Application of SPME/GC-MS To Characterize Metabolites in the Biodesulfurization of Organosulfur Model Compounds in Bitumen[†]

T A N Y A   M A C P H E R S O N , [‡]
C H A R L E S   W .   G R E E R , [‡]   E D W A R D   Z H O U , [‡]
A L I S O N   M .   J O N E S , [‡]   G E S I N E   W I S S E , [‡]
P E T E R   C .   K .   L A U , [‡]   B R U C E   S A N K E Y , [§]
M A T T H E W   J .   G R O S S M A N , [‖]   A N D
J A L A L   H A W A R I * , [‡]

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A combined solid-phase microextraction/GC-MS analytical technique was used to monitor the formation of metabolites in the biodesulfurization of the bitumen model organosulfur compounds, dibenzothiophene (DBT) and the dialkylated derivative 4,6-diethyldibenzothiophene (DEDBT), by *Rhodococcus* sp. strain ECRD-1. In the case of DBT, the following metabolites were detected: DBT 5-oxide (sulfoxide), DBT 5,5-dioxide (sulfone), dibenz[*c,e*][1,2]oxathiin 6-oxide (sultine), dibenz[*c,e*][1,2]oxathiin 6,6-dioxide (sultone), and the end product, 2-hydroxybiphenyl (2-HBP), whereas, with DEDBT, 4,6-DEDBT 5-oxide, 4,6-diethyldibenz-[*c,e*][1,2]oxathiin 6-oxide (sultine), and 2-hydroxy-3,3′-diethylbiphenyl (HDEBP) as the final product, were identified. A time course study for the formation and disappearance of DBT and DEDBT metabolites was used to construct desulfurization pathways, which in both cases, involved the formation of the corresponding sulfoxides.

Introduction

Solid-phase microextraction (SPME) is a solventless and rapid extraction technique that uses polymer-coated fibers for the extraction of organic compounds from an aqueous or gaseous phase sample followed by thermal desorption in the injection port of a gas chromatograph for subsequent detection and quantification. The technique is known for its speed and sensitivity which enables detection in the microgram per liter range (*1–5*).

Although SPME has been widely used for the trace analysis of organic compounds in several aqueous based matrixes, little is known on the applicability of the technique for monitoring organic biotransformations in biological matrixes (*6*). Until recently, lengthy sample preparation and separation techniques (*e.g.*, liquid/liquid extraction followed by chromatographic cleanup procedures) were required to isolate and identify intermediates produced during biotransformation processes (*5, 7*). When such intermediates are formed in trace amounts, the previously mentioned traditional techniques are not practical or fast enough for their detection, thus, leading to the loss of valuable information on the transformation pathways.

The main objective of this study was to apply SPME in combination with GC/MS to identify metabolites formed during desulfurization by *Rhodococcus* sp. strain ECRD-1, of two model thiophenic compounds commonly found in fossil fuel, i.e., DBT and DEDBT (*8, 9*). In Canada, reserves of fossil fuel such as bitumen are extremely large, but the fuel value is low due in part to the high organic sulfur content, which upon combustion, can release sulfur dioxide into the atmosphere causing acid rain. To increase the fuel value without causing harm to the environment, the crude oil must be desulfurized without an excessive reduction of its calorific value (*10–12*). Several studies have described products that are generated from these model compounds using different microorganisms under both aerobic and anaerobic conditions. For example, through extensive GC/FTIR/MS analysis, Olson et al. (*13*) reported the formation of key metabolites including dibenz[*c,e*][1,2]oxathiin 6-oxide (sultine) and dibenz-[*c,e*][1,2]oxathiin 6,6-dioxide (sultone) during the desulfurization of DBT by *Rhodococcus* sp. strain IGTS8.

The present work describes the utility of SPME/GC-MS in the identification of key metabolites formed during the desulfurization of DBT and DEDBT using *Rhodococcus* sp. strain ECRD-1. A time profile of the appearance and disappearance of the detected metabolites was used to elucidate the desulfurization pathway of these model organosulfur compounds by *Rhodococcus* sp. strain ECRD-1.

Materials and Methods

Dibenzothiophene (DBT) and DBT 5,5-dioxide (sulfone) were from Aldrich, (Milwaukee, WI). DBT 5-oxide (sulfoxide) was from ICN Biomedicals, Inc., (High Wycombe, U.K.). Dibenz-[*c,e*][1,2]oxathiin 6-oxide (DBT-sultine), dibenz[*c,e*][1,2]-oxathiin 6,6-dioxide (DBT-sultone), 4,6-diethyl dibenzothiophene (DEDBT), 3-methyl-dibenzothiophene (MDBT), and 4,6-diethyl dibenzothiophene 5,5-dioxide (DEDBT-sulfone) were from Exxon, NJ. The 2-hydroxybiphenyl (2-HBP) was from Sigma (St. Louis, MO). *Rhodococcus* sp. strain ECRD-1 was obtained from the American Type Culture Collection (ATCC 55305).

Conditions for the biodesulfurization study. *Rhodococcus* sp. strain ECRD-1 was grown in a minimal salts medium (MSM) which contained 0.4 g of KH_2PO_4, 1.6 g of K_2HPO_4, 1.55 g of NH_4Cl, 0.165 g of $MgCl_2 \cdot 6H_2O$, 0.09 g·$CaCl_2 \cdot 2H_2O$, 5 g of sodium acetate, and 5 g of glucose/L of distilled water (pH 7.0). After autoclaving, the MSM received 1.0 mL of Pfennig's vitamins, 5.0 mL of Modified Wolfe's minerals, and 0.5 mg of $Na_2WO_4 \cdot 2H_2O/L$ (Pfennig's vitamins was composed of 50 mg of ρ-aminobenzoic acid, 50 mg of vitamin B-12, 10 mg of biotin, and 100 mg of thiamine per liter of distilled water). Modified Wolfe's minerals was composed of 1.5 g of nitrilotriacetic acid, 5.1 g of $MgCl_2 \cdot 6H_2O$, 0.66 g of $MnCl_2 \cdot 2H_2O$, 1.0 g of NaCl, 1.0 g of $FeCl_3 \cdot 6H_2O$, 0.1 g of $CaCl_2 \cdot 6H_2O$, 0.01 g of $CuCl_2 \cdot 6H_2O$, 0.08 g of $ZnCl_2$, 0.05 g of $AlCl_3$, and 0.04 g of $Na_2MoO_4 \cdot 2H_2O/L$ of distilled water (pH 6.5). The sulfur substrate, DBT or DEDBT, was added as a sterile solution in HPLC-grade ethanol to give a final amount added to each flask of 10 mg/L. The amounts added were in excess of the aqueous solubility to ensure that substrate did not become limiting during the assay. Cells from a plate culture were transferred to a 10 mL volume of MSM and
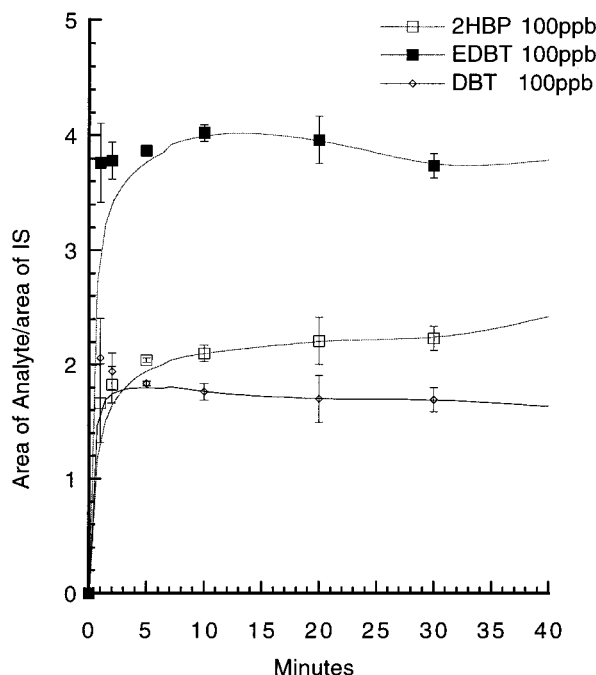

FIGURE 1. Representative adsorption isotherms of DBT, EDBT, and 2-HBP using polyacrylate SPME. IS is the internal standard 3-methyl-dibenzothiophene (MDBT) (100 ppb).
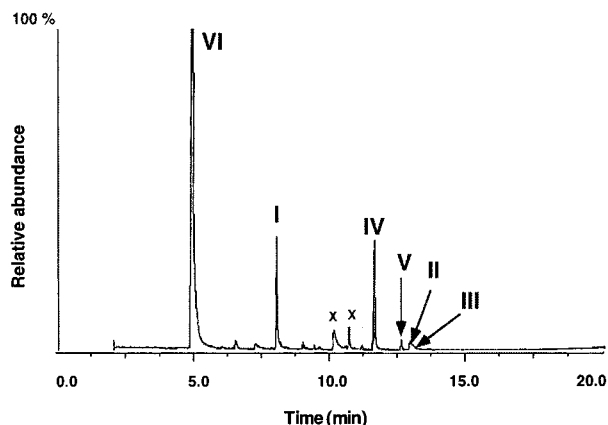

FIGURE 2. A typical SPME/GC/MS total ion chromatogram of biodesulfurization of DBT by *Rhodococcus* sp. strain ECRD-1. The metabolites are identified as follows: DBT (I), DBT 5-oxide (II), DBT 5,5-dioxide (III), DBT-sultine (IV), DBT-sultone (V), and 2-HBP (VI). x is unidentified.
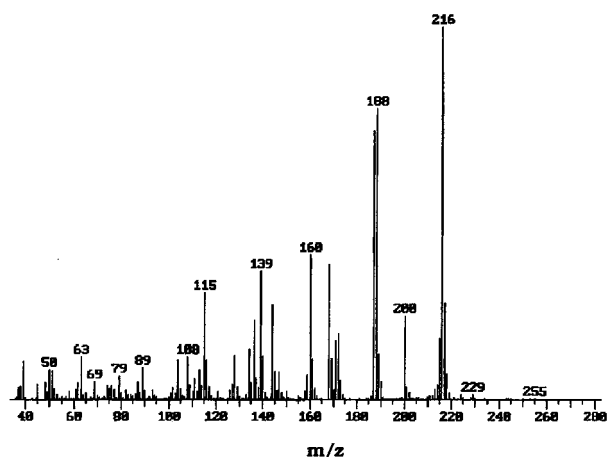

FIGURE 3. A typical SPME/GC/MS chromatogram of the metabolite dibenzo[*c*,*e*][1,2]oxathiin 6-oxide (sultine) formed during the biodesulfurization of DBT by *Rhodococcus* sp. strain ECRD-1.

incubated at 27 °C on a shaker at 240 rpm for 4 days. When a large volume of washed cells was required, 500 mL of starting culture was used. The cells were harvested from the culture medium, washed with phosphate buffer, and resuspended in MSM to perform a growing cell assay in 1 L Erlenmeyer flasks incubated at 240 rpm and 27 °C. Control flasks containing autoclaved cells were incubated under the same conditions to determine if any degradation of the substrate occurred abiotically. The cultures were sampled at intervals by removing an aliquot (2 mL) for SPME/GC-MS analysis and for the determination of the OD_{600} in a UV–vis spectrophotometer.

Solid-Phase Microextraction Followed by GC/MS. A fused silica fiber coated with an 85 μm polyacrylate polymer (Supelco, Bellefonte, PA) was conditioned by placing it inside the injection port of a GC/MS at 300 °C until a blank background was produced (about 2 h). At each sampling time, 2 mL aliquots of the cell suspension were acidified with H_3PO_4 (pH 2) and filtered with a Millex-HV 0.45 μm filter to remove cells and undissolved substrate. Analytes were adsorbed directly from the MSM filtrate onto the fiber and then thermally desorbed inside the GC injector for analysis by GC/MS. Thermodynamic equilibrium for the partitioning of DBT and its final metabolite 2-HBP between the SPME sorbent and the aqueous phase was achieved in less than 20 min (Figure 1). A 20 min adsorption time followed by a 10 min desorption were found appropriate for reproducible analyses. Recovery was determined using 4-ethyl DBT (85%) as the recovery standard. The response for both DBT and 2-HBP was linear ($R = 0.998$ and 0.997, respectively), over the following concentrations: 20, 50, 100, 200, 400, and 800 ppb.

A time study, to monitor the formation and disappearance of metabolites during desulfurization, was carried out as follows: culture samples, prepared as described above, were taken at $T = 0$ and at 20 min and then at either 30 or 40 min intervals for the first 6 h followed by samplings at 24 and 72 h.

A Varian GC/MS equipped with a Saturn II ion trap detector (transfer line temperature 220 °C) was connected to a DB-5 capillary column (15 m × 0.25 mm id × 0.25 μm film). A splitless injection was used for the first 6 min, followed by split injection (ratio 1/10) for the remainder of the GC program. The carrier gas was helium, and the temperature of the injection port was 250 °C. The initial oven temperature (100 °C) was increased at a rate of 7 °C/min to 210 °C, followed by 15 °C/min to a final temperature of 280 °C. The mass spectrum was obtained using an electron impact of 70 eV with a filament emission current of 30 mA, a mass range of 20–300 amu and a scan rate of 2 scans/s. Metabolites were identified by comparison with authentic standards, and the profile of their formation was followed by their area counts. Positive chemical ionization (PCI) with CH_4 gas was used to characterize the DEDBT metabolites that did not have authentic standards.

Results and Discussion

Metabolites from the Desulfurization of DBT by *Rhodococcus* sp. Strain ECRD-1. A typical SPME/GC/MS total ion chromatogram of DBT undergoing desulfurization, containing the starting material and several other intermediate products, is shown in Figure 2. One metabolite, observed at a retention time (rt) of 13.40 min, was identified by its mass spectrum and by comparison with a reference compound as DBT-sulfoxide. The metabolite showed a molecular ion at m/z 200 amu and a base peak at m/z 184 corresponding to the loss of an oxygen radical (16 amu). A second
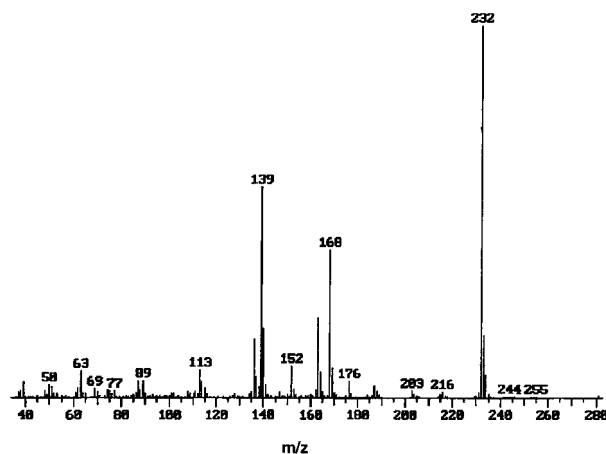
FIGURE 4. A typical SPME/GC/MS chromatogram of the metabolite dibenzo[c,e][1,2]oxathiin 6,6-oxide (sultone) formed during the biodesulfurization of DBT by *Rhodococcus* sp. strain ECRD-1.
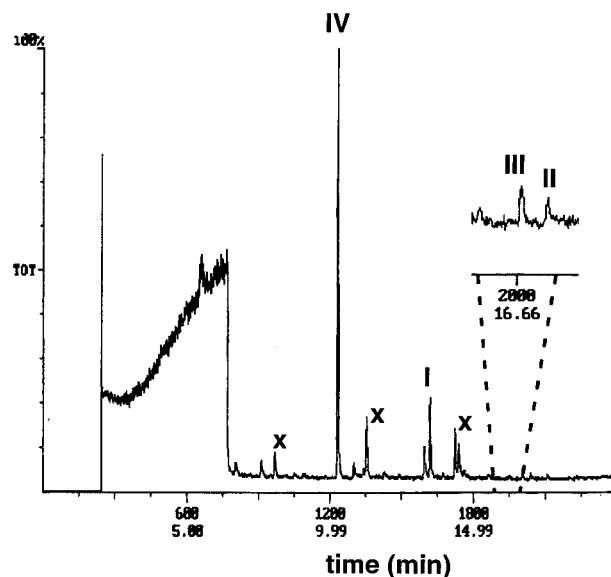

FIGURE 5. A typical SPME/GC/MS total ion chromatogram of the biodesulfurization of 4,6-DEDBT by *Rhodococcus* sp. strain ECRD-1. The metabolites are identified as follows: DEDBT (I), DEDBT 5-oxide (II), DEDBT-sultine (III), and HDEBP (IV). x is unidentified.
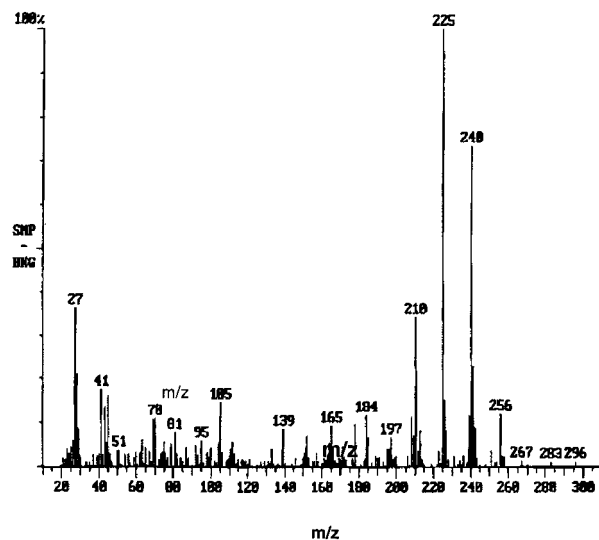

FIGURE 6. A typical SPME/GC/MS chromatogram of the metabolite DEDBT 5-oxide.

FIGURE 7. A typical profile of DBT metabolites detected in a resting/ growing cell assay with *Rhodococcus* sp. strain ECRD-1. Culture density (OD_{600}), accumulation of the final metabolite (2-HBP) (A), and the formation and disappearance of intermediates (B) were monitored for 72 h. Panel C expands the time axis for the first 6 h. Relative concentration was measured using DBT as an internal standard.

metabolite, observed at a rt of 13.50 min, was identified as DBT-sulfone, by its mass spectrum and by comparison with an authentic standard. The metabolite showed a molecular ion at m/z 210 amu, which was also the base peak. The presence of DBT-sultine at a rt of 12.23 min was confirmed by using an authentic standard. Also, the highest observed mass ion, m/z ratio of 216, corresponded to the molecular ion of DBT-sultine. There was another mass fragment at m/z 200 corresponding to the loss of an oxygen radical (16 amu) and the base peak appeared at m/z 187 (see Figure 3). DBT-sultone, with a rt of 13.24 min, was also confirmed by using an authentic standard. The highest mass fragment ion observed was 232 amu and was also the base peak (see Figure 4). The final product, 2-hydroxybiphenyl, was observed at a rt of 5.23 min and was identified with a standard compound. The observation of the highest fragment ion and base peak at a m/z 170 confirmed that the molecular weight was the same as that of the standard. As Figure 2 shows, DBT-sultine, DBT-sultone, and 2-HBP have their peaks highly resolved and pose no identification problem. DBT-sulfoxide and DBT-sulfone were found severely over-lapped and were identified by a selective ion retrieval (SIR)

method using the base peak of the sulfoxide (m/z 184 amu) and the molecular ion (m/z 216 amu) of the sulfone.

Metabolites from the Desulfurization of DEDBT by *Rhodococcus* sp. Strain ECRD-1. Figure 5 represents a typical SPME/GC-MS total ion chromatogram of DEDBT undergoing biodesulfurization. One metabolite, observed at a rt of 16.57 min, was tentatively identified as 4,6-diethyl dibenzothio-phene-sulfoxide (4,6-DEDBT 5-oxide) based on its mass spectrum (Figure 6). For example, the highest observed mass fragment (256 amu) was found to represent the molecular weight (m/z 256) of the suspected metabolite. Loss of an oxygen radical (16 amu), gave the corresponding thiophene (m/z 240) which fragmented in a fashion similar to the parent

m/z = 272   - H →   m/z = 271

- SO

m/z = 224   - CO

- H ←   m/z = 244
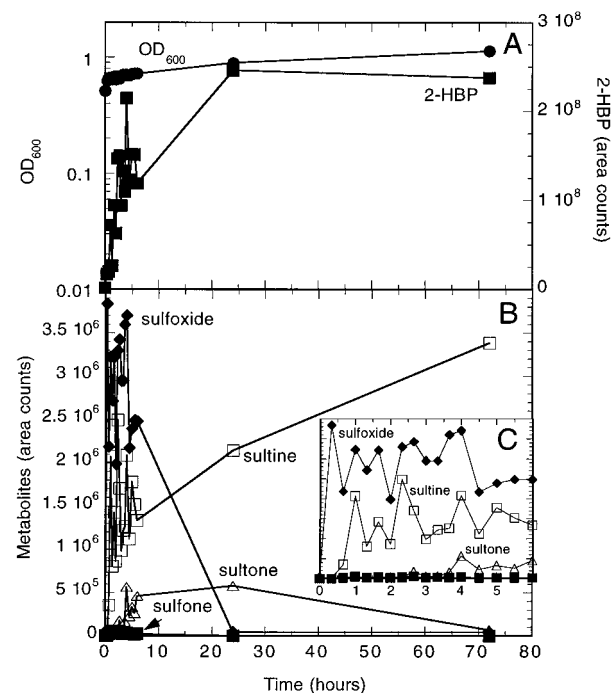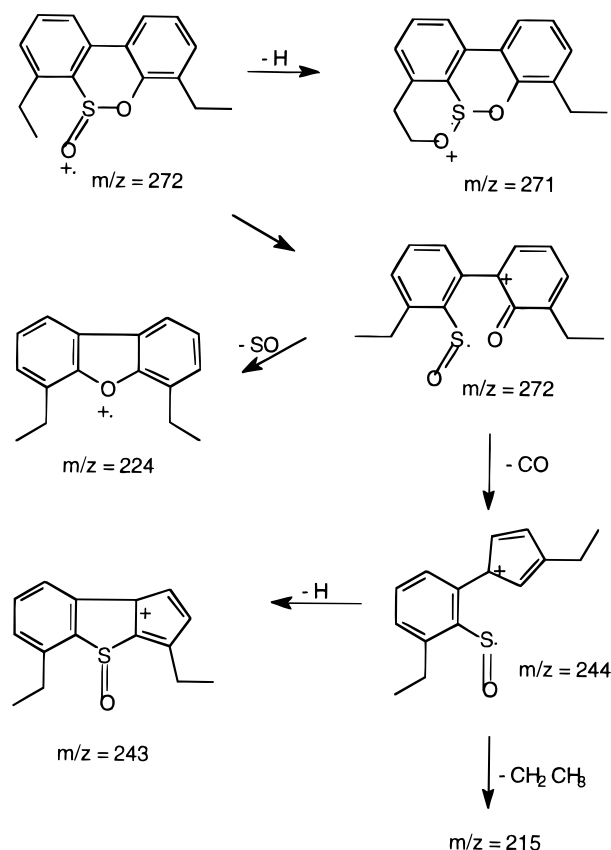
m/z = 243   - CH$_2$CH$_3$

m/z = 272

m/z = 215

FIGURE 8. A typical profile of DEDBT metabolites detected in a resting/growing cell assay with *Rhodococcus* sp. strain ECRD-1. Culture density (OD$_{600}$), accumulation of the final metabolite (HDEBP) (A), and the formation and disappearance of intermediates (B) were monitored for 9 days.

DEDBT compound. Other ion fragments observed at m/z 225 corresponded to the loss of both a CH$_3$ and O radical, while a fragment ion at m/z 210 corresponded to the loss of an additional CH$_3$- radical.

A second metabolite observed at a rt of 16.40 min was also tentatively identified as the sultine derivative of DEDBT, 4,6-diethyldibenz[*c,e*][1,2]oxathiin 5-oxide, identified from its mass fragmentation pattern (Scheme 1). The highest observed mass ion at m/z 272 provided evidence of the molecular weight of the metabolite (m/z 272 amu). A fragment ion was observed at m/z 256 and was attributed to the loss of an O radical (16 amu). There were also fragment ions at m/z 271, 224, 244, and 243 and a base peak at m/z 215, corresponding to the loss of a H atom, SO, CO, CO + H, and CO + CH$_2$CH$_3$ radicals, respectively.

The final product was observed at a rt of 10.15 min and was tentatively identified from its mass spectrum and mass fragmentation pattern as 2-hydroxy-3,3'-diethylbiphenyl (HDEBP). It gave a molecular ion at m/z 226 and had a base peak at m/z 211 and a prominent fragment ion at 197 amu corresponding to the loss of a CH$_3$ and a CH$_2$CH$_3$ group, respectively. The mass fragmentation pattern observed for the HDEBP product is similar to that reported earlier by Lee et al. (*9*).

Time Profiles of Metabolites Detected during the Growth of Strain ECRD-1 on DBT and DEDBT: Metabolic Pathways. After establishing the suitability of SPME/GC-MS for the direct detection of metabolites formed during biodesulfurization, a growing cell assay was set up with strain ECRD-1 and DBT or DEDBT to look for the formation and disappearance of the corresponding metabolites with time.

In the case of DBT, growing cell assays with a relatively high initial OD$_{600}$ (0.9) were used to produce sufficient amounts of metabolites for time profiling. The mass rang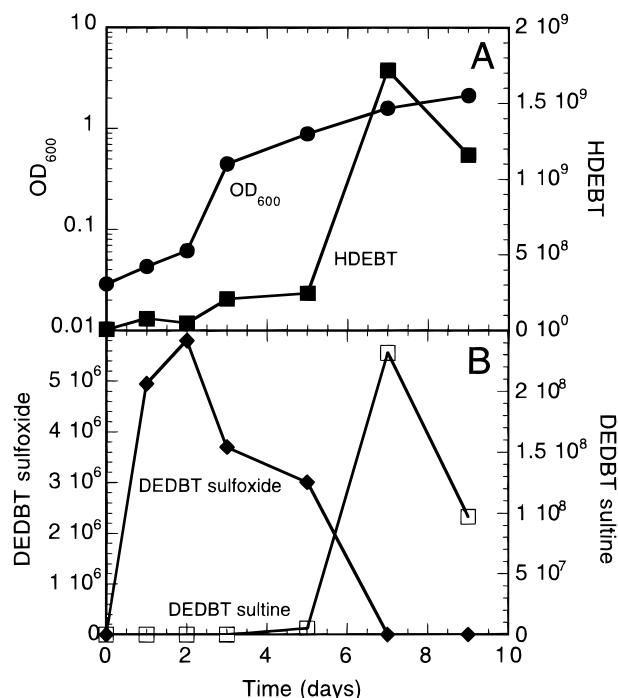e 20−300 amu was scanned repeatedly every 0.5 s to give a total ion current chromatogram for each sample, and the metabolite peaks were integrated. Selected ion retrieval (SIR) was utilized to retrieve the mass ions of the base peak of DBT-sulfoxide (m/z 184) and of the molecular ion of DBT-sulfone (m/z 216) for quantitation. The peak area integration counts were graphed to determine the appearance and disappearance of the DBT metabolites over the course of the experiment (Figure 7).

DBT-sulfoxide first appeared at 20 min, and then it decreased rapidly to little more than a trace after 24 h (Figure 7, panels B and C). DBT-sulfone concentrations remained low throughout the experiment (Figure 7). However, the raw data indicated that the sulfone first appeared in the sample after 1 h. DBT-sultine appeared at 1 h and continued to accumulate for the duration of the experiment. DBT-sultone first appeared at 3 h and 40 min and didn't decrease until 72 h (Figure 7B). The concentration of the end-product, 2-HBP, increased rapidly and reached a plateau after 24 h (Figure 7A). The SPME/GC-MS data shown in Figure 7 does not include DBT because the amount of DBT added to the culture medium at $t = 0$ was in excess of its water solubility by roughly 2 orders of magnitude. Consequently, the treated culture medium was first filtered to eliminate undissolved DBT for subsequent analysis by SPME. The principle behind the performance of the SPME analytical technique is that the target analyte must first achieve a thermodynamic equilibrium in its distribution between the polymeric coating of the SPME fiber and the bulk aqueous phase (see Figure 1).

The data indicate a stepwise metabolism of DBT showing that DBT-sulfoxide was the first metabolite formed. The second metabolite, probably DBT-sulfone, seemed to be converted rapidly to DBT-sultine, the acid rearranged product of the corresponding sulfinic acid, and this was followed by the appearance of DBT-sultone, the acid rearranged product of the corresponding sulfinic acid. 2-HBP was the final product, and its final concentration was 2 orders of magnitude higher than any other metabolite.

SCHEME 2. Proposed Metabolic Pathway of Biodesulfurization of DBT by *Rhodococcus* sp. Strain ECRD-1[a]
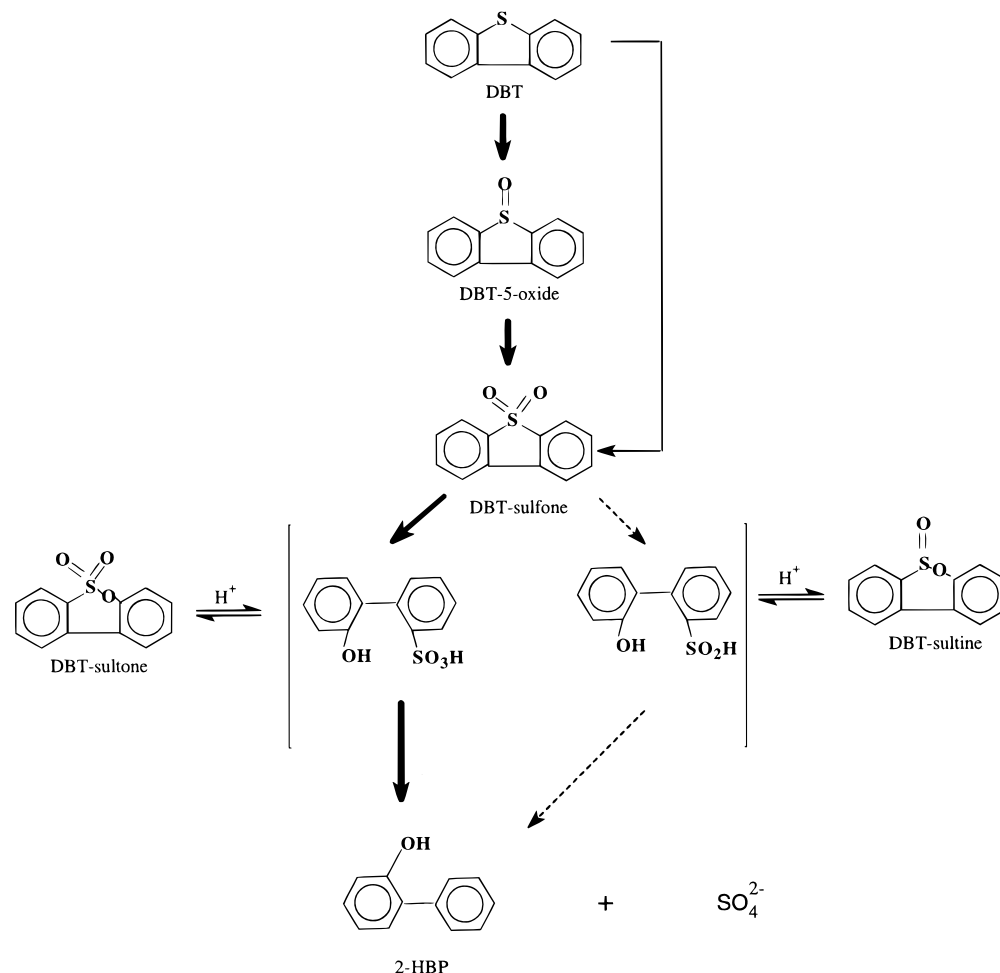
[a] The bold arrows represent the actual sequential conversion observed by the SPME/GC-MS time study which resembles the theoretical 4S pathway (*13*). The dashed arrow represents the possible second route to 2-HBP via the sulfinic acid. The bracketed Intermediates (2-hydroxybiphenyl sulfinic acid and 2-hydroxybiphenyl sulfonic acid) were not observed directly by SPME, but were inferred from the detection of their acid (pH 2) cyclicized derivatives, DBT-sultine and DBT-sultone.

Several studies have described the use of *Rhodococcus* sp. strain IGTS8 to desulfurize DBT to 2-HBP (*14−20*). Although there is a general consensus that desulfurization of DBT to 2-HBP proceeds in a stepwise fashion, there seems to be a variation among literature reports on the nature of the metabolites and the order in which they appear. Piddington et al. (*15*) reported that DBT is first converted to the corresponding sulfone, which in turn is desulfurized to produce 2-HBP as the end product. Lei and Tu (*18*) isolated an enzyme from the same strain that catalyzes the conversion of DBT to the corresponding sulfoxide and subsequently to the sulfone. Whereas, Olson et al. (*13*) and Denome et al. (*16*) reported that the desulfurization of DBT by the same *Rhodococcus* sp. IGTS8 produced several intermediates including DBT-sulfoxide, DBT sulfone, 2′-hydroxybiphenyl-2-sulfonic and 2′-hydroxybiphenyl-2-sulfinic acid before producing 2-HBP. All four intermediates detected by Olson et al. (*13*), i.e., DBT-sulfoxide, DBT-sulfone, the sultone, and the sultine were detected by the present SPME study. 2′-Hydroxybiphenyl-2-sulfonic acid and 2′-hydroxybiphenyl-2-sulfinic acid were not observed as acids but as the corresponding cyclicized derivatives, i.e., the sultone and the sultine, because of the acidic conditions (pH 2) employed in preparing the sample (*13*).

The time profile showing the relationship among various detected intermediates is best described in Scheme 2, which closely resembles a hypothetically constructed pathway, known as the 4S desulfurization pathway (*13*). For example,

the SPME data showed the following sequence, DBT → DBT-sulfoxide → DBT-sulfone → DBT-sultine → DBT-sultone → 2-HBP, whereas the 4S pathway depicts desulfurization to proceed through the following sequence, DBT, DBT-sulfoxide, DBT-sulfone, DBT-sulfonate (a precursor to the corresponding sultone), and finally 2-HBP (*13, 15*).

The genes that have been cloned to date from the desulfurization pathway of IGTS8 account for the production of DBT-sulfoxide, DBT-sulfone, 2′-hydroxybiphenyl-2-sulfinate (HBPS), and 2-HBP (*17*). Further investigations will be required to determine if other gene products are required for complete expression of the desulfurization pathway.

A time course of metabolites was also obtained for the desulfurization of the sterically hindered DBT analogue, DEDBT, by strain ECRD-1 (Figure 8). Although there was some similarity to the DBT metabolite profile, some interesting differences were observed. The most obvious difference was the length of time required before the metabolite concentrations peaked, typically days instead of hours. This may be due to the growing cell assay conditions used in the present study, starting with cells at a low optical density ($OD_{600} = 0.029$), or it may have been caused by a steric effect from the two bulky ethyl groups on the aromatic rings of the substrate. The other obvious difference was the complete absence of DEDBT-sulfone and DEDBT-sultone, although traces of the sulfone derivative were observed when using an enrichment culture.

SCHEME 3. Proposed Metabolic Pathway for the Biodesulfurization of 4,6-DEDBT by *Rhodococcus* sp. Strain ECRD-1[a]
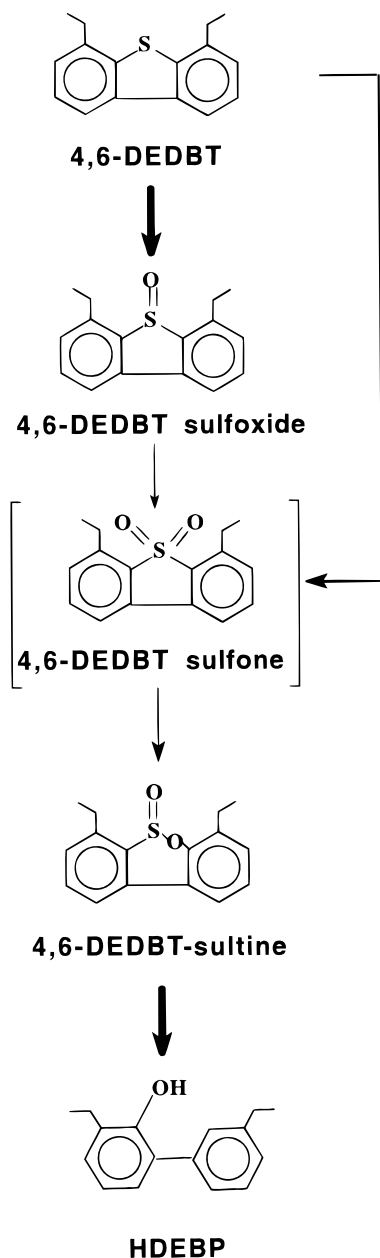
4,6-DEDBT

4,6-DEDBT sulfoxide

4,6-DEDBT sulfone

4,6-DEDBT-sultine

HDEBP

[a] The bold arrows represent the actual sequential conversion observed by the SPME/GC-MS time study.

The metabolite, tentatively identified as DEDBT-sulfoxide, peaked at 2 days, after which it decreased and was no longer detected after 7 days. After 5 days, as the DEDBT-sulfoxide concentration decreased, a second metabolite, suggested to be DEDBT-sultine, appeared and peaked at day 7. The end-product, identified as 2-hydroxy-3,3′-diethylbiphenyl (HDEBP), was detected at a low concentration at $T = 0$ and continued to accumulate for the first 7 days. The DEDBT-sulfone might have been formed in trace amounts that converted rapidly to the next intermediate, as was the case with DBT-sulfone. In an earlier study, Lee et al. (*9*) reported the formation of DEDBT-sulfoxide, DEDBT-sulfone, and HDEBP from the desulfurization of DEDBT using the same strain. From the preceding discussion, the biodesulfurization of DEDBT by strain ECRD-1 can be reasonably profiled as shown in Scheme 3.

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