DOE/PC/88855--T8

ENZYMATIC DESULFURIZATION OF COAL

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Sixth Quarterly Report

DynaGen Report No. 2477
DynaGen Project No. DOE-12
DOE Contract No. DE-AC22-88PC88855

Submitted to:

Mr. David Boron
Project Manager,
U.S. Department of Energy
P.O. Box 10940, Building 922
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Pittsburgh, Pennsylvania 15236-0940

Prepared by:

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DYNAGEN, INC.

December 14, 1989

Submitted by:

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Cambridge, Massachusetts 02139

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Section 1

INTRODUCTION

1.1 Review of Program Goals

Numerous studies are underway to develop biological processes for the removal of both mineral and organic sulfur from coal. To remove the organic sulfur which is covalently bound, various research groups are studying strains of bacteria and fungi which can be induced to utilize organic sulfur compounds as feedstocks.

A consideration of industrial scale-up and operational requirements indicates that microbial ingestion of sulfur may produce technical difficulties that can be circumvented by the use of extracellular (i.e., secreted) or purified enzymes rather than whole microbes. For example, a 20,000 ton/day coal process would require about 200 tons of microbes to achieve a 1 percent removal of organic sulfur. If this sulfur is incorporated into the microbe, the daunting task of separating the fuel from the sulfur-enriched organisms presents added cost and process requirements.

Our current efforts to develop clean coal technology center around solving processing problems through the use of enzymes rather than live organisms for chemical catalysis. Enzymes will not accumulate sulfur; they will not add significant bulk in processing; they will be selective in their activity; and they will be functional in organic solvents, alleviating the need for the addition of water to coal.

The question of catalyst specificity has not previously been sufficiently addressed in considering pre-combustion coal desulfurization. The energy of coal is held primarily in carbon-carbon bonds; the organic matrix also contains oxygen, sulfur, and nitrogen. When coal is used as a fuel, thermal energy is gained through the addition of oxygen to the organic molecules. Complete oxidation breaks all bonds to carbon, adding oxygens to produce carbon dioxide and inorganic oxides and acids such as H_2SO_4. The aim of pre-combustion desulfurization is to achieve selective oxidative cleavage

of sulfur-carbon bonds, releasing sulfuric acid. Ideally, this is accomplished without affecting the stability of the carbon-carbon bonds, thus preserving most of the energy content of the coal. A secondary issue is the disruption or "solubilization" of the coal matrix, deemed necessary by some to provide access of the catalyst to the sulfur sites. Little is actually known about the need for "solubilization" in coal treatment with various catalysts.

Our experimental approach focuses on the use of enzymes which catalyze the addition of oxygen to organic compounds. In tailoring the application of these enzymes to coal processing, we are particularly interested in ensuring that oxidation occurs at sulfur and not at carbon-carbon bonds. For example, in the model coal compound DBT which we use to evaluate processes, there are three specific types of oxidation sites: carbon-carbon bonds in the phenyl-rings; carbon-sulfur bonds in the thiophene ring; and sulfur itself. Previous studies with DBT have shown that the reaction most frequently observed in microbial oxidative pathways is one in which DBT is oxidized at ring carbons. These reactions, as we have said, are accompanied by a considerable decrease in the energy content of the compound.

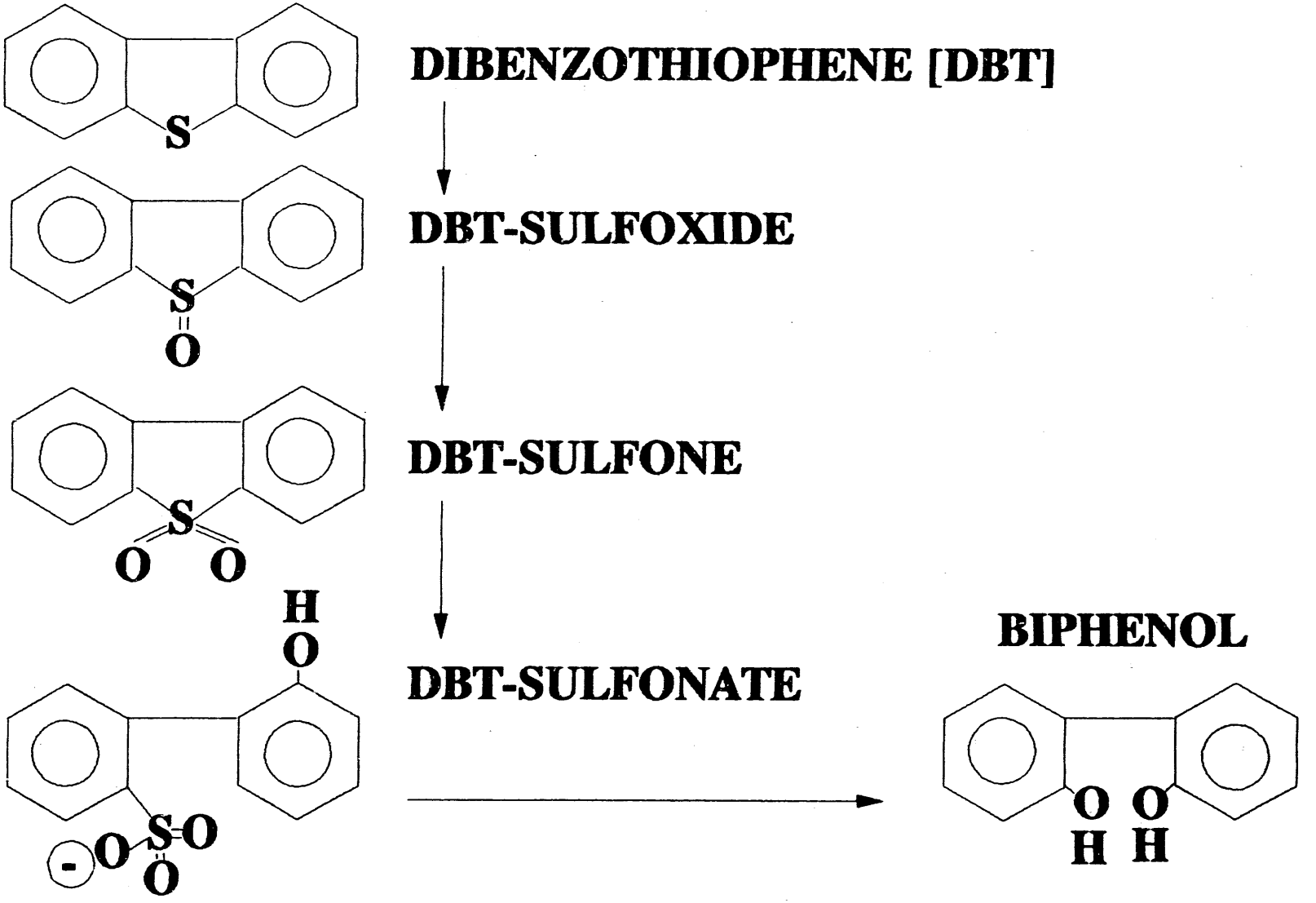
In addition, microbial pathways have been identified in which the sulfur atom is sequentially oxidized to sulfoxide, to sulfone, to sulfonate, and finally to sulfuric acid. This "4S" pathway of DBT oxidation, shown in Figure 1.1, adds one or two hydroxyls to the phenyl rings, but leaves them otherwise intact. In this case, the fuel value of the desulfurized compounds is largely retained. Identification of the multi-step ("4S") microbial reaction pathway has encouraged us to examine enzyme processes which will selectively catalyze oxidation at sulfur. We are evaluating the potential of commercially available enzymes to perform this function, and also seeking to isolate "4S" pathway enzymes from a microbial source which produces "4S" products.

1.2 Review of Earlier Results

Our technical progress in the first quarter can be summarized as follows. We worked with laccase and horseradish peroxidase in buffer and in aqueous organic solvents. After establishing the activity of our enzymes

Figure 1.1

DBT AND ITS SULFUR OXIDATION PRODUCTS



in buffer, many tests of activity against standard substrates in hydrated dioxane and hydrated DMF media were made. In both solvents, some evidence of activity against dibenzothiophene [DBT] was observed. We also investigated spectral and chromatographic methods of identification of the compounds in the "4S" pathway.

In the second quarter, the screening of media for the enzyme reactions with DBT was expanded. Changes in buffer were examined and several more hydrophobic solvents were utilized. An extensive amount of data was obtained by gas chromatography, utilizing a method which identifies the products of the "4-S" pathway. Particular success was noted with peroxidase in new solvents. It seemed that the high concentrations of DBT often utilized for easy detection with the GC might inhibit enzyme activity. The reactivity of DBT with H_2O_2 at varying concentrations was measured and it was shown that at the levels utilized, little if any oxidation occurred.

In the third quarter, we obtained important results both with the development of our understanding of the enzyme reaction systems, and also with the microbial work at Woods Hole. In the latter case, we received from Dr. Bazylinski (from Dr. Jannasch's group) two pure cultures which thrive in the presence of DBT. One of these produces a colored product indicative of DBT oxidation.

In Dr. Marquis' laboratory at Boston University, kinetic studies with three enzymes (laccase, horseradish peroxidase, and sulfatase) were made to evaluate the inhibition of these enzymes by our model coal compounds and their sulfur oxidation products. The inhibitions observed, interpreted tentatively as a measure of binding in the substrate active site, have implications for the planning of efficacious coal processing.

In the fourth quarter, we reported evidence of stability of laccase in hydrated ethylacetate and hydrated acetonitrile for at least five days. Our attempts to identify reaction products in the reaction of laccase with DBT were unsuccessful. We had tested laccase in these media with DBT sulfone and no sulfur oxidation was observed. The reversibility of binding of DBT, EPS, and their sulfur oxidation products to horseradish peroxidase, laccase, and sulfatase was shown. The work with the microorganisms (GB-1

and GB-2) from the hydrothermal vents was shown in the fourth quarter to be quite fruitful. We were able to show production of DBT sulfoxide and DBT sulfone when GB-1 was grown in the presence of DBT.

During the fifth quarter, we completed more extensive testing of laccase, horseradish peroxidase, and sulfatase in hydrated organic solvents. We found that under the conditions used, the conversion of DBT to "4S" oxidation products was seen, although the yield was low. Other, as yet unidentified, oxidation products are also observed; these are most likely molecules in which carbon-carbon bond cleavage has occurred. We also began work on the isolation of the microbial enzymes from GB-1 shown to produce "4S" products.

1.3 Summary of Sixth Quarter Results

This report covers the period of September 16, 1989 to December 15, 1989. The greatest success in this quarter has been in the fractionation of GB-1, with the isolation of intracellular and membrane fractions in addition to the extracellular fraction previously utilized. We see apparent production of DBT sulfoxide and sulfone with extracellular fraction in organic solvents and intracellular fraction seems to be similarly active. The assays with laccase and horseradish peroxidase continue to give ambiguous results because of the very low levels of products observed.

Section 2

MATERIALS AND METHODS

2.1 Materials

The commercial enzymes used in our experiments include laccase (No. L-5510) and horseradish peroxidase (No. P-8125) which were both obtained from Sigma Chemical Company. Dimethyl formamide (DMF) was from Burdick and Jackson (No. 076). Acetonitrile (No. A998-4) and ethyl acetate (No. E-195) were both HPLC grade and were obtained from Fisher Scientific.

The syringe filter units used for aliquots removed from the assay samples were 0.2 μ pore size, 3 mm diameter Nylon 66 membranes in a polypropylene housing. These disposable syringe filter units were purchased from Rainin Instrument Company (No. 38-159). Larger Diameter (13 mm) and pore size (0.45 μ) syringe filter units (Rainin No. 38-154) were also used to pre-filter aliquots from microbial enzyme samples that contained suspended material.

The samples for GC are derivatized with Tri-Sil/BSA; Formula D (in DMF) from Pierce Chemical Company (No. 49010).

2.2 Analytical Methods

2.2.1 Gas Chromatography

It may be recalled from prior reports that the GC analytical techniques were not carried out effectively. In this quarter, we have taken several steps to improve our analysis. One change which has improved our results is the use of a derivatizing reagent. This has eliminated the difficulties with reproducibility which we had been experiencing. In addition, we have switched our gas chromatograph from a packed column to a capillary column. The column chosen is a SPB(TM)-1 from Supelco, which is similar to that commonly used on GC-MS equipment. The column has the following characteristics:

- dimethylpolysiloxane phase;
- bonded phase;
- nonpolar;
- dimensions: 15 m length, .2 mm I.D., and .2 μ film thickness; and
- useful in the separation of solvents, petroleum products, and many other industrial chemicals including acidic, basic, and other active compounds.

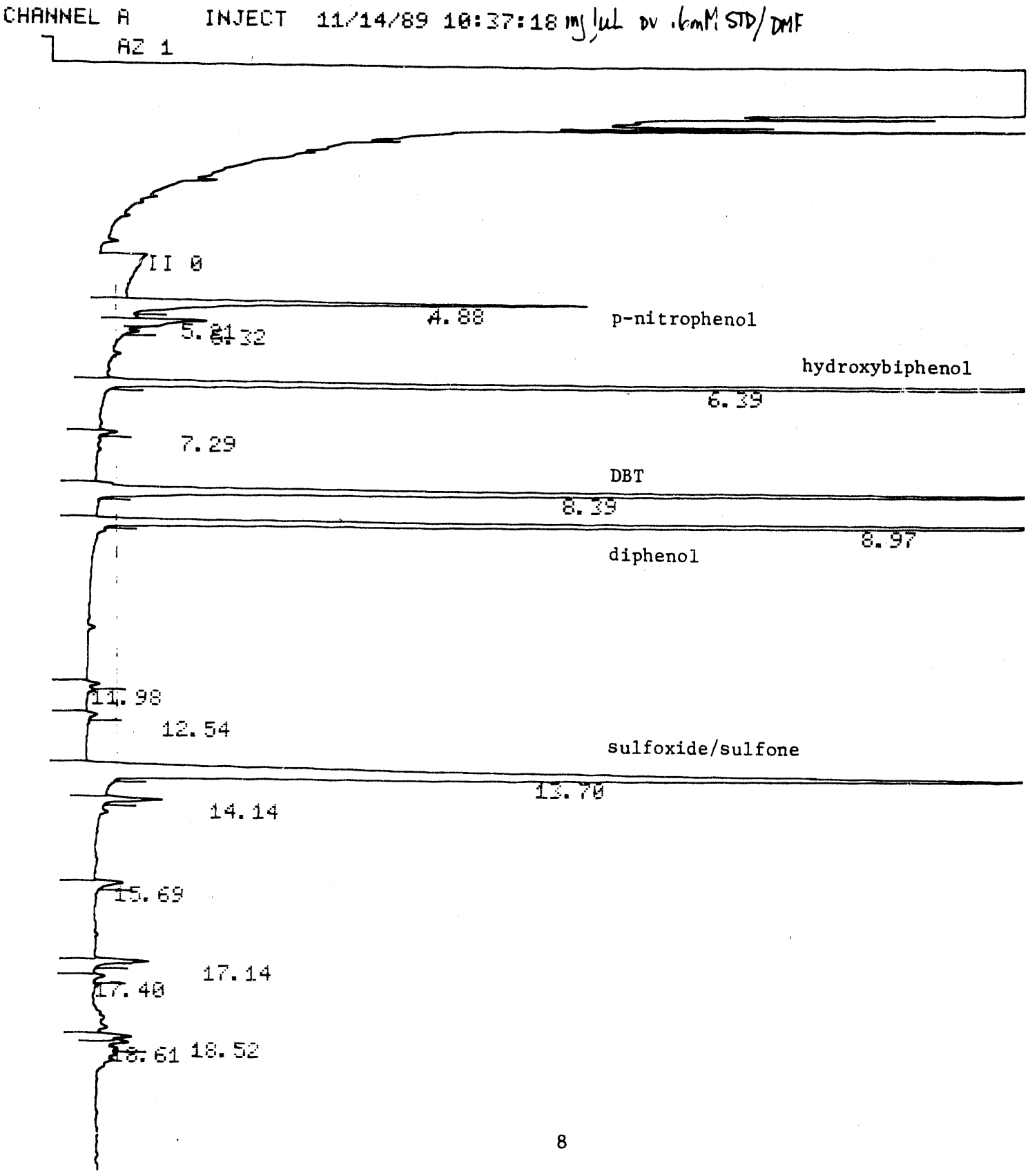
With the use of splitting techniques (split ratio ~150:1, 70 cc/min), we are able to get excellent separation of the compounds of interest without overshadowing by solvent peaks, while leaving time before our earliest peak for detection of unknowns. The conditions utilized are: initial injector temperature, 260°C; initial column temperature, 120°C; detector temperature, 260°C; hold at 120°C for 2 min. (turn on splitter after 0.75 min); and raised at 6°C/min up to 240°C. The program has a turn around time of 30 minutes. A chromatogram which shows the separation of a standard mixture is shown in Figure 2.1. In this standard, p-nitrophenol is added as a reference and the solution of compounds (each 1.2 mM) is diluted 50% with derivatizing reagent.

Samples are dried and either resuspended in a smaller volume of DMF prior to being derivatized with TMS-BSA in DMF; or by adding TMS-BSA in DMF to the dry sample, and heating at 65°C for 15 minutes.

2.2.2 GC Mass Spectrometry

The GC program in use at DynaGen was transferred to the GC-MS (Hewlett-Packard 5088A) at Boston University School of Medicine. The electron multiplier voltage was 200 V above the daily autotune value. The transfer line temperature was 200°C and the source temperature was 200°C. Although the retention times were slightly longer and the sensitivity seemed to be less (on the GC chromatogram) the program was satisfactory. We were able to run all standards and collect chromatograms from the centers of the major peaks. Perhaps because of the use of chemical ionization, the

FIGURE 2.1
GC STANDARD
(1.0uL OF A SOLUTION OF 1.2 mM EACH DBT, DBT SULFOXIDE, DBT-SULFONE, O-HYDROXYBIPHENYL, O-O'DIPHENOL, AND NITROPHENOL)



fragment patterns were not very clean, although in every case except that of DBT-sulfoxide, the mass ion could be observed. The actual chromatograms and scans are shown in Figures 2.2 to 2.6. The fragmentation patterns did not seem to be good enough for use with SIMS (single ion mass spectrometry) and, thus, the samples were analyzed in the same way as the standards.

Our samples were much more dilute than the standards. The scans taken at the retention times at which the standards appeared, in the case of DBT and the marker compound nitrophenol, were acceptable. The other product peaks which we had readily observed by GC and/or HPLC, e.g. sulfoxide, sulfone, o-hydroxyl biphenyl, were not clearly seen. When scans were taken at the previously established retention times we found either only low molecular weight fragments or (in one sample) a very low level of fragments which were not good enough to give conclusive identification. We are presently repeating the work with more concentrated samples in the SIM mode to look for low concentrations of the compounds of interest.

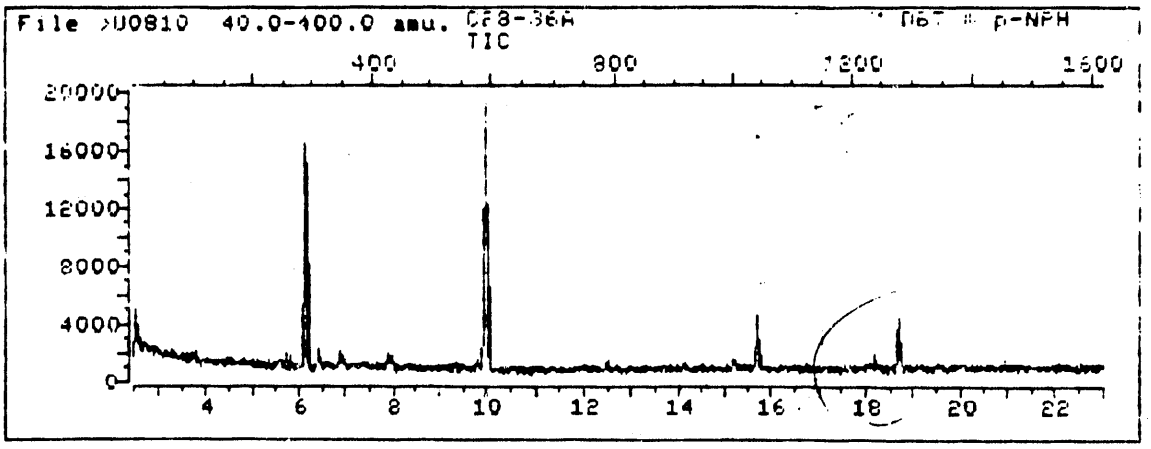
2.2.3 HPLC

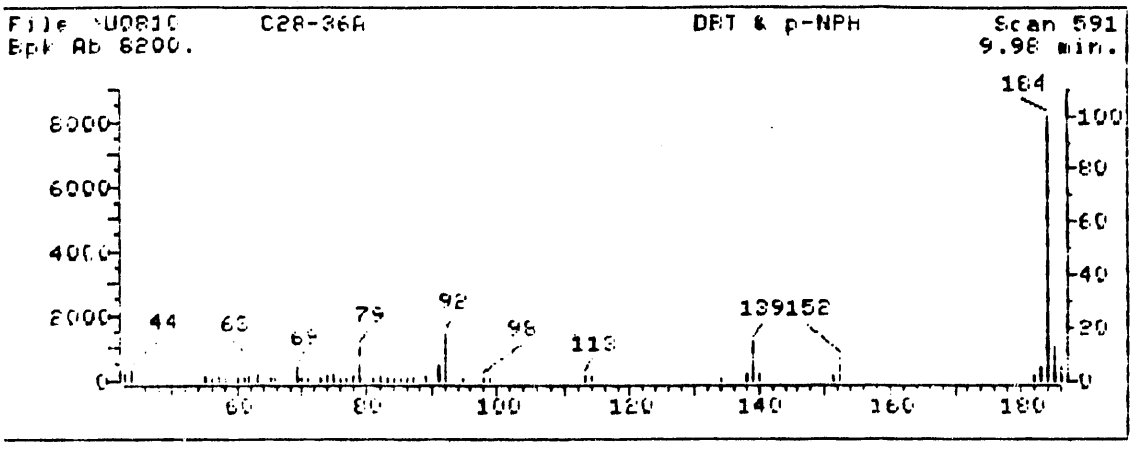
HPLC is performed on a Waters system with a C18 resolve column. The method has been modified so as to reduce the program time by about 10 minutes. The modification is in the mobile phase concentration. Previously, THF:CH_3CN:water (23:17:60) was being used. This has been changed to THF:CH_3CN:water (23:20:57). DBT, the peak that elutes last, now has a retention time of ~19 minutes (as opposed to ~30 minutes). A standard chromatogram is shown in Figure 2.7. DBT sulfoxide and DBT sulfone remain separated in this method.

2.3 Fractionation of GB-1

In our initial experiments, we had worked with whole cells of GB-1 and also with the extracellular fraction (ECF) obtained by centrifugation of the culture broth. Both had shown activity against DBT and we had demonstrated by HPLC the presence of DBT-sulfoxide and some DBT-sulfone. In order to determine whether the intracellular and/or membrane fractions of GB-1 can also oxidize DBT, we have now isolated these fractions as well. The fractionation procedure is outlined in Figure 2.8.

FIGURE 2.2
SELECTED GC-MS DATA FROM A DBT (DIBENZOTHIOPHENE) STANDARD
(0.5 uL OF A 1.2 mM SOLUTION)
MWT - DBT = 184





File: >U0810 Scan #: 591 Retn. time: 9.98

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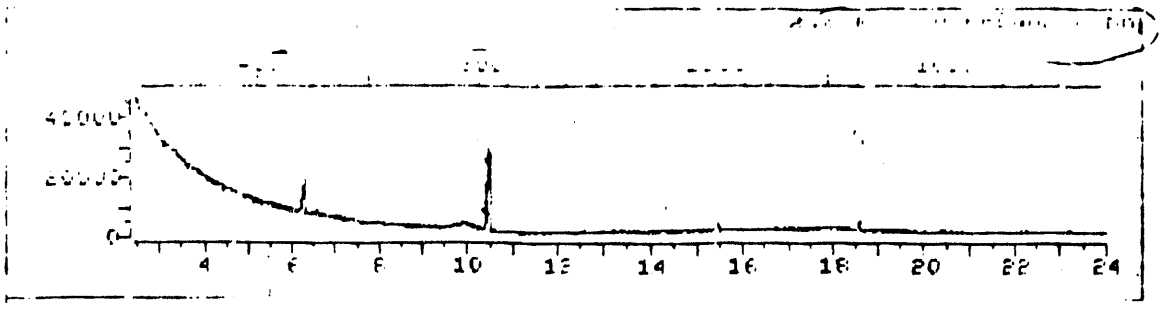
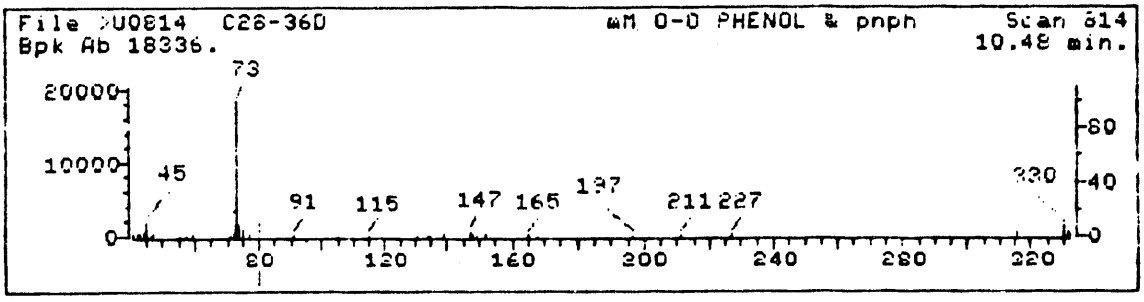
42.90	2.171	63.00	2.622	77.00	.854	89.00	1.524	139.15	14.629
43.90	3.951	65.00	.598	78.00	1.122	91.00	5.720	140.15	2.098
44.90	1.488	65.90	1.220	79.00	12.598	92.00	17.305	151.15	1.634
55.00	1.134	69.00	4.439	81.00	.622	95.00	.354	152.15	8.317
56.00	.659	70.00	1.171	82.00	1.220	96.00	.695	182.00	1.463
57.00	1.500	71.00	.793	83.00	.659	99.00	.634	183.15	5.122
57.90	.866	73.00	1.037	84.00	.317	113.00	1.695	184.15	100.000
59.90	.573	74.00	1.671	85.00	.720	114.00	1.317	185.15	12.329
61.00	1.037	75.00	2.549	86.00	.732	134.00	.732	186.15	5.134
61.90	1.305	76.00	.720	87.00	1.610	138.15	2.049		

FIGURE 2.3
SELECTED GC-MS DATA FROM AN O-O'DIPHENOL STANDARD
(0.5 uL OF A 1.2 mM SOLUTION)
MWT - O-O'DIPHENOL = 186; DERIVATIZED = 330





File: >U0814 Scan #: 814 Retn. time: 10.48

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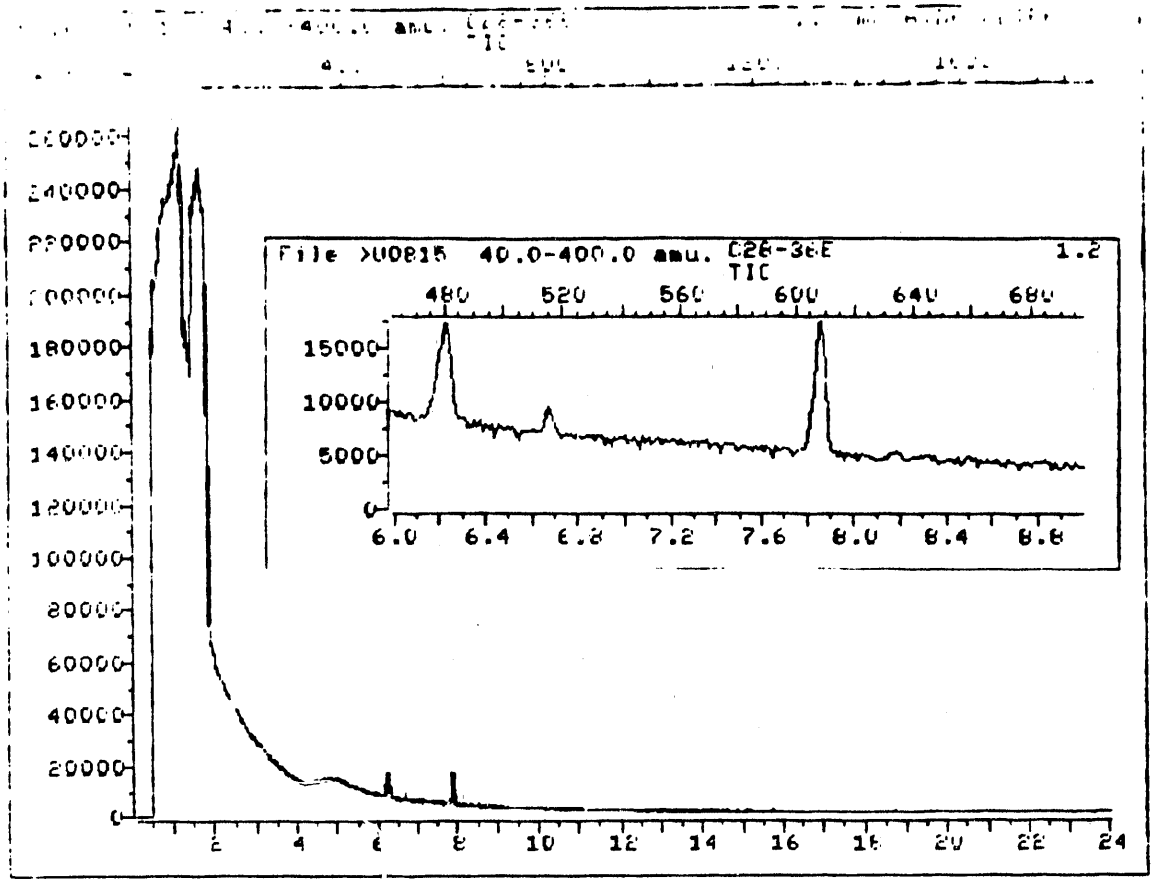
40.90	.414	57.00	.354	77.00	.496	139.15	.474	197.00	.153
41.90	.545	59.00	.960	91.00	.436	147.15	3.218	211.15	.556
42.90	1.211	71.00	.376	105.00	.202	148.15	.409	227.15	.878
43.90	3.523	72.00	.644	115.00	.278	149.15	.295	315.25	2.122
44.90	9.969	73.00	100.000	131.00	.289	152.15	.371	330.25	7.253
45.90	.322	74.00	7.592	134.00	.076	165.15	.502	331.25	2.149
46.90	.513	75.00	5.214	135.00	.153	168.15	.349	332.25	.660
54.90	.278								

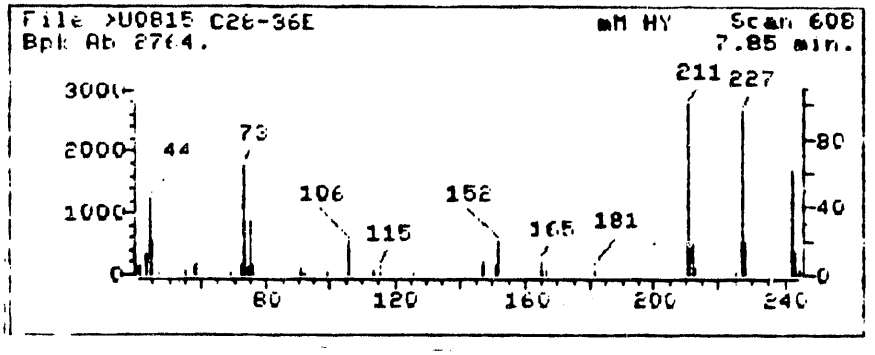
FIGURE 2.4

SELECTED GC-MS DATA FROM AN O-HYDROXYBIPHENYL STANDARD

(0.5 uL OF A 1.2 mM SOLUTION)

MWT - O-HYDROXYBIPHENYL = 170; DERIVATIZED = 242





File: >U0815 Scan #: 608 Retn. time: 7.85e file ion range

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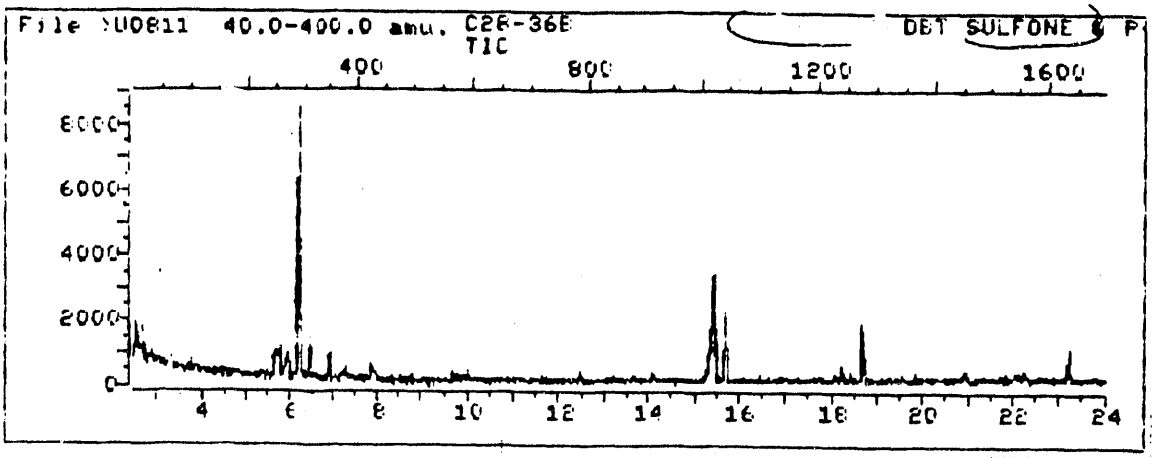
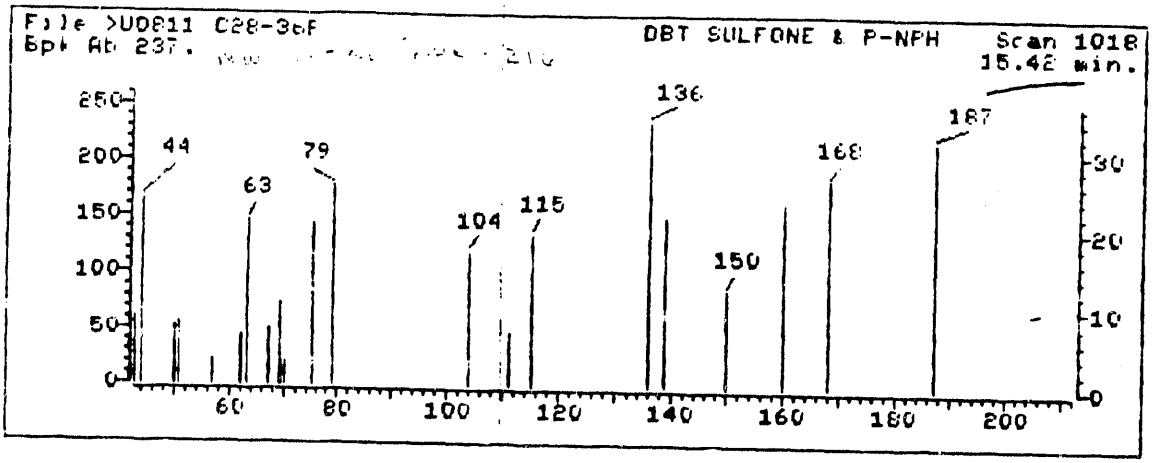
40.90	4.667	72.00	4.522	91.00	3.148	151.15	5.355	213.15	4.342
42.90	12.156	73.00	63.206	92.00	1.049	152.15	19.247	225.15	1.122
44.00	43.777	74.00	3.654	99.00	1.447	153.15	3.799	227.15	96.093
44.90	19.139	75.00	31.548	105.90	19.103	165.15	7.996	228.15	19.428
46.90	5.753	76.00	4.450	113.65	2.856	166.15	2.315	229.15	4.269
54.90	2.026	77.00	6.042	115.15	4.703	181.15	2.894	242.15	60.528
57.90	4.450	82.00	.289	126.00	.977	211.15	100.000	243.25	12.373
58.90	6.223	83.00	1.483	147.15	7.055	212.15	18.198	244.15	2.677
69.00	.941								

FIGURE 2.5
SELECTED GC-MS DATA FROM A DBT-SULFONE STANDARD
(0.5 uL OF A 1.2 mM SOLUTION)
MWT - DBT-SULFONE = 216





>U0811 C28-36B
1018 NRM

DBT SULFONE & P-NPH

reg	type	# pts	scan#	range: amu\r.t.	base	file	ion range
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File: >U0811 Scan #: 1018 Retn. time: 15.420 >U0811
MGR : DR,2

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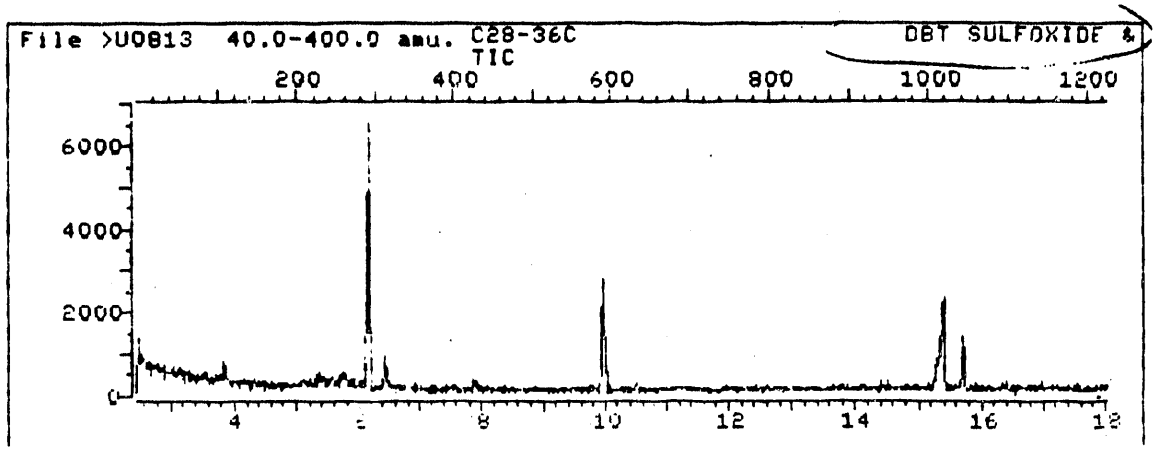
42.90	8.689	62.00	6.125	75.00	20.655	115.00	19.231	160.15	23.504
43.90	23.504	63.00	20.798	79.00	25.356	136.00	33.761	168.15	26.496
49.90	7.407	67.00	6.980	104.00	16.952	139.15	21.510	187.15	31.481
50.90	7.977	69.00	10.541	111.00	6.695	150.15	12.393	216.15	100.000
56.90	2.991	69.75	2.707						

FIGURE 2.6
SELECTED GC-MS DATA FROM A DBT-SULFOXIDE STANDARD
(0.5 uL OF A 1.2 mM SOLUTION)
MWT - DBT-SULFOXIDE = 200



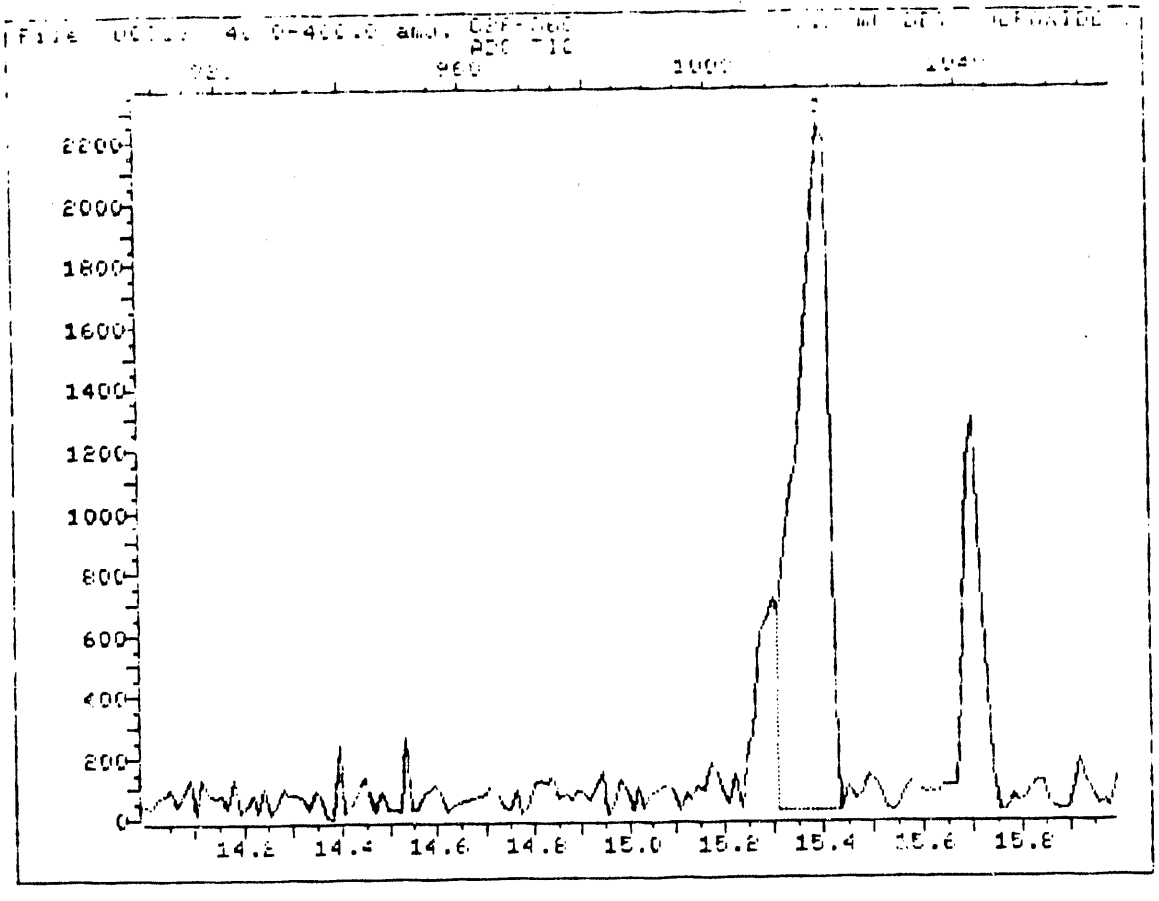
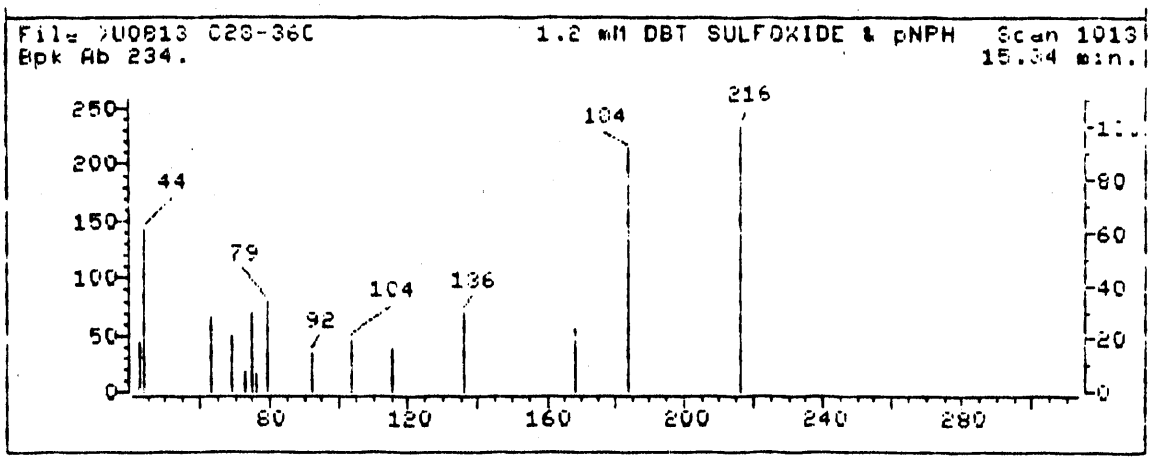
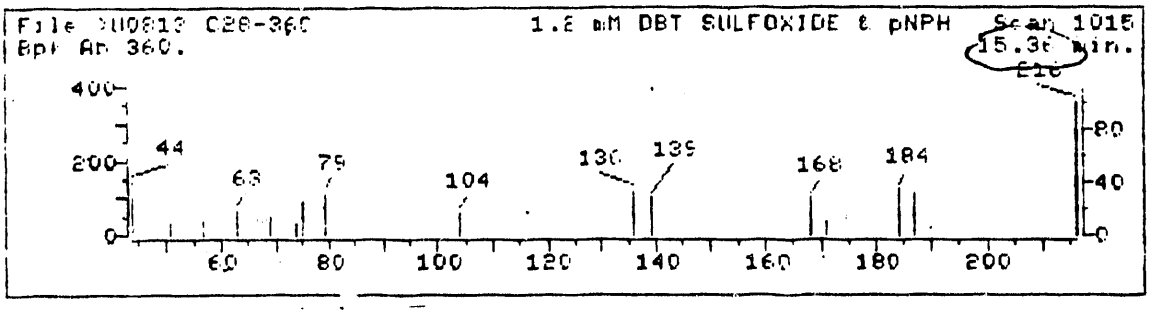


FIGURE 2.6 (CONTINUED)
SELECTED GC-MS DATA FROM A DBT-SULFOXIDE STANDARD
(0.5 uL OF A 1.2 mM SOLUTION)
MWT - DBT-SULFOXIDE = 200



File: >U0813 Scan #: 1013 Retn. time: 15.34

m/z	Int.	m/z	Int.	m/z	Int.	m/z	Int.	m/z	Int.
42.90	18.376	69.00	21.368	75.90	6.838	104.00	19.658	168.15	23.504
43.90	60.256	73.00	7.692	79.00	33.761	115.00	16.239	184.15	91.453
63.00	28.205	75.00	29.915	92.00	14.530	136.00	29.315	216.15	100.000



File: >U0813 Scan #: 1015 Retn. time: 15.36
working...

m/z	Int.	m/z	Int.	m/z	Int.	m/z	Int.	m/z	Int.
43.90	41.667	69.00	12.778	79.00	30.278	139.15	27.778	184.15	35.000
50.90	8.611	73.90	8.889	104.00	17.500	168.15	28.056	187.15	31.111
56.90	10.278	75.00	25.278	136.00	33.889	171.00	10.833	216.15	100.000
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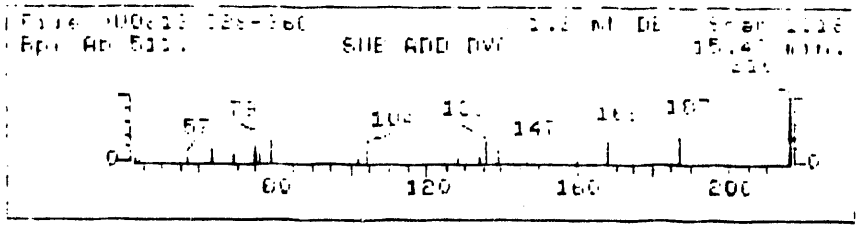
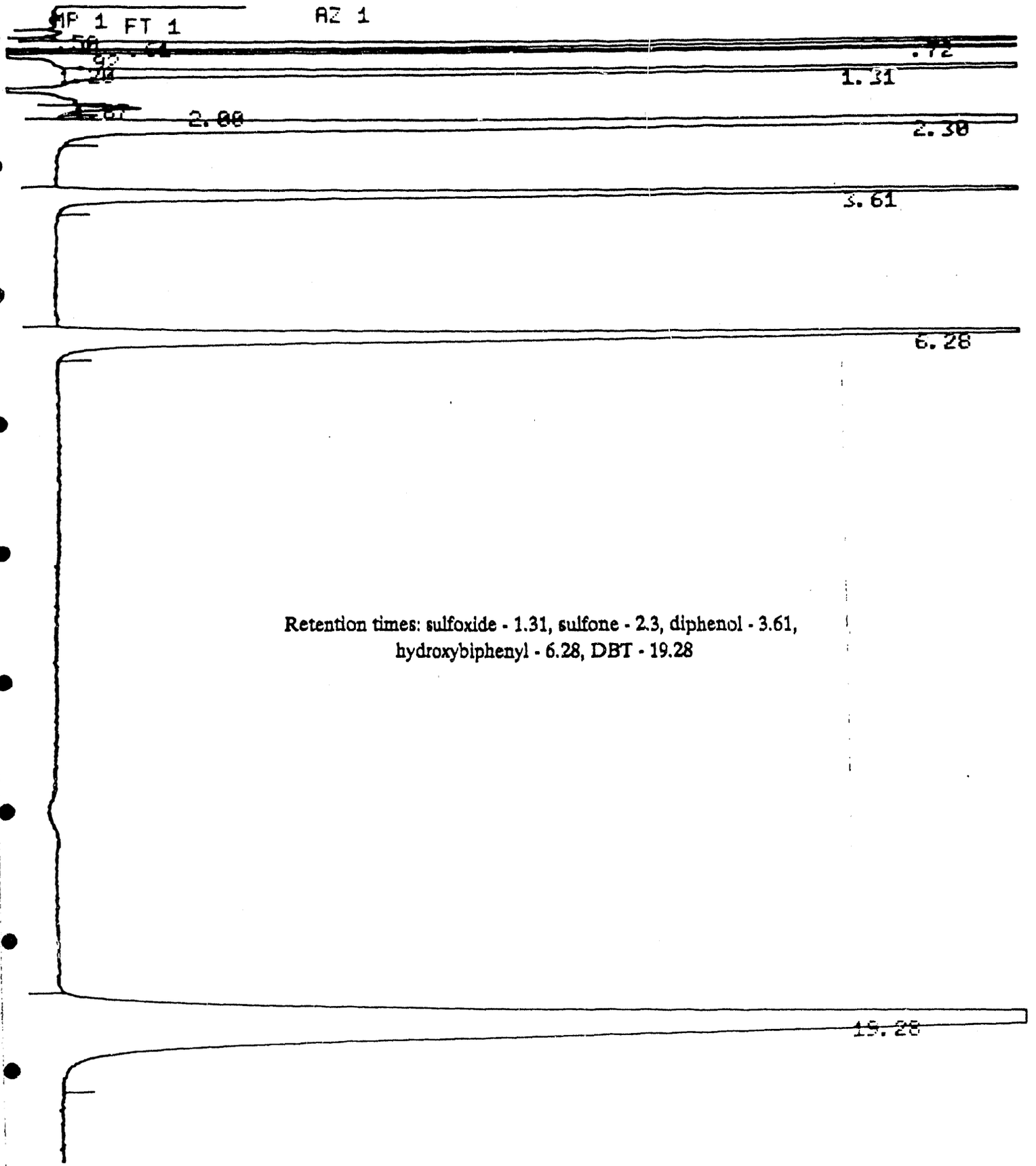
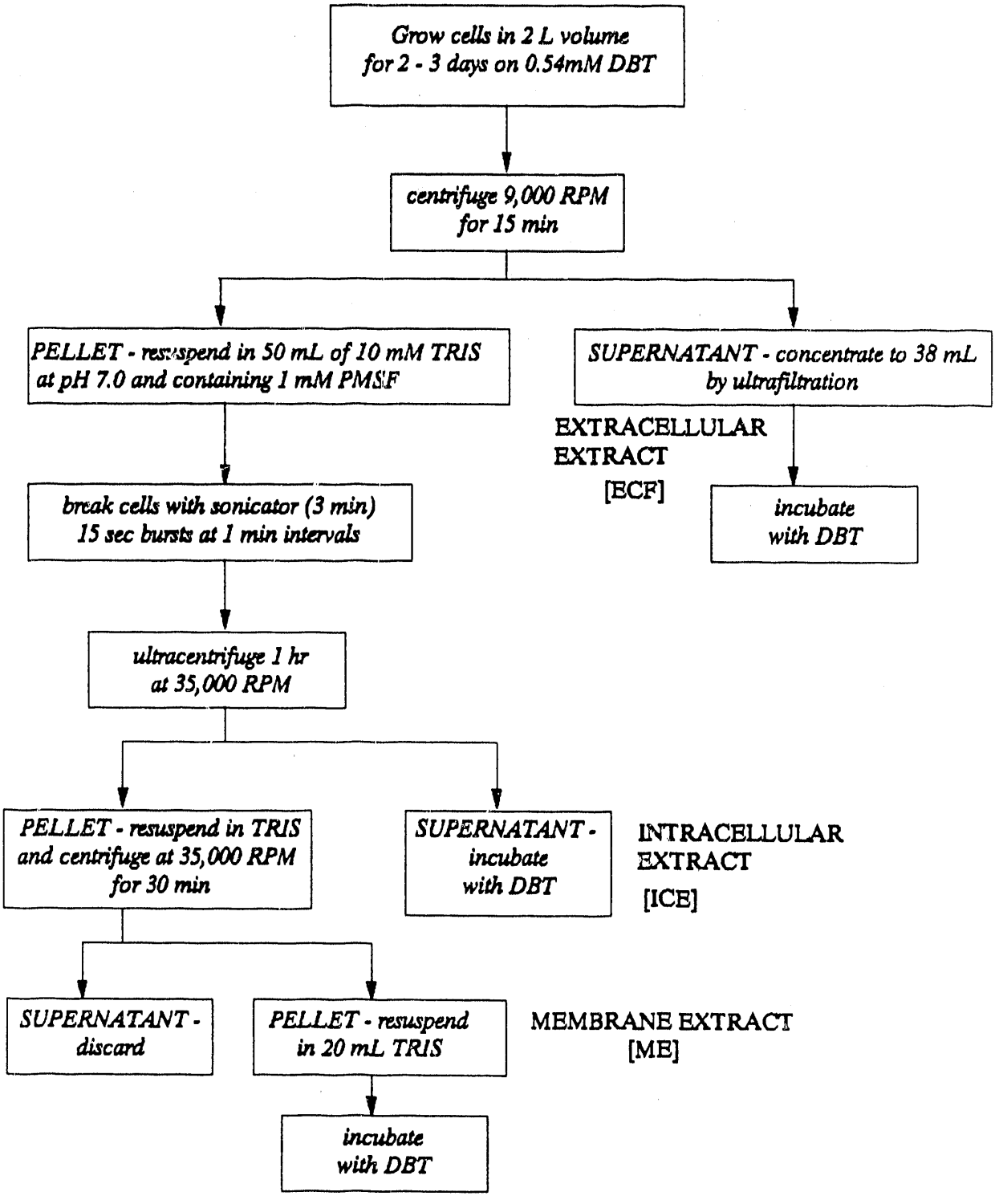


FIGURE 2.7
HPLC STANDARD
(4.0uL OF A SOLUTION OF 0.1 mM EACH DBT, DBT SULFOXIDE, DBT-SULFONE, O-HYDROXYBIPHENYL, AND O-O'DIPHENOL)



Retention times: sulfoxide - 1.31, sulfone - 2.3, diphenol - 3.61, hydroxybiphenyl - 6.28, DBT - 19.28

FIGURE 2.8
FLOW CHART SHOWING INITIAL PROCESS FOR ISOLATING SULFUR-OXIDIZING ENZYMES FROM GB-1



GB-1 was grown in artificial sea water medium with 0.54 mm DBT. The culture was grown at 25°C with shaking. At the end of 72 hours, the cells were harvested by centrifugation at 9,000 rpm for 15 minutes. The pellet was washed in buffer and resuspended in 50 ml of 10 mM Tris buffer, pH 7.0 containing 0.5 ml of a 100 mM ethanolic solution of phenyl methyl sulfonyl fluoride (PMSF), a protease inhibitor. The pellet fraction was sonicated in the cold for 3 minutes using 15 second bursts at 1 minute intervals. The cells were examined microscopically to check for lysis. This fraction was then ultracentrifuged at 35,000 rpm for 1 hour at 4°C. The supernatant was labelled ICF and treated as the intracellular fraction. The pellet was washed with Tris buffer and centrifuged again. The supernatant was discarded, the pellet was resuspended in 1.0 ml of Tris buffer, pH 7.0, and labelled as the membrane fraction (MF).

The final extracellular fraction (ECF) was obtained by subjecting the supernatant from the 72 hour culture broth to ultrafiltration. An Amicon Model 8050 ultrafiltration cell (50 ml volume) was used with a Pharmacia Omega Series 43 mm ultrafiltration membrane with a 10,000 molecular weight cut-off. Ice cold water was constantly passed around the ultrafiltration cell using a peristaltic pump. Nitrogen was used to pressurize the cell at ~20 psi. The fraction with molecular weight greater than 10,000 that had accumulated within the ultrafiltration cell and was labelled extracellular fraction (ECF). In this manner, about 1.8 liters of culture supernatant was concentrated down to 47 ml of ECF. All three fractions were distributed into 4 ml aliquots in vials and frozen at -20°C until used.

2.4 Assay Procedures

2.4.1 Microbial Assay Procedures

The ECG, ICF, and MF were assayed for their activity against DBT in the aqueous phase as well as in acetonitrile and ethyl acetate. The ECF was also assayed in DMF.

For the assay in the aqueous phase, 1 ml each of the ECF, ICF, and MF were incubated with 1 mg of DBT in 8 ml screw cap tubes. The tubes were capped and incubated horizontally at 25°C in a shaker. Control tubes

included fractions incubated without DBT, and Tris buffer and medium concentrated by ultrafiltration (as done to obtain the ECF) incubated with DBT. The tubes were harvested after 7 days.

Assays in organic solvent were set-up as follows: a DBT solution was prepared in organic solvent so as to give a final DBT concentration of 0.05 mM. To 0.9 ml of this solution was added 0.1 ml of ECF, ICF, or MF, and the tubes were capped and incubated as described before. Controls without DBT and of buffer and medium concentrate with DBT were also included.

An additional experiment included incubation of ECF, ICF, and MF in 50% acetonitrile with a final DBT concentration of 0.1 mM. Tubes were incubated as before with shaking at 25°C. Controls included ECF, ICF, and MF in 50% acetonitrile without DBT.

2.4.2 Commercial Enzyme Assay Procedures

Table 3.3 summarizes the following experiments.

2.4.2.1 First Experiment

This experiment was performed at Boston University and the analytical tasks were performed at Boston University for the GC/MS work while the GC and HPLC analysis were carried out at DynaGen. A stock solution of 0.1 mM DBT in acetonitrile was prepared and 9.9 mls was added to each of 3 glass scintillation vials. To one vial was added 100 µl of 1 mg/ml HRP in distilled water. To the second vial 100 µl of 1 mg/ml laccase in distilled water was added. As a control, 100 µl of distilled water was added to the third vial. The three vials were placed on a rotating shaker at room temperature.

At 1, 24, and 48 hours, 500 µl was removed from each sample and filtered through a 0.2 µ Nylon 66 syringe filter unit. After seven days a final aliquot was taken from each sample. These aliquots were screened by GC and HPLC for the presence of DBT oxidation products. The results of this analysis can be found in Table 3.4.

2.4.2.2 Second Experiment

Since there had been early indications of the presence of DBT oxidation products in the aliquots taken in the first experiment, a repeat was started using more samples in order to confirm the preliminary findings. A new stock solution of 0.1 mM DBT in acetonitrile was prepared. A sample of this stock solution was filtered through a 0.2 μ filter unit and retained as a time = 0 control.

To each of nine screw cap tubes, a total of 4.95 ml of the 0.1 mM DBT stock was added. To each of three glass scintillation vials, 9.9 ml of this stock was added. To three of the screw cap tubes containing DBT, 50 μl of 1 mg/ml laccase in distilled water was added. Similarly, 50 μl of 1 mg/ml HRP in distilled water was added to another three screw cap tubes containing DBT. 100 μl of the HRP solution was added to two of the scintillation vials. Finally, 50 μl of distilled water was added to the last set of three screw cap tubes and 100 μl distilled water was added to the last scintillation vial. The samples were placed on a shaker at room temperature for rapid mixing.

500 μl aliquots were removed at 1, 24, and 48 hours and filtered through the 0.2 μ filter units. Larger aliquots of ~ 3 mls were removed and filtered at time ≈ 168 hours. In order to have the products of interest be as concentrated as possible for detection, the 168 hour aliquots were evaporated to dryness prior to analysis. Each aliquot was placed 100 μl at a time in a test tube in a heating block at 60°C. A gentle stream of N_2 was blown over the liquid to help speed the evaporation of solvent to dryness. This was repeated until a total of 500 μl of each aliquot had been reduced to dryness. The dried sample was then redissolved in 50 μl of acetonitrile. These concentrated aliquots were then analyzed by GC and HPLC at DynaGen and some were taken to Boston University for analysis by GC/MS. Table 3.5 summarizes the results of the GC and HPLC analysis. The significant decrease in the concentration of DBT from the initial value of 0.1 mM to that which is seen in Table 3.5 suggests that DBT was lost, probably in the drying/evaporation process.

2.4.2.3 Experiments Three and Three A

A private communication from staff at Boston University had indicated that the HRP used in the first and second experiments might not have been as active as previously thought. To correct this possibility, a new lot of HRP was obtained for this round of experiments. Stock solutions of DBT at 0.2 mM were prepared using the following solvents: acetonitrile, DMF, and ethyl acetate. A stock of 0.1 mM DBT in acetonitrile was also used.

As a repeat of the HRP trial of the second experiment, 4.95 ml of 0.1 mM DBT in acetonitrile was added to each of four screw cap test tubes. To two of the tubes, 50 µl of HRP (1 mg/ml in distilled water) solution was added while 50 µl of distilled water was added to the second pair of tubes.

For each of the 0.2 mM DBT stock solutions in the three solvents, 2.5 ml was added to each of four screw cap test tubes. For each set, 2,450 µl distilled water and 50 µl of HRP (10 mg/ml) in distilled water were added to two tubes while 2,500 µl distilled water was added to the second pair of tubes in each set. All four sets of tubes were shaken to mix and a 0.5 ml aliquot removed and filtered through a 0.2 µ syringe filter unit for a time = 0 sample. All sixteen tubes were placed in a shaker housed in a constant temperature room with the temperature at 24°C. Depending on the sample set, an intermediate time aliquot (1 ml) was removed from each tube at time = 2 or 3 days. In the case of the 50% ethyl acetate samples, the tubes were allowed to stand and the aqueous and organic layers were allowed to separate before 0.5 mls were removed from each layer. These aliquots will be referred to as the sample for Experiment Three.

Since the initial results of Experiment One had indicated HRP activity against DBT without use of the enzymes co-factor, H_2O_2, no H_2O_2 had been added to the samples in subsequent experiments. In order to correct this possible omission, it was decided to modify Experiment Three on Day 2 or 3 by adding enough H_2O_2 to each of the tubes to have the final concentration of 0.05 mM in each sample tube. This was accomplished as follows.

After the withdrawal of the intermediate time aliquots in Experiment 3, 17.3 µl of 0.0101 M H_2O_2 solution (31% H_2O_2 diluted 1,000 times) was added to the remaining 3.5 ml of sample in each tube. The tubes were returned to the shaker. Aliquots were taken on day = 5 or 6 and analyzed. The aliquots taken after the addition of the H_2O_2 will be referred to as Experiment Three A.

Section 3

RESULTS AND DISCUSSION

3.1 Assays With Microbial Enzymes

The fractions obtained as described in Section 2.3 were assayed in the combinations shown in Table 3.1. The results are summarized in Table 3.2. Results by both GC and HPLC are given qualitatively. The samples were dried and resuspended for analysis and some material was apparently lost in this process. Thus, the quantities measured were variable. Because the Tris buffer seems to effect the GC retention times, we rely more on the HPLC data for these samples.

The assays of extracellular fractions in 99% acetonitrile and in 50% DMF show the presence of small amounts of DBT sulfoxide and DBT sulfone. Sulfoxide was also seen in aqueous assays of the ECF. In 99% ethylacetate, the ECF assay contained DBT-sulfoxide. The intracellular fraction appeared to produce DBT sulfoxide, DBT-sulfone, and o-hydroxy biphenyl in 99% acetonitrile and a small amount of DBT-sulfone in 99% ethylacetate. The membrane fraction assays routinely show the presence of DBT-sulfoxide in very small amounts. Our controls also occasionally show very small levels of sulfoxide or sulfone which we believe may be residual from the initial cell growth on DBT.

3.2 Assays With Commercial Enzymes

Many assays with laccase and horseradish peroxidase in hydrated organic solvents were carried out. An outline of the assays performed is given in Table 3.3. Most assays were analyzed by both GC and HPLC. Some samples were additionally analyzed by GC-MS however these tests are being repeated before reporting. The GC and HPLC results are summarized in Tables 3.4, 3.5, and 3.6.

In the tests performed at Boston University (first experiment), we found evidence of DBT sulfoxide and DBT-sulfone production by HRP without peroxide at seven days. Additionally, we saw sulfoxide and o-hydroxy biphe-

TABLE 3.1

ASSAYS PERFORMED ON GB-1 FRACTIONS

DURING THE 6th QUARTER

FRACTION/ MEDIUM	EXTRA-CELLULAR FRACTION	INTRA-CELLULAR FRACTION	MEM-BRANE FRACTION	CONCEN-TATED MEDIA	TRIS
Neat, DBT[1 mg/ml]	C	C	C	C	C
neat (no DBT)	C	C	C		
99% DMF, DBT [0.1 mM]	C				
99% ACN, DBT [0.05mM]	C	C	C	C	C
99% ACN (no DBT)	C	C	C		
99% ETOAC, DBT [0.05mM]	C	C	C	C	C
99% ETOAC (no DBT)	C	C	C		
50% ACN, DBT [0.1mM]	C	C	C		
50% ACN	+	C	C		

DBT = dibenzothiophene

DMF = dimethylforamide

ACN = acetonitrile

ETOAC = ethylacetate

("C" means that assay has been completed)

TABLE 3.2
ANALYSIS OF GB-1 FRACTION ASSAYS
DURING THE 6th QUARTER

			REACTION PRODUCTS OBSERVED					
FRAC-TION/ SOLVENT	DBT [mM]	METHOD	O-O'-DIPHENOL	DBT	DBT-SULFOXIDE*	DBT-SULFONE*	DBT-OXIDE\ONE**	O-HYDROXY-BIPHENYL
ICF		GC	-	+			-	+
ACN	0.05	HPLC		+	+			+
ICF		GC	-	-			-	+
ACN	0.0	HPLC	-	-	+	-		
ICF		GC	N.D.					
ETOAC	0.05	HPLC	-	+	+	+		
ICF		GC	N.D.					
ETOAC	0.0	HPLC	-	-	+	-		+
ECF		GC	-	+			+	+
ACN	0.05	HPLC	-	+	+	+		+
ECF		GC	-	-		+	+	+
ACN	0.0	HPLC	-	-	+	-		-
ECF		GC	N.D.					
ETOAC	0.05	HPLC	-	+		-		+
ECF		GC	N.D.					
ETOAC	0.0	HPLC	-	-	-	-		-
MF		GC	-	+			+	-
ACN	0.05	HPLC	-	+	+	-		-
MF		GC	N.D.					
ACN	0.0	HPLC	-		+	-		-
MF		GC	N.D.					
ETOAC	0.05	HPLC	-		+	-		-
MF		GC	N.D.					
ETOAC	0.0	HPLC	-		+	-		-
TRIS		GC	IN BOTH SYSTEMS - MANY PEAKS BUT NOT STDS					
ACN	0.0	HPLC						
TRIS		GC	IN BOTH SYSTEMS - MANY PEAKS BUT NOT STDS					
ETOAC	0.0	HPLC						
MED		GC	N.D.					
ETOAC	0.05	HPLC	-	+		+		-

Continued on next page

TABLE 3.2(CONTINUED)

ANALYSIS OF GB-1 FRACTION ASSAYS

			REACTION PRODUCTS OBSERVED					
FRACTION/ SOLVENT	DBT [mM]	METHOD	O-O'-DIPHENOL	DBT	DBT-SULFOXIDE*	DBT-SULFONE*	DBT-OXIDE\ONE**	O-HYDROXY-BIPHENYL
ECF NEAT	5.43	GC	-	-****			+	-
		HPLC	-	-****	++	-		-
ECF NEAT	0.0	GC	N.D.	-				→
		HPLC	-	-	+	-		-
ECF 50% DMF	5.43	GC	N.D.					→
		HPLC	-	+	+	+		-
ICF NEAT	5.43	GC	N.D.					→
		HPLC	-	-****	-	-		-
ICF NEAT	0.0	GC	N.D.					→
		HPLC	-	+	+***	-		-
MF NEAT	5.43	GC	N.D.					→
		HPLC	-	-****	-	-		-
MF NEAT	0.0	GC	N.D.					→
		HPLC	-	+		-		-

("+" means that the compound was observed,
"-" means that it was not observed)

*HPLC only, **GC only

***Detected on extraction of ICF with methylene chloride and concentration

****Not detected because it is filtered prior to injection

ECF = extracellular fraction; ICF = intracellular fraction;
MF = membrane fraction
DBT = dibenzothiophene, GC = gas chromatography, HPLC = high performance liquid chromatography, N.D. = not done,

TABLE 3.3

ASSAYS PERFORMED WITH LACCASE AND HORSERADISH PEROXIDASE DURING THE 6th QUARTER

MEDIUM	DBT [mM]	LACCASE	HORSERADISH PEROXIDASE	NO ENZYME	COMMENTS
99% ACN	0.1mM	C 0.01 mg LAC/ml	C 0.01 mg HRP/ml	C	Performed at B.U. First Experiment
99% ACN	0.1mM	C 0.01 mg LAC/ml	C 0.01 mg HRP/ml	C	Second Experiment
99% ACN	0.1mM		I.P. 0.01 mg HRP/ml	I.P.	new lot of HRP Third Experiment
99% ACN H2O2 [0.05mM]	0.1mM		I.P. 0.01 mg HRP/ml	I.P.	Experiment Three-A
50% ACN	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Third Experiment
50% ACN H2O2 [0.05mM]	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Experiment Three-A
50% DMF	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Third Experiment
50% DMF H2O2 [0.05mM]	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Experiment Three-A
50% ETOAC	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Third Experiment
50% ETOAC H2O2 [0.05mM]	0.1mM		I.P. 0.1 mg HRP/ml	I.P.	Experiment Three-A

ABBREVIATIONS: C = EXPERIMENT COMPLETED; I.P. = EXPERIMENT IN PROGRESS
HRP = HORSERADISH PEROXIDASE; DBT = DIBENZOTHIOPHENE; DMF = DIMETHYLFORAMIDE;
LAC = LACCASE; ACN = ACETONITRILE
IN ALL MEDIA, THE UNTITLED COMPONENT IS DISTILLED WATER
ALL CONCENTRATIONS ARE EXPRESSED AS THE FINAL CONCENTRATIONS IN THE ASSAY

TABLE 3.4

ANALYSIS OF LACCASE AND HORSERADISH PEROXIDASE IN ACETONITRILE - FIRST EXPERIMENT

			REACTION PRODUCTS OBSERVED					
ENZYME	TIME	METHOD	O-O'-DIPHENOL [mM]	DBT [mM]	DBT-SULFOXIDE* [mM]	DBT-SULFONE* [mM]	DBT-OXIDE\ONE** [mM]	O-HYDROXY-BIPHENYL [mM]
HRP	1hr	GC	N.D. →					
HRP	1hr	HPLC	0.0	0.076	0.0	0.0	N.D.	N.D.
HRP	24hr	GC	N.D. →					
HRP	24hr	HPLC	0.0	0.087	0.0	0.0	N.D.	N.D.
HRP	48hr	GC	0.002	0.08	N.D.	N.D.	0.0	0.0
HRP	48hr	HPLC	0.0	0.029	0.012	0.0	N.D.	N.D.
HRP	168hr	GC	0.0	0.16	N.D.	N.D.	0.44	0.0
HRP	168hr	HPLC	0.0	0.022	0.06	0.035	N.D.	N.D.
LAC	1hr	GC	N.D. →					
LAC	1hr	HPLC	0.0	0.058	0.0	0.0	N.D.	N.D.
LAC	24hr	GC	N.D. →					
LAC	24hr	HPLC	0.0	0.093	0.0	0.0	N.D.	N.D.
LAC	48hr	GC	N.D. →					
LAC	48hr	HPLC	0.0	0.07	0.0	0.0	N.D.	N.D.
LAC	168hr	GC	0.01	0.05	N.D.	N.D.	0.02	0.01
LAC	168hr	HPLC	0.0	0.046	0.071	0.0	N.D.	N.D.
CN	1hr	GC	N.D. →					
CN	1hr	HPLC	0.0	0.068	0.0	0.0	N.D.	N.D.
CN	24hr	GC	N.D. →					
CN	24hr	HPLC	0.0	0.89	0.0	0.0	N.D.	N.D.
CN	48hr	GC	N.D. →					
CN	48hr	HPLC	0.0	0.073	0.0	0.0	N.D.	N.D.
CN	168hr	GC	0.0	0.017	N.D.	N.D.	0.0	0.0
CN	168hr	HPLC	0.0	0.049	0.0	0.0	N.D.	N.D.

*HPLC only, **GC only

HRP = horseradish peroxidase, LAC = laccase, CN = no enzyme, DBT = dibenzothiophene, GC = gas chromatography, HPLC = high performance liquid chromatography, N.D. = not done

0.1 ml of enzyme in water or water alone was added to 9.9 ml of acetonitrile with DBT at a final concentration of 0.1mM. The mixtures were placed in sealed scintillation vials and held at room temperature with shaking. Aliquots were removed at the times indicated.

TABLE 3.5

ANALYSIS OF LACCASE AND HORSERADISH PEROXIDASE IN ACETONITRILE - SECOND EXPERIMENT

			REACTION PRODUCTS OBSERVED					
ENZYME	TIME	METHOD	O-O'-DIPHENOL [mM]	DBT [mM]	DBT-SULFOXIDE* [mM]	DBT-SULFONE* [mM]	DBT-ONE/OXIDE** [mM]	O-HYDROXY-BIPHENYL [mM]
HRP	168hr	GC	0.0	0.05	N.D.	N.D.	0.0	0.0
HRP	168hr	HPLC	0.0	0.052	0.0	0.0	N.D.	0.0
HRP	168hr	GC	0.0	0.06	N.D.	N.D.	0.0	0.0
HRP	168hr	HPLC	0.0	0.048	0.0	TRACE	N.D.	0.0
HRP	168hr	GC	0.0	0.02	N.D.	N.D.	0.0	0.0
HRP	168hr	HPLC	0.0	0.038	0.0	0.0	N.D.	0.0
***HRP	168hr	GC	0.0	0.02	N.D.	N.D.	0.0	0.0
***HRP	168hr	HPLC	0.0	0.04	0.0	0.0	N.D.	0.0
***HRP	168hr	GC	0.0	0.02	N.D.	N.D.	0.0	0.0
***HRP	168hr	HPLC	0.0	0.039	0.0	TRACE	N.D.	0.0
LAC	168hr	GC	0.0	0.04	N.D.	N.D.	0.0	0.0
LAC	168hr	HPLC	0.0	0.055	0.0	TRACE	N.D.	0.0
LAC	168hr	GC	0.0	0.007	N.D.	N.D.	0.0	0.0
LAC	168hr	HPLC	0.0	0.027	0.0	TRACE	N.D.	0.0
***CN	168hr	GC	0.0	0.02	N.D.	N.D.	0.0	0.0
***CN	168hr	HPLC	0.0	0.037	TRACE	TRACE	N.D.	0.0
CN	168hr	GC	0.0	0.017	N.D.	N.D.	0.0	0.0
CN	168hr	HPLC	0.0	0.049	0.0	0.0	N.D.	0.0

*HPLC only, **GC only, ***in scintillation vial

HRP = horseradish peroxidase, LAC = laccase, CN = no enzyme, DBT = dibenzothiophene, GC = gas chromatography, HPLC = high performance liquid chromatography, N.D. = not done,

0.05 ml of enzyme in water or water alone was added to 4.95 ml of acetonitrile with DBT at a final concentration of 0.1mM. The mixtures were placed in sealed tubes or scintillation vials and held at room temperature with shaking. Aliquots were removed at the times indicated.

Results for separate reactors are shown

nyl in the 168 hour laccase experiment. One occurrence in this test was that scintillation vials were used as reactors and after 7 days some of the cap liners had loosened.

In the second experiment, most reactors were tubes with teflon liners, but two sets of reactors were made with scintillation vials. We also observed cap liner loosening. In this experiment at 168 hours, a trace of DBT sulfone was seen in two HPR tube reactors, in two LAC reactors, and in one HRP vial reactor. The control vial reactor showed both DBT sulfoxide and DBT sulfone. The quantities observed were so low that we are unable to make conclusive commentary on the results of Experiment Two.

Experiments Three and Three A are being analyzed now. We will concentrate the samples to a greater extent and will give greater attention to quantitation. We expect the results of these experiments, analyzed by GC, HPLC, and GC-MS to give conclusive evidence of the true reactivity of the laccase and HRP. It should also be noted that Experiments Three and Three A include assays at 50% solvent levels. It has come to our attention that such a composition is a common method of utilization of HRP and other enzymes.

Section 4

PLANS AND PROGRESS ASSESSMENT

4.1 Evaluation of Progress

Our progress with enzyme isolation from GB-1 has been good. One two-liter batch has been separated into three fractions, e.g., extracellular, intracellular, and membrane. Using these concentrated materials, we have been able to carry out assays in buffer and in organic solvents. Work with commercial enzymes has also given indication of success.

The recurrent problem area in our work has been in product analysis. We have made real progress in utilizing HPLC and GC to obtain comparable and quantitative analysis of DBT and oxidation products to levels of around .01 mM concentration. Since we have found assays to be most successful at less than 1 mM, we still have difficulty with analyses without sample concentration. We are able to carry out the concentrations, however, there seems to be concomittant loss of sample in the process.

Detection of very low amounts of expected compounds is theoretically possible with GC-MS. We have been proceeding with this method and expect to have results by early January which give conclusive evidence confirming results in this report.

4.2 Future Plans

In the Seventh Quarter, we will be focusing first on GC-MS confirmation of our results. When this method of analysis is supplying us with reliable data, we will continue with the enzymes, or enzyme fractions, which are known to give desired products. Assays with pure sulfoxide or sulfone as substrate will elucidate the active pathways of the enzymes and reactions with coal will begin to show the practicality of enzyme processing for organic sulfur removal.

END

DATE
FILMED
2/27/92