniques and some speculations regarding the biochemical and genetic bases. *In Dev. Indust. Microb*. Vol. 31, suppl. 5: 103-14.

Monticello D J. (1985). Microbial desulfurization of fossil fuels. *Ann. Rev. Microbiol*. 39: 371-89.

Monticello D J. (1993). Biocatalitic desulfurization of petroleum and middle distillates. *Environ. Prog*. 12: 1-4.

Omori T, L Monna, K Saiki, T Kodama. (1992). Desulfurization of dibenzothiophene by Corynebacterium sp. strain SY1. *Appl. Environ. Microbiol*. 58 (3): 911-5.

Ohshiro T, Y Hine, Y Izumi. (1994). Enzymatic desulfurization of dibenzothiophene by a cell-free system of *Rhodococcus erythropolis D-1.FEMS Microbiol. Lett*. 118: 341-4.

Ohshiro T, Kanabashi Y, Izumi Y. (1995). Involvement of flavin coenzyme in dibenzothiophene degrading enzyme system from *Rhodococcus erythropolis D-1. Biosci. Biotech. Biochem*. 59: 1349-51.

Ohshiro T, Hirata T, Izumi Y. (1996). Desulfurization of dibenzothiophene derivatives by whole cells of *Rhodococcus erythropolis H-2. FEMS Microbiol. Lett.* 142: 65-70.

Purdy R F, J E Lepo, B Ward. (1993). Biodesulfurization of organic-sulfur compounds. *Curr. Microb*. 27: 219-22.

Van Afferden M, J Giralt, A Bordons. (1994). Desulfurization of dibenzothiophene by bacteria. *World J. Microbiol. Biotechnol*. 10: 510-6.

Wang P, S Krawiec. (1994). Desulfurization of dibenzothiophene to 2-Hydroxybiphenyl by some newly isolated bacterial strains. *Arch. Microbiol*. 161: 266-71.