Sulfur-selective desulfurization of dibenzothiophene and diesel oil by newly isolated *Rhodococcus* sp. strains

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Received 11 March 2002; received in revised form 12 August 2002; accepted 15 August 2002

First published online 10 September 2002

**Abstract**

New desulfurizing bacteria able to convert dibenzothiophene into 2-hydroxybiphenyl and sulfate were isolated from contaminated soils collected in Mexican refineries. Random amplified polymorphic DNA analysis showed they were different from previously reported *Rhodococcus erythropolis* desulfurizing strains. According to 16S rRNA gene sequencing and fatty acid analyses, these new isolates belonged to the genus *Rhodococcus*. These strains could desulfurize 4,6-dimethyldibenzothiophene which is one of the most difficult dibenzothiophene derivatives to remove by hydrodesulfurization. A deeply hydrodesulfurized diesel oil containing significant amounts of 4,6-dimethyldibenzothiophene was treated with *Rhodococcus* sp. IMP-802 cells. Up to 60% of the total sulfur was removed and all the 4,6-dimethyldibenzothiophene disappeared as a result of this treatment.

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**Keywords:** Biodesulfurization; Dibenzothiophene; 4,6-Dimethyldibenzothiophene; Diesel oil; *Rhodococcus*

**1. Introduction**

Combustion of petroleum derived fuels leads to the atmospheric emission of sulfur oxides which are the major cause of acid rain. Strict government regulations on sulfur content in fuels have been implemented worldwide to control these emissions [1]. Recently, the Environmental Protection Agency of the United States proposed a 97% reduction of the sulfur content in diesel to less than 15 ppm by 2006 down from the current specification of 500 ppm [2]. It is expected that similar regulations will be adopted in other countries. In Mexico, 59% of the oil produced is of Maya type which is a heavy crude with a high sulfur content, the proportion of this type of crudes is increasing in the Mexican refineries feedstocks. In fractions used to produce diesel, most of the sulfur is found in dibenzothiophene (DBT) and alkyl substituted DBT derivatives [3]. DBT derivatives in which the sulfur atom is sterically hindered by substitutions in positions 4 and 6 are the most difficult to remove by hydrodesulfurization (HDS) and 4,6-dimethyldibenzothiophene (4,6-DMDBT) has been shown to be particularly recalcitrant to HDS [4]. HDS is the technology presently used in refineries for the pre-combustion desulfurization of fuels. It involves the use of metallic catalysts at high pressures and temperatures. Higher temperatures and pressures as well as new catalysts will be needed to desulfurize the most recalcitrant molecules, leading to increased operation and capital costs as well as more CO₂ emissions. New technologies complementary to HDS are therefore needed to remove the sulfur from the most recalcitrant molecules and meet the specifications on sulfur content in diesel. A possible approach is the biodesulfurization (BDS) of fuels. The aerobic bacterium *Rhodococcus erythropolis* strain IGTS8 is able to selectively remove the sulfur from DBT without degrading the carbon skeleton of this molecule [5]. This prototype strain has been the most extensively studied desulfurizing bacterium and it is the basis of the commercial process proposed by the North American Energy Biosystems Corporation [6]. *R. erythropolis* IGTS8 and all BDS processes derived from this strain are patented. The energetic value...
of fuels treated with this strain is not affected since DBT is not degraded but only transformed into 2-hydroxybiphenyl (2-HBP) and sulfate. Within the last decade a number of other desulfurizing bacteria following the same metabolic pathway as *R. erythropolis* IGTS8 have been isolated. These include principally several strains of *R. erythropolis* [7–10], *Paenibacillus* sp. [11], *Gordona* sp. [12], *Nocardiadsp.* [13], *Sphingomonas* sp. [14] and *Bacillus subtilis* [15].

In this work, new desulfurizing bacteria native from Mexican oil refineries. The following strains were used isolated from hydrocarbon-contaminated soils from different Mexican oil refineries. The following strains were used for comparison: *R. erythropolis* IGTS8 and are also able to desulfurize 4,6-DMDBT and diesel oil.

**2. Materials and methods**

2.1. Source of bacteria

The DBT desulfurizing bacteria used in this study were isolated from hydrocarbon-contaminated soils from different Mexican oil refineries. The following strains were used for comparison: *R. erythropolis* IGTS8 (ATCC 53968) and *R. erythropolis* X309 (ATCC 55309).

2.2. Isolation and culture conditions

The minimal salt medium (MSM) was used for the isolation and cultivation of DBT desulfurizing strains [16]. DBT, dibenzothiophene sulfone (DBTS) and 4,6-DMDBT were dissolved in hexane (50 mM) and added to the sterilized MSM. For solid media preparation, MSM agar plates were coated with 1 ml of DBT solution. Cultures were incubated on a rotary shaker at 180 rpm at 30°C. Initial enrichments cultures were prepared by adding soil samples to MSM supplemented with 14 mM of dimethylsulfoxide. Colonies of DBT-degrading bacteria were then obtained by plating the enrichment cultures on DBT-coated MSM agar plates.

2.3. Identification of the isolates

Preliminary characterization of bacterial strains were made by macroscopic observation, microscopy and Gram staining. Fatty acid analyses were conducted at Microbial ID Inc. (Newark, DE, USA). Sequencing of the 16S rRNA genes and sequence analyses were performed by MIDI Labs (Newark, DE, USA).

2.4. Genomic DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was isolated from 15 ml of stationary phase cells grown in MSM supplemented with DBT by using a modified cetyltrimethylammoniumbromide method [17]. Bacteria were incubated at 37°C for 1 h with 375 U of mutanolysin before adding the lysozyme to improve cell lysis [18]. Random amplified polymorphic DNA (RAPD)-PCRs were performed using Ready-To-Go RAPD analysis beads and primers 1, 2 and 6 (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The PCR primers for the amplification of the desulfurization genes were designed using DNA sequences from strain IGTS8 (GenBank accession number U08850). PCR primers for *dszC* gene amplification were *dszC*1, *dszC*4, *dszC*3 were used. Amplification of the desulfurization genes were designed with the manufacturer’s recommendations. The PCR conditions were as follows: one cycle of 1 min at 95°C; 30 cycles consisting of 1 min at 95°C, 1 min at 60°C and 1.5 (for *dszC*) or 4 min (for *dszABC*) at 72°C; and a final cycle of 10 min at 72°C.

2.5. Desulfurization reactions

For DBT, DBTS and 4,6-DMDBT utilization experiments, shake-flask cultures consisting of 20 ml of MSM containing 1.5 mM DBT, 1.5 mM DBTS or 1.2 mM 4,6-DMDBT in 250-ml Erlenmeyer flasks were performed. Cultures were incubated on a rotary shaker at 180 rpm at 30°C and samples were taken on day 7 for analysis. Diesel desulfurization was performed in 500-ml Erlenmeyer flasks containing 95 ml of MMS, 5 g l⁻¹ of biomass and 5 ml of diesel oil during 7 days at 30°C with an agitation of 180 rpm. Deeply hydrosulfurized diesel oil was obtained from a Mexican refinery. Biomass was obtained from fermentator scale cultures in MMS supplemented with 1.2 mM 4,6-DMDBT.

2.6. Analytical procedures

Determination of accumulated phenolic compounds was performed by the Gibb’s assay [19]. 100 μl of fresh solution of Gibb’s reagent prepared in ethanol was mixed with 1 ml of culture supernatants. Positive reactions developed blue to purple color after 1 h of incubation at room temperature. DBT, DBTS, 4,6-DMDBT and their metabolites were analyzed by high-performance liquid chromatography (HPLC) using a Hewlett-Packard (model HP1100) system, with a SRP-18 Waters column. Elution was performed with a 80/20 (v/v) acetonitrile/water mobile phase at 1 ml min⁻¹ and detection was realized with an UV detector at 225 nm. For HPLC analysis, cultures were extracted with equal volumes of acetonitrile. DBT and 4,6-DMDBT metabolites were also analyzed by gas chromatography–mass spectrometry (GC–MS) using a Hew-
lett-Packard (model 6890) chromatograph coupled to a mass spectrometer. The gas chromatograph was equipped with a HP capillary column HP-5% phenyl methyl siloxane (30 m × 250 μm × 0.25 μm). The oven temperature was programmed from 100°C to 270°C with an increasing rate of 7°C min⁻¹. For GC–MS analysis, cultures were acidified to pH 2 with HCl and extracted with toluene. Extracts were dried over anhydrous Na₂SO₄ and concentrated by evaporation. Total sulfur was determined in culture supernatants by X-ray fluorescence with an Horiba sulfur analyzer (model SLFA-1100H).

The distribution of organosulfur compounds in diesel was determined by GC using a Hewlett-Packard (model 6890) chromatograph coupled to a flame ionization detector and a sulfur chemiluminescence detector (SCD) (Siever 355). 1 μl filtered oil samples was injected into a dimethyl siloxane column (30 m × 320 μm × 0.25 μm). The injector and detector temperatures were set to 280 and 300°C respectively. Chromatography was accomplished over 60 min by using an oven temperature program which started at 90°C and was then ramped to 300°C at 4°C min⁻¹ and held for 5 min at this temperature.

3. Results and discussion

3.1. Isolation of the DBT desulfurizing strains

The DBT desulfurizing strains were obtained from hydrocarbon-contaminated soils originating from several Mexican oil refineries by standard culture enrichment techniques using DBT as the sole source of sulfur. Individual colonies forming zones of clearing when grown on DBT-coated agar plates indicated DBT degradation. Four strains named IMP-S02, IMP-S06, IMP-S24 and IMP-A17 were selected to continue this study since they presented the highest desulfurizing activity and were able to selectively remove the sulfur atom from DBT. These four isolates were aerobic, Gram-positive, non-motile and non-spor-forming cocci and short rods. Rough with entire margin orange colonies were produced on DBT minimal medium. Microscopic observations showed that the isolates presented a morphologic cycle consisting of cocci and short rod cells which formed filaments with elementary branching. These filaments then fragmented into cocci and short rods. The strains had strongly hydrophobic surfaces and preferentially adhered to the glass walls of the flasks. Contrary to R. erythropolis IGTS8, they did not show a mucoidal morphology.

3.2. Characterization of the isolates

The strains were characterized by RAPD analysis in order to differentiate them from the already patented desulfurizing strains. One primer of the three tested produced distinct fingerprints between the four isolates and the control desulfurizing strains (R. erythropolis IGTS8 and X309). Differences were observed in the size and intensity of the bands (Fig. 1). Thus, RAPD analysis had the capacity to distinguish between the four newly isolated bacteria and R. erythropolis strains, as demonstrated by the different banding patterns obtained. So, on the basis of these analyses these new isolates were different from the prototype desulfurizing strains, at the genomic DNA level.

The strains were then identified by sequencing the first 500 bases of their 16S rRNA genes. The four isolates were identical at the 16S rRNA level and a 99.8% identity was found with Rhodococcus globerulus. The only difference between these strains and R. globerulus was found at position 400 at which there was a T instead of a C. Fatty acid analyses revealed that the four isolates had similar membrane fatty acid compositions. Palmitic acid (16:0) and oleic acid (18:1 ω9c) comprised around 30 and 20% of the membrane, respectively. Analyses of the data indicated that the four strains belonged to the species R. globerulus. Complete sequencing of the best desulfurizing strain, IMP-S02, was then performed. Analysis of the obtained 1513-bp sequence revealed that this isolate was closely related to both R. erythropolis ( genetic difference: 0.56%) and R. globerulus ( genetic difference: 0.76%). Lack of resolving power of 16S rRNA at the species level has been recently underlined for nearly identical or heterogeneous species [20]. We will therefore continue to refer to our isolates as Rhodococcus sp. IMP-S02, IMP-S06, IMP-S24 and IMP-A17. Other desulfurizing bacteria different from Rhodococcus sp. have been reported, however this genus is of particular interest due to its capacity to metabolize hydrophobic substrates [21].

3.3. Metabolism of DBT and 4,6-DMDBT

The desulfurizing activity of the isolates was studied in MMS medium containing DBT as sole source of sulfur. Phenolic compounds accumulated in the medium as indi-

Fig. 1. RAPD profiles generated with primer 2 for several DBT desulfurizing strains. Lane 1, 1-kb molecular mass marker; lane 2, R. erythropolis IGTS8; lane 3, IMP-S02; lane 4, IMP-S06; lane 5, IMP-S24; lane 6, IMP-A17; lane 8, R. erythropolis X309. Electrophoretic separation was performed in a 1.5% agarose gel in TBE buffer.
cated by the production of a purple color in the presence of Gibb’s reagent. Sulfate ions were also detected by capillary electrophoresis in the culture supernatants while they were not present in the initial culture medium (data not shown). HPLC analysis of culture extracts revealed the presence of 2-HBP and DBTS after comparison with chromatographs of standards of these compounds. GC–MS analysis of the same culture extracts confirmed that the end metabolite of DBT desulfurization was 2-HBP. The detected metabolite had a molecular mass of 170 and its mass ion (m/z) corresponded to the 2-HBP (Fig. 2). The isolates were also able to use DBTS as sole of sulfur. So, DBT desulfurization was assumed to proceed as DBT $\rightarrow$ DBTS $\rightarrow$ 2-HBP + sulfate. This metabolic pathway is similar to that of the prototype strain *R. erythropolis* IGTS8 in which DBT is desulfurized by selective cleavage of its C–S bonds, conserving its C–C bonds. Additionally,
the strains were also screened by PCR for the presence of the *dszC* gene encoding the DBT monoxygenase DszC catalyzing the oxidation of DBT into DBTS and the *dszABC* operon encoding the complete BDS pathway in *R. erythropolis* IGTS8. For this, oligonucleotide primers derived from IGTS8 sequences were used. The four new isolates produced the same 1.2- and 3.7-kb DNA fragments for *dszC* and *dszABC* as strain IGTS8, respectively. Endonuclease digestion of these amplified fragment using several 6- and 4-base recognition site enzymes produced identical restriction patterns (data not shown). The *dszABC* operon was therefore also present in the new *Rhodococcus* sp. desulfurizing strains confirming that these isolates had the same desulfurization pathway as *R. erythropolis* IGTS8.

The isolates were then tested for their ability to desulfurize 4,6-DMDBT which is one of the most refractory compounds to HDS. Strains grew well in MMS containing 4,6-DMDBT as the sole source of sulfur suggesting that they could efficiently desulfurize this compound. Again phenolic compounds and sulfate were detected in culture extracts. GC–MS of culture extracts showed that the 4,6-DMDBT lost its sulfur atom and was metabolized to the 2-hydroxy-3,3′dimethyl biphenyl (2-HDMBP) (Fig. 3).

### 3.4. Desulfurization of diesel oil

Desulfurization tests were performed with a deeply hydrodesulfurized diesel oil from a Mexican refinery using the IMP-S02 strain since this strain exhibited the highest desulfurizing activity. Initial sulfur content of this fraction was 500 ppm. The control sample consisted in sterile MMS supplemented with diesel. After incubation, the diesel fraction was recovered and its total sulfur content determined. The sulfur content of the oil after treatment with IMP-S02 was 200 ppm. So this strain was able to remove 60% of the sulfur present in the oil. Diesel was also analyzed by GC equipped with a sulfur-specific detector before and after treatment with the IMP-S02 strain. Fig. 4 shows that sulfur compounds with longer retention times than DBT were desulfurized. Those compounds are DBT derivatives which are generally recalcitrant to HDS. In particular the complete 4,6-DMDBT peak disappeared. In HDS, this compound has a reactivity more than 10 times lower than DBT.

### Acknowledgements

This work was supported by the Project D.00020 ‘Tecnologia complementaria al proceso de hidrodesulfuración de diesel’ of the Mexican Institute of Petroleum.

### References


