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

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Abstract

A dibenzothiophene (DBT) degrading bacterium, which utilizes DBT as the sole source of sulfur, was isolated from soil contaminated with crude 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 (DBTO₂), 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 DBTO₂ 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-combustion 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),
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 x 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 OD600. 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) Na2CO3 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 DBTO₂ 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 DBTO₂ 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 DBTO₂ as sole sources of sulfur in two separate experiments and accumulation of HBP from DBT or DBTO₂ was monitored throughout the growth cycle using the Gibbs assay (Fig. 1). In both DBT and DBTO₂ 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 DBTO₂ degradation by this strain. It was also observed that formation of HBP started earlier in DBTO₂ 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 DBTO₂ as an intermediate (Isbester, 1986; Kraweic, 1990). This may suggest that DBTO₂ 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 DBTO₂ media con-

![Figure 1. Growth of *Rhodococcus sp.* Strain FMF on DBT and DBTO₂ as sole source of sulfur and formation of HPD during growth.](image-url)
taining 20 mM MgSO₄ (Kayser, 1993; Purdy, 1993). These results indicate and suggest that the strain FMF can selectively desulfurize organo sulfur compounds (DBT & DBTO₂) without destroying the carbon skeleton via a pathway similar to that of 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 DBTO₂ 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**


