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Desulphurization of dibenzothiophene by bacteria

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Four out of 187 strains, from enrichment cultures of dibenzothiophene (DBT), grew on DBT or thiophene 2-carboxylate as S sources. The four isolates, presumptively identified as *Agrobacterium* sp., *Xanthomonas* sp. and *Corynebacterium* spp., individually and together desulphurized DBT, producing 2-hydroxybiphenyl and sulphate.

Key words: Bacterial desulphurization, dibenzothiophene, organic sulphur.

Coal, one of the world's most abundant fossil energy resources, is mainly used to produce electric power, although its combustion causes serious environmental problems due to its S content. During combustion, several gases containing oxides of S are given off into the atmosphere, including SO_2 which is oxidized to SO_3 and H_2SO_4.

Organic S in coal is covalently attached to a molecular matrix as thiols, sulphides and thiophenes (Kargi 1982), and is difficult to remove physically or chemically, in contrast to pyrite or inorganic S. Microbial desulphurization systems can selectively remove organic S from coal as sulphate at low energy cost, prior to combustion. The complex structure of coal and limitations in the relevant analytical techniques present technical problems in analysing biodesulphurization. For example, organic S is measured indirectly as the difference between total S and pyritic S, whereas all sulphate in the cultural media is considered a product of organic S oxidation, with no correction for any free sulphate which may be present in the raw material.

Accordingly, thiophenes, especially dibenzothiophene (DBT), which is the most abundant one (Monticello & Finnerty 1985), have been selected to study microbial desulphurization because they represent 40 to 60% of the organic S in coal (Olson & Kelly 1991).

Several bacteria capable of metabolizing DBT have already been isolated (Nakatani *et al.* 1968; Yamada *et al.* 1968; Hou & Laskin 1976; Laborde & Gibson 1977; Monticello *et al.* 1985; Foght & Westlake 1988), but no desulphurization was achieved by their use, since S was retained in the metabolites of the DBT degradation. Metabolic studies on DBT degradation have been carried out with *Pseudomonas* (Kodama *et al.* 1973; Hou & Laskin 1976; Foght & Westlake 1988) and *Beijerinckia* strains (Laborde & Gibson 1977). The degradation can produce heterocyclic organic S compounds which are potentially carcinogenic (Monticello & Finnerty 1985). There have been few recent studies on the biodesulphurization of DBT by a pathway in which S is oxidized to free sulphate (Kilbane 1990; Omori *et al.* 1992; Gallagher *et al.* 1993).

DBT desulphurization and biotransformation, by several bacteria capable of growing on organic S, were the subjects of the present study.

Materials and Methods

Media and Growth Conditions

Basal salt (BS) broth contained (g/l): KH_2PO_4, 1; $MgCl_2.6H_2O$, 1; NH_4Cl, 2; $CaCl_2$, 0.001; and $FeSO_4.7H_2O$, 0.001. For enrichment and isolation of strains, BS broth was supplemented with 1 g DBT/l (BSD) and, where indicated, 0.5 g yeast extract/l (BSDY). The final pH was 7.0. Liquid cultures were grown at 30°C on a gyratory shaker at 150 rev/min. Agar (2% w/v) was added to all broths as required. Isolated strains were maintained in Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) (Difco).

Enrichment, Isolation and Identification of DBT-utilizing Bacteria

Four soil samples were collected from a coal-mining area and five from old residues of coal mixed with soil at Berguedà (Catalonia, Spain). Samples were suspended (10% w/v) in 100 ml BSDY broth. Every 4 to 5 days, a subsample (10 ml) of the culture was

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inoculated into fresh medium, over a period of 18 days. At each step, plates of BSD agar were streaked. Isolated colonies were obtained on BSD agar plates or BS agar containing 0.15 g thiophene-2-carboxylate (T2C)/l (BST). Strains which grew on both media were inoculated into BSDY broth and selected by their relatively rapid growth and the amount of sulphate produced after 10 days' incubation. These strains were then grown on BST containing 0.5 g yeast extract/l (BSTY); the cells were harvested and inoculated successively in the same medium, with the yeast extract concentration decreasing from 0.5 to 0.025 g/l. The selected strains were characterized morphologically and biochemically and identified according to Krieg & Holt (1984). The API 20 NE series of tests (Biomérieux, France) were used on some isolates.

Desulphurization Experiment

The selected strains were plated on BS agar containing 1 g glucose and 0.1 g DBT per litre (BSDG). From these plates, isolates were grown, in duplicate, in BSDG broth without DBT (BSG), centrifuged at 12,000 × ***g*** and re-inoculated into BSG containing 0.001 g $FeCl_3.6H_2O$/l instead of the $FeSO_4$, i.e. a medium without a source of S. After 3 days, the cells were harvested, washed twice in saline solution (8.5 g NaCl/l) and inoculated again into 500 ml BSG broth. If the cells did not grow, 40 ml was withdrawn from each flask and divided equally between two flasks. Distilled water (0.1 ml) was added to one and 0.4 M Na_2SO_4 (0.1 ml) to the other. These two flasks were used as negative and positive controls of growth, respectively. DBT (0.05 g/l) dissolved in 0.1% (v/v) dimethylformamide was added to the remaining 460 ml. All incubations were for 37 days. During growth, samples were removed periodically for measurement of bacterial growth, sulphate and DBT and its metabolites. All glassware was scrupulously cleaned with HCl to prevent spurious growth on contaminant S.

Extraction of DBT and its Metabolites

Samples (10 ml) of cultures were acidified with 0.1 M HCl and extracted with an equal volume of dichloromethane. Phenanthrene (0.05 g/l) was used as internal standard. The mixture was mixed for 15 min and the organic fraction collected. After evaporation of the dichloromethane, the residue was redissolved in 0.5 ml dichloromethane, and analysed by GC.

Analytical Methods

Quantification of DBT and its possible metabolites, such as DBT sulphoxide, DBT sulphone, 2-hydroxybiphenyl (2PP), biphenyl and 2,2'-biphenol (2,2'-BP), was carried out by GC on a Hewlett Packard gas chromatograph (Model HP 5890A), using a HP-1 semi-capillary polymethyl siloxane column (5 m × 540 μm) and a flame-ionization detector. The temperature gradient was from 110°C to 170°C at 5°C/min and the injector and detector temperatures were both 250°C. Helium was the carrier gas. Calibrations and quantifications were based on the pentadecane used as internal standard for the injection volume. When necessary, GC-MS, using a Hewlett Packard spectrometer (Model HP 5988A) a capillary column (25 m × 0.52 μm) and the same temperature programme, was performed. Mass spectra of commercial compounds and DBT sulphoxide, prepared as described below, were compared to those produced by bacterial cultures. TLC was carried out on silica gel containing a 254 nm fluorescent indicator, using heptane/acetone (1:1) as solvent (Faison *et al.* 1991). Products were detected by viewing under short-wave u.v. light and identified by comparison with standards. Glucose was measured enzymatically (Carrol *et al.* 1970). Bacterial growth during selection of strains was monitored by measuring the absorbance at 440 nm. The maximum values obtained were about 0.5. In desulphurization assays, growth was quantified by direct microscopic counts, using a Neubauer chamber. Soluble sulphate was measured in culture supernatants (12,000 × ***g***), by precipitation with $BaCl_2$, after stabilization by gelatin (3 g/l) (Tabatabai 1974).

Synthesis of DBT Sulphoxide

DBT sulphoxide was prepared as described by Gilman & Esmay (1952), i.e. oxidation of DBT with H_2O_2 in glacial acetic acid, and its identity was confirmed by GC-MS, TLC and determination of the melting point, which was 183.2°C.

Chemicals

All chemicals used were of reagent grade and were purchased from Merck, Aldrich Chemical Co. or Sigma.

Results

Isolation and Selection of Strains

From nine DBT enrichment cultures, 187 bacterial strains were isolated and these were reduced to 33 according to their growth on BSD or BST agar plates. Twelve strains were selected from the 33, based on their relatively rapid growth and sulphate production in BSD broth, and then four of these (MC401, MC402, MC501 and MC701) were selected for further study because of their relatively rapid growth on BST containing 0.05 g yeast extract.

Desulphurization of DBT

Strains MC401, MC402, MC501 and MC701 were incubated individually and together in duplicate, in BSG broth. DBT was added to cultures when bacteria did not grow on BSG without a S source in 3 days. Cell-free BSG was incubated as a control.

All the bacterial cultures produced an extractable metabolite during exponential growth, with a GC retention time (RT) of 2.72 min, similar to that of authentic 2PP (Figure 1). This peak was not observed in the sterile control or in zero-time cultures. DBT, phenanthrene and pentadecane peaks were detected in all cultures, with RT of 5.80, 6.22 and 3.14 min, respectively. Another peak, with a RT of 1.62 min, appeared in all DBT-containing samples, including those from the sterile control. This compound is probably a thermal degradation product of DBT produced during analysis and has not yet been identified.

A minor component, with a RT of 11 min, appeared when cultures were incubated with DBT (Figure 1). It probably corresponds to DBT sulphone or DBT sulphoxide. On TLC analysis, only DBT ($R_f = 0.78$) was detected in all cultures at zero time, whereas DBT sulphoxide ($R_f = 0.44$) and 2PP ($R_f = 0.54$) were only detected after 24 or 30 days growth of the bacteria.

Growth and production of DBT and 2PP by strain MC501 are shown in Figure 2, and data obtained for all four strains and their mixture are shown in Table 1. 2PP

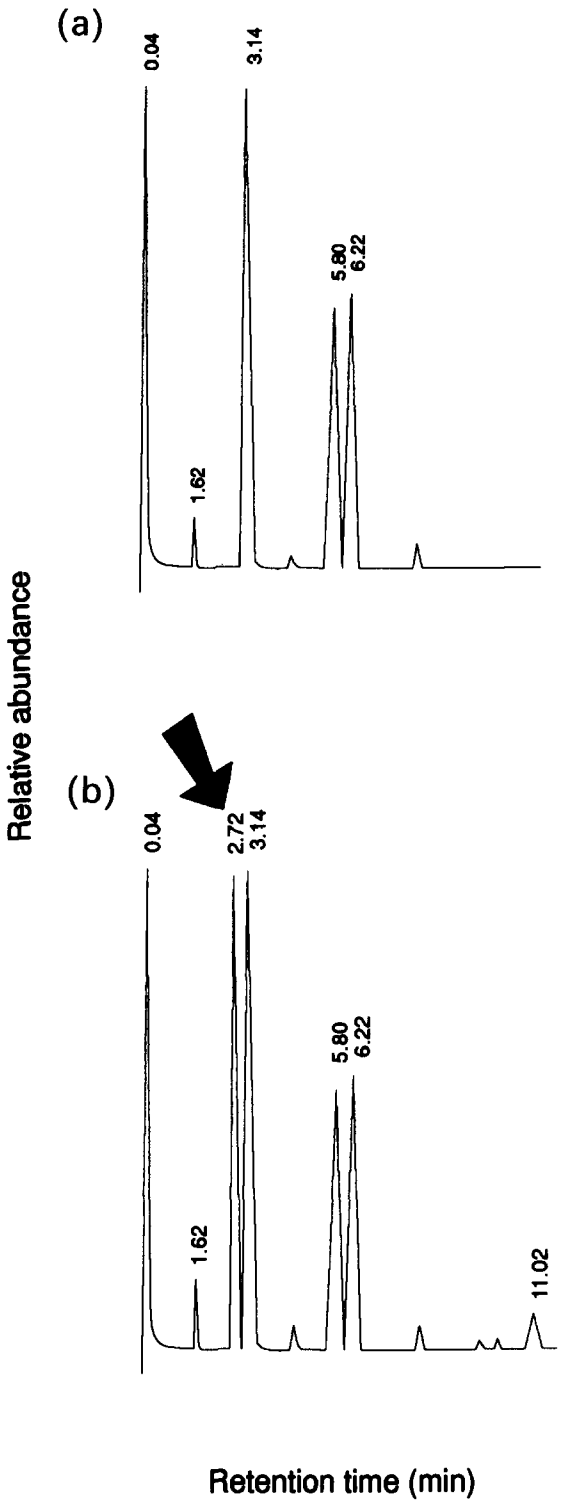

Figure 1. Gas chromatograms of strain MC501 grown in basal salt medium containing glucose (1 g/l) and dibenzothiophene (0.05 g/l) as sole sulphur source at (a) zero time and (b) after 24 days' incubation. Bacterial formation of 2 hydroxybiphenyl (2PP) is indicated by an arrow. Pentadecane (retention time 3.14 min) and phenantrene (retention time 6.22 min) were used as injection and extraction controls, respectively. GC of authentic dibenzothiophene without cells showed exactly the same pattern as (a).

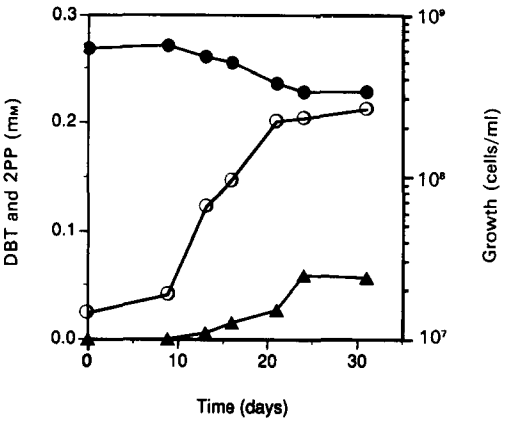

Figure 2. Growth (○), utilization of dibenzothiophene (DBT; ●) and formation of 2-hydroxybiphenyl (2PP; ▲) by strain MC501 in basal salt medium containing glucose (1 g/l) and DBT (0.271 mM).

reached peak concentrations after 24 to 29 days incubation, depending on the strain. A slight decrease in its concentration occurred in the stationary phase of growth with strains MC501 and MC401. The final concentration of glucose was less than 0.04 g/l for all strains, indicating that all of it was consumed, so some 2PP could have been used as a C source. Sulphate was not detected. A reasonable stoichiometry existed between residual DBT and the 2PP formed.

Strain MC501 and the mixed culture produced the highest 2PP concentrations. Mass spectra of the DBT sulphoxide produced by both of these cultures show the same molecular peak (184) as the compound synthesized in the laboratory (Figure 3); DBT sulphone, with a molecular peak of 216 (data now shown), was not detected in either culture. The 2PP produced by the bacteria also had the same molecular peak as that of the authentic compound (Figure 4). Therefore, it was concluded that strains MC401, MC402, MC501, MC701 degraded DBT by the so-called 4S pathway (Kilbane 1990), which includes four compounds containing S (DBT, DBT sulphoxide, DBT sulphone and sulphate). These strains desulphurize DBT with the formation of 2PP but not of biphenyl nor 2,2'-BP (Figure 5).

Identification of the Selected Strains

Morphological and biochemical characteristics of strains MC401 and MC402 suggested that they were *Corynebacterium* spp. (Table 2), whereas MC501 and MC701 were Gram-negative, aerobic, non-fermentative rods. The latter strains were tested using the API 20 NE series of 20 biochemical tests designed for use with non-enteric Gram-negative bacteria. On the basis of the results of these tests, MC501 and MC701 were presumptively identified as *Agrobacterium* and *Xanthomonas* spp., respectively (Table 2).

Gram stains of colonies from the mixed culture after 37 days incubation revealed a preponderance of Gram-negative rods, presumably the *Agrobacterium* MC501 and

Table 1. Growth of selected strains and formation of 2-hydroxybiphenyl(2PP) with dibenzothiophene (DBT) as S source.

Strain	Time (days)	Cell density (cells/ml)	2PP (mM)	Residual DBT* (mM)
MC401	29	$3.7 + 10^8$	0.043	0.190
MC402	28	$3.1 + 10^8$	0.043	0.222
MC501	24	$2.4 + 10^8$	0.059	0.228
MC701	24	$2.7 + 10^8$	0.039	0.217
Mixed culture (all four strains)	27	$2.8 + 10^8$	0.046	0.222

* The initial level of DBT was 0.271 mM.

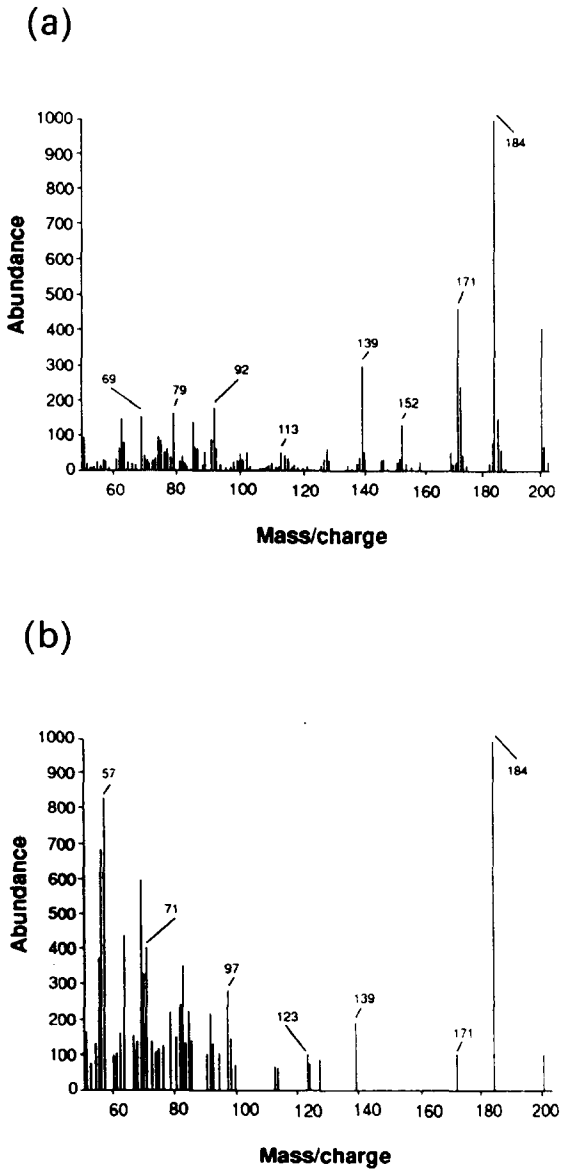

Figure 3. Mass spectra of (a) authentic dibenzothiopene sulphoxide (DBT sulphoxide) and (b) DBT sulphoxide produced by strain MC5O1 during DBT desulphurization.

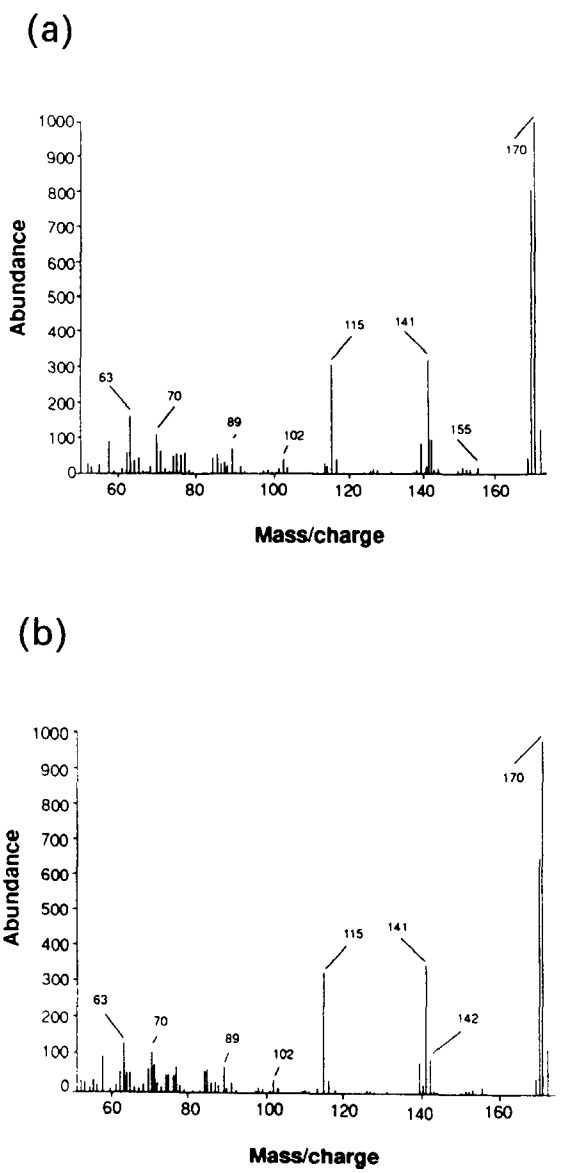

Figure 4. Mass spectra of (a) authentic 2-hydroxybiphenyl (2PP) and (b) 2PP produced by strain MC501 during dibenzothiophene (DBT) desulphurization.

Table 2. Characteristics of the selected strains.

Characteristic	Strains	
	MC401 and MC402	MC501 and MC701
Gram stain	+	−
Morphology	Irregular rod, coryneform, not mycelial,	Small rod
Spores	−	−
Motility	−	+
Aerobic	+	+
Fermentation of sugars	−	−
Catalase	+	+
Oxidase	−	−
Reduction of nitrate	−	−
Indole from tryptophan	−	−
Esculine hydrolysis	nd	+
Gelatine hydrolysis	nd	−
β-Galactosidase	nd	+
Utilization of:		
Glucose	+	+
Maltose	+	+
Mannose	nd	+
Mannitol	nd	+
N-Acetylglucosamine	nd	+
Caprate	nd	−
Adipate	nd	−
Malate	nd	+
Citrate	nd	+
Phenylacetate	nd	−

* In addition MC501 was urease positive and could utilize arabinose, mannitol and gluconate, whereas MC701 did not. On the other hand, MC701 hydrolized gelatine.
nd — Not determined.

Xanthomonas MC701, with a small proportion of Gram-positive coryneform bacteria. Oxidative Gram-negative bacteria are included in the same group as most soil and degradative microorganisms. i.e. as *Beijerinkia* (Laborde & Gibson 1977), *Pseudomonas* and *Acinetobacter* (Isbister & Kobylinski 1985). A strain of *Corynebacterium* which oxidizes DBT by the S pathway has recently been reported (Omori *et al.* 1992).

Discussion

The present biodesulphurization study was carried out using DBT as sole S source and glucose as the main C source, in order to leave the C structure of DBT unchanged, because it is desirable, from the point of view of coal desulphurization, that the S atom be removed specifically, to minimize energy loss. Bacterial growth was due to oxidation of DBT, since no growth was detected in its absence. DBT was stable at 30°C since no metabolites were detected as a result of abiotic degradation; a loss of DBT was observed in experiments with thermophilic *Sulfolobus* at 68°C (Constantí *et al.* 1992).

The results obtained from GC and TLC analyses suggested that the selected strains were able to transform DBT by the 4S pathway (Figure 5) and MS analysis of the degradation products confirmed this hypothesis. Once the cells had grown, the cultures became red in colour, but u.v. spectral analysis did not show soluble DBT metabolites containing S, such as 3-hydroxy-2-formyl-benzothiophene or 2-(3-hydroxy)-thianaphthenyl-2-oxo-3-butenoic acid, the coloured products of the oxidative pathway of DBT metabolism by *Pseudomonas jianii* (Kodama *et al.* 1973). It was therefore concluded that the present isolates do not degrade DBT by this pathway.

When biodesulphurization of DBT follows the 4S pathway, several aromatic compounds (biphenyl, 2,2′-BP and 2PP) are formed (Figure 5). Kilbane (1990) reported production of 2,2′-BP by strain IGTS7. In the present study, 2PP was formed by strains MC401, MC402, MC501 and MC701. This product was also reported during DBT degradation by *Pseudomonas* CB1 (Isbister & Kobylinski 1985), but the results were not reproducible (Agus *et al.* 1988). DBT degradation to sulphoxide and sulphone derivatives, without further desulphurization, has been reported (Mormile & Atlas 1988; Van Afferden *et al.* 1988; Stoner *et*

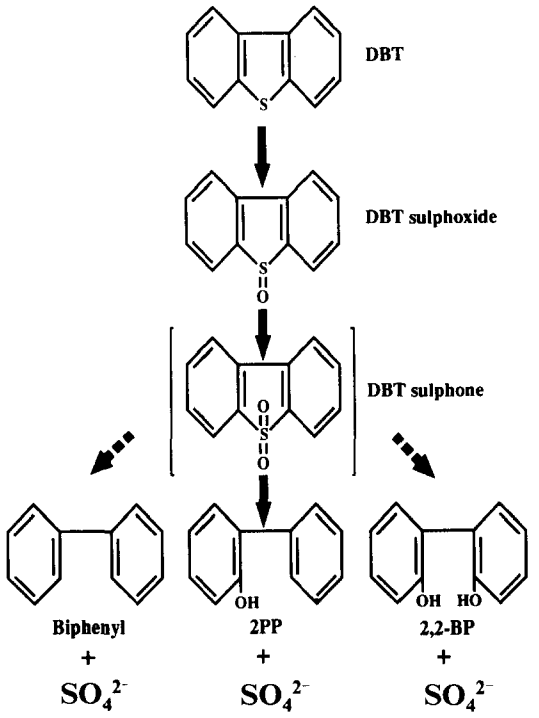

Figure 5. Proposed pathway (from Kilbane, 1990) for desulphurization of dibenzothiophene (DBT). The structure shown in brackets is proposed as an intermediate and has not been isolated. 2PP—2-Hydroxybiphenyl; 2,2-BP—2,2'-biphenol.

Afferden *et al.* (1990), was able to remove S from DBT with sulphate formation, with the aromatic carbon structure being reduced to benzoate.

According to the 4S pathway, sulphate should be released into the medium on DBT oxidation, but no significant accumulation was detected. It has been suggested that bacteria could reutilize the SO_4 derived from DBT as a source of S for growth (Omori *et al.* 1992), so that SO_4 production would then be almost insignificant. Nevertheless, we found small amounts of SO_4 (up to 0.02 mM) in more recent biodesulphurization experiments (data not shown), proving that organic S is oxidized to SO_4.

Since 2PP and sulphates seem to be the last products of DBT desulphurization by strains MC401, MC402, MC501 and MC701, no DBT mineralization to CO_2 occurred. In the case of DBT transformation, an organic compound without the S atom was formed, preserving its carbon structure. The recalcitrance of DBT to oxidation disappeared when no other S source was added to the medium.

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