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ENZYMATIC DESULFURIZATION OF COAL

Fourth Qurterly Report

Holometrix Report No. 2469
Holometrix Project No. DOE-12
DOE Contract No. DE-AC22-88PC88855

Submitted to:

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June 16, 1989

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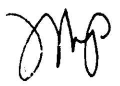
MASTER



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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 emphasize the advantages of enzymatic desulfurization techniques and have specifically addressed the potential of using partially-purified extracellular microbial enzymes as well as commercially available enzymes. Our work is focused on the treatment of "model" organic sulfur compounds such as dibenzothiophene (DBT) and ethylphenylsulfide (EPS). Furthermore, we are designing experiments to facilitate the enzymatic process by means of a hydrated organic solvent matrix.

During the first year of this project, our laboratories have pursued primarily the multi-step, enzymatic breakdown of DBT and the development of the Klibanov-type hydrated solvent reaction system. Previous studies with the aromatic sulfur compound DBT have shown that there are two general biological pathways for the oxidative breakdown of this compound. In the reaction most frequently observed in microbial oxidative pathways,

DBT is oxidized at a ring carbon, and the reaction is accompanied by a considerable decrease in the free energy of the compound. Our work is focused on oxidation at the sulfur with consequent liberation of inorganic sulfate. The identification of this multi-step ("4S") reaction pathway has led us to examine each of the oxidized sulfur intermediates, as well as the desulfurized product. These compounds are illustrated in Figure 1.1.

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 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.

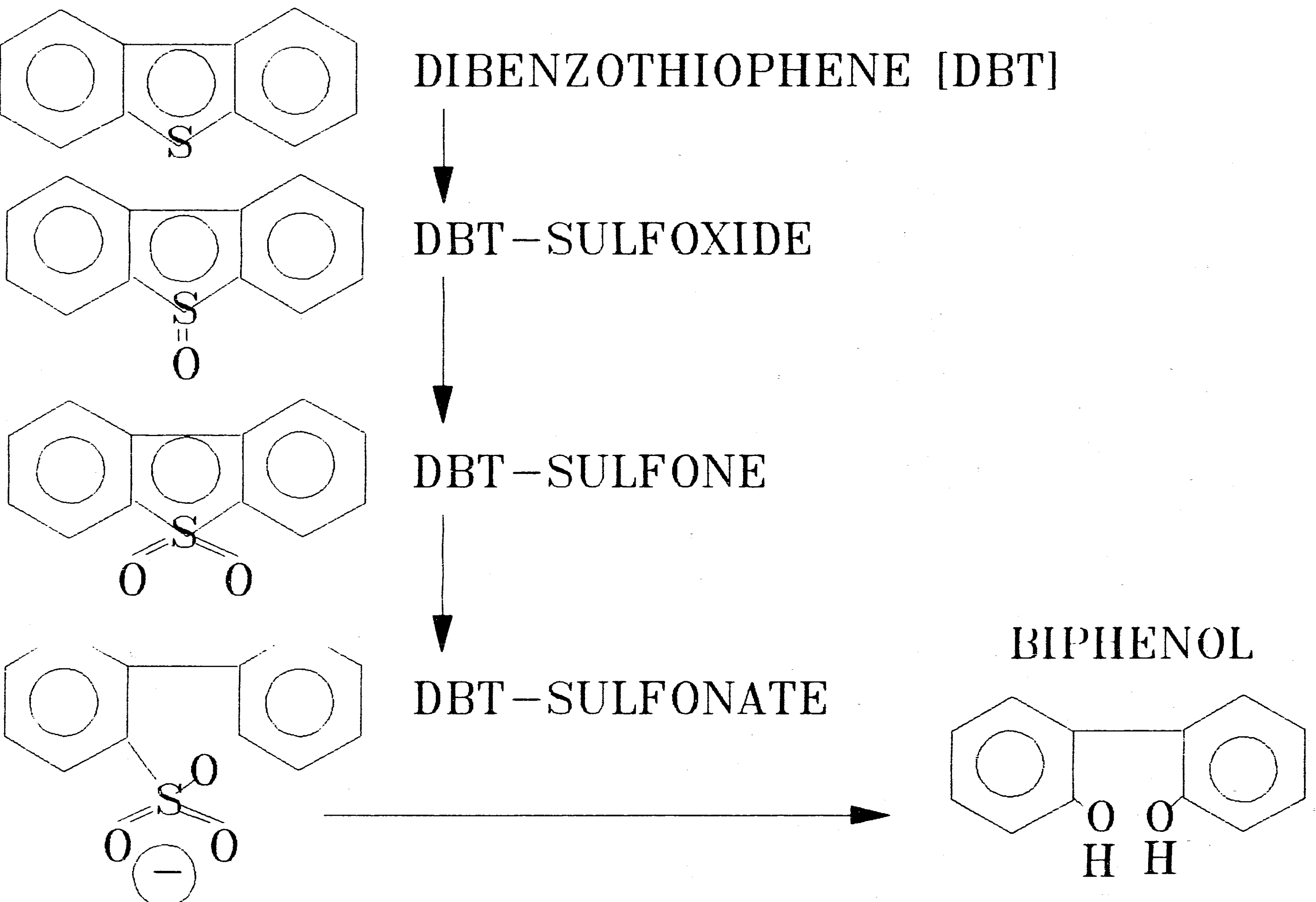
In the second quarter, the screening of media for the enzyme reactions with DBT was expanded. Changes in buffer were examined and several more hydrophobic solvents were utilized. An extensive amount of data was obtained by gas chromatography, utilizing a method which identifies the products of the "4-S" 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, and degradation.

DBT-ring

Figure 1.1

DBT AND ITS SULFUR OXIDATION PRODUCTS



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.

1.3 Summary of Fourth Quarter Results

This report covers the period of March 16, 1989 to June 15, 1989. We report 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 have been unsuccessful. We have 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 has been shown.

The work with the microorganisms (GB-1 and GB-2) from the hydrothermal vents has been fruitful. We are able to show production of DBT sulfoxide and DBT sulfone when GB-1 is grown in the presence of DBT.

Section 2

MICROBES - EXPERIMENTAL RESULTS AND DISCUSSION

2.1 Introduction

Studies of organic desulfurization of coal using microorganisms or enzymes have been in progress in many laboratories for several years. Under a contract from Holometrix, Jannasch and coworkers at the Woods Hole Oceanographic Institute isolated two microorganisms (GB-1 and GB-2) from Guaymas Basin hydrothermal vents that were able to grow in the presence of dibenzothiophene (DBT). Subsequent work with these cultures has continued at Holometrix.

2.2 Studies of Growth Conditions

GB-1 and GB-2 were grown in artificial sea water medium containing DBT and supplemented with 0.05% yeast extract (YE). Within 24 hours of incubation in the presence of DBT, the GB-1 culture medium turned red while the GB-2 culture remained colorless. GB-1 grows without YE and with DBT as sole carbon source and sole sulfur source. GB-2 does not grow without YE supplementation or without another carbon source. Both cultures are aerobic and do not grow above 28°C. Improved growth is obtained with shaking. The red-colored compound(s) seem to be cell-bound/cell-associated since by centrifuging the culture and extracting the supernatant, the red compound(s) was lost in the cell pellet. The red color was found not to be pH-dependent. No further studies have been conducted to identify this compound(s).

2.3 Analysis of Cell-Free Extract Activity Vs. DBT

During the initial growth experiments, GB-1 and GB-2 were grown without DBT in media supplemented with YE. After 48 hours and 96 hours of incubation, the cultures were centrifuged. The activity of the cell-free extracts (CFEs) were tested at 25°C against 0.1% DBT at pH 5.0, 6.0, 7.0, and 8.0. GB-1 CFE at pH 6.0 and 7.0 turned red in color within 24 hours of

incubation with DBT. No obvious change was observed with GB-2 CFE. Analysis of products from such CFE reactions are yet to be performed and similar experiments also will be set up with shaking of the reaction mixtures.

2.4 Analysis of DBT-Degradation Products Extracted from Media

Cells were grown as described in Section 2.2 with DBT and YE. Preliminary experiments involved extraction of growth media at various times with methylene chloride (with and without acidification prior to extraction). Concentrated extracts were examined for their absorption spectra by UV spectroscopy, and it was found that the spectra were obscured by the media components and the multiplicity of DBT-derived products. The absorption maximum of the red-colored compound was at 528 nm.

With the conditions described in the Third Quarterly Report (Holometrix Report No. 2465), it was possible to separate DBT, biphenol, and DBT sulfoxide using gas chromatography (GC). DBT sulfone has the same retention time (RT) as DBT sulfoxide under the conditions used. Analysis of samples by GC was hindered by problems with material carryover from one sample injection to the next.

The next set of experiments involved the use of thin-layer chromatography (TLC) for the identification of DBT and its sulfur oxidation products in the media extractions. Cultures grown on DBT (0.01% and 0.05% DBT concentrations were tested) were acidified to pH 2.1 to 2.3 and extracted in an equal volume of methylene chloride. The methylene chloride extract was evaporated to dryness and the material was resuspended in a small volume of methylene chloride. Ten microliters of this was then spotted for each plate.

Three different systems were used:

(1) alumina plates (Eastman Kodak 13252) with fluorescence indicator (No. 6063) were used with ethanol:water:ammonium hydroxide (2:15:1) as eluent;

(2) silica gel plates -60F254 (Merck No. 5628) with chloroform: acetone (80:20) as eluent; and

(3) silica gel plates -60F_{254} (Merck No. 5628) with benzene: chloroform (97:3) as eluent.

With System 1, of the four "4S" pathway products, only biphenol fluoresces (with a purple fluorescence). When GB-1 was grown in DBT-medium supplemented with yeast extract and extracted, concentrated and observed by this system, a compound that fluoresced purple was observed. The Rf of this compound was different, however, from that of biphenol (Figure 2.1). When two-dimensional TLC was done with this sample and compared to a two-dimensional run of biphenol, however, the Rf values were very close. When biphenol was added to the sample and this was run on alumina plates, it was clear that the fluorescing compound in the sample was different from biphenol.

On silica gel plates with chloroform:acetone (80:20) as eluent, all four "4S" pathway oxidation products are visible under UV light. In this system, the same GB-1 sample gave a fluorescence at an Rf similar to that of the DBT sulfoxide. Other compounds in the mixture were observable with visible light. No compounds were observed with GB-2 cultures on TLC.

On silica gel plates with benzene:methanol (97:3) as eluent, better separation was observed between DBT sulfoxide and biphenol. It was otherwise similar to System 2. It was obvious that GB-1 growing on DBT produced compounds of interest and a more quantitative analytical method was required.

Recently, we have developed a HPLC method that gives excellent resolution of DBT and its "4S" oxidation products. The column and HPLC conditions used are shown in Figures 2.2a and 2.2b, along with two chromatograms of "4S" standards. Unfortunately, although good resolution of the "4S" oxidation products is obtained with this system, extraneous peaks are observed in the vicinity of the DBT peak even with injection of solvent alone. It is possible to reduce the number of such peaks by modification of the gradient program but we have been unsuccessful in obtaining a cleaner

Figure 2.1a

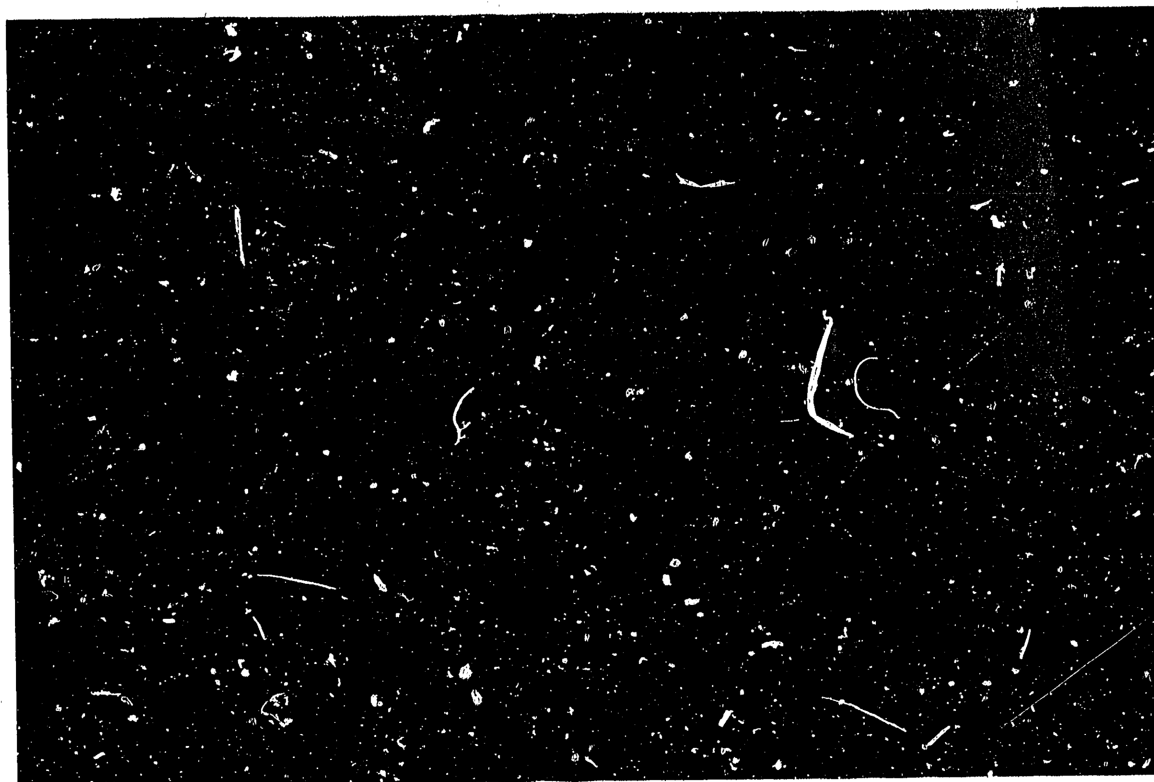
TLC ANALYSIS OF TWO SAMPLES EXTRACTED FROM GB-1 GROWTH MEDIA

(The plate is Kodak alumina 13252 with fluorescent indicator. The eluent is ethanol:water:ammonium hydroxide 2:15:1.)

Sample 1: GB-1 was grown on 0.05% DBT and harvested at 65 hours. After acidification to pH 2.2, the media was extracted with an equal volume of methylene chloride (100 ml). The solvent was reduced to 3 ml before analysis.

Sample 2: GB-1 was grown on 0.01% DBT. The harvesting and extraction conditions are identical to Sample 1.

The plate in visible light



The samples from left to right are: 1) Sample 2 + biphenol; 2) Sample 1 + biphenol; 3) Sample 2; 4) biphenol; 5) Sample 1; 6) DBT; and 7) DBT sulfoxide.

Figure 2.1b

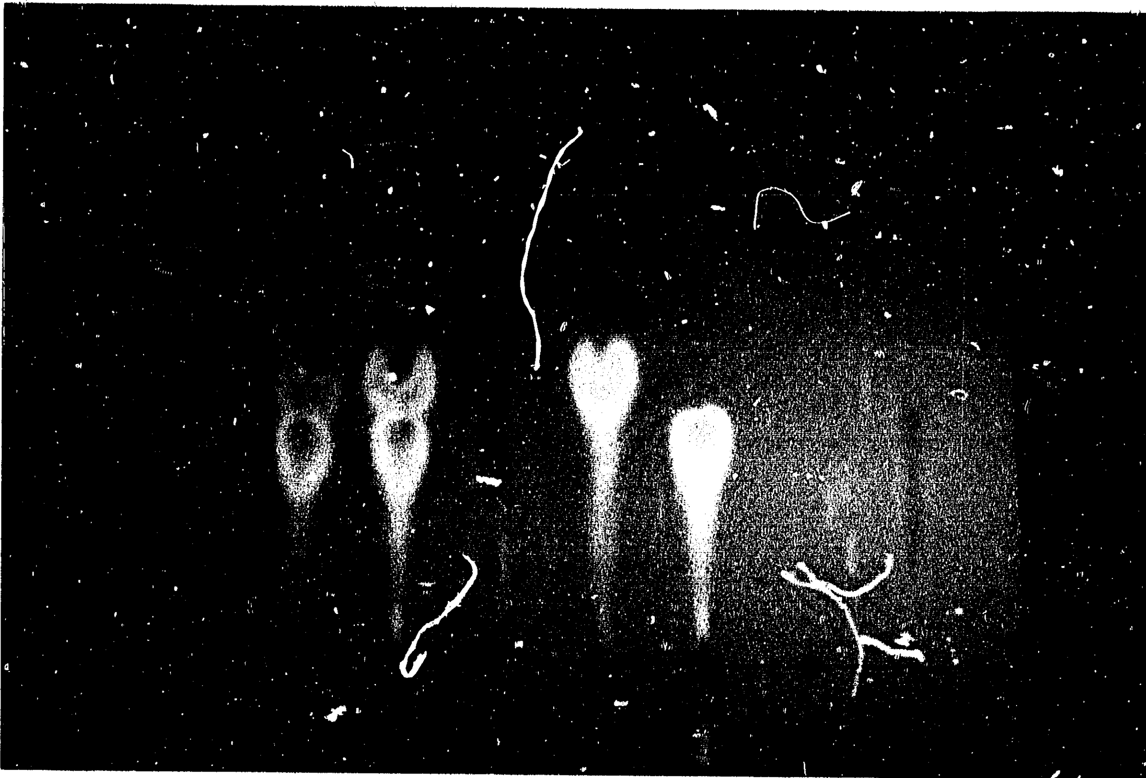
TLC ANALYSIS OF TWO SAMPLES EXTRACTED FROM GB-1 GROWTH MEDIA

(The plate is Kodak alumina 13252 with fluorescent indicator. The eluent is ethanol:water:ammonium hydroxide 2:15:1.)

Sample 1: GB-1 was grown on 0.05% DBT and harvested at 65 hours. After acidification to pH 2.2, the media was extracted with an equal volume of methylene chloride (100 ml). The solvent was reduced to 3 ml before analysis.

Sample 2: GB-1 was grown on 0.01% DBT. The harvesting and extraction conditions are identical to Sample 1.

The plate in UV light



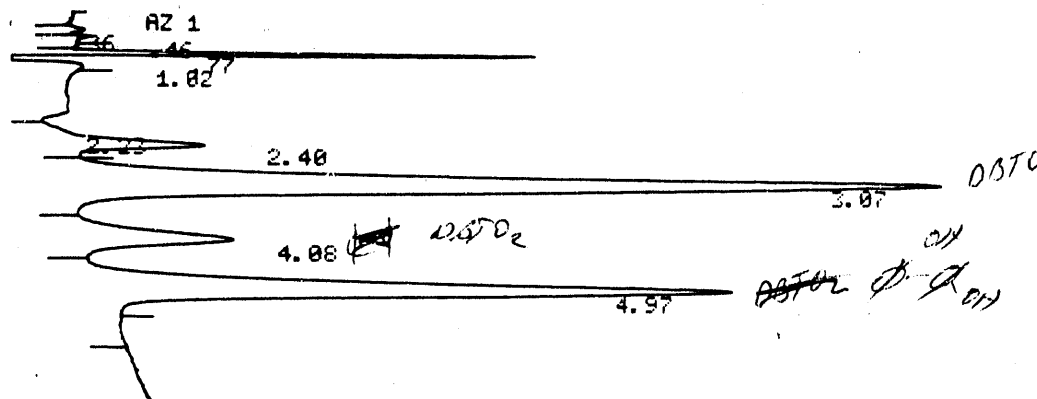
The samples from left to right are: 1) Sample 2 + biphenol; 2) Sample 1 + biphenol; 3) Sample 2; 4) biphenol; 5) Sample 1; 6) DBT; and 7) DBT sulfoxide.

HPLC conditions are as follows:

- Column : Waters resolve 5 micron spherical C18
- Injection Volume: 2 μl
- Eluent A : water
- Eluent B : acetonitrile:water (70:30)
- Gradient Program:

TIME (min)	FLOW RATE (ml/min)	%A	%B	CURVE
Initial	2.0	60	40	*
10	2.0	30	70	6
15	2.0	0	100	6
20	2.0	60	40	6

- HPLC System : Waters model 510 pump system (2); Waters U6K injector; Waters lambda-max model 481LC spectrophotometer (λ = 254 nm AUFS = 0.01; Response time = 0.5A); Waters automated gradient controller; and Spectra Physics SP 4270 integrator.

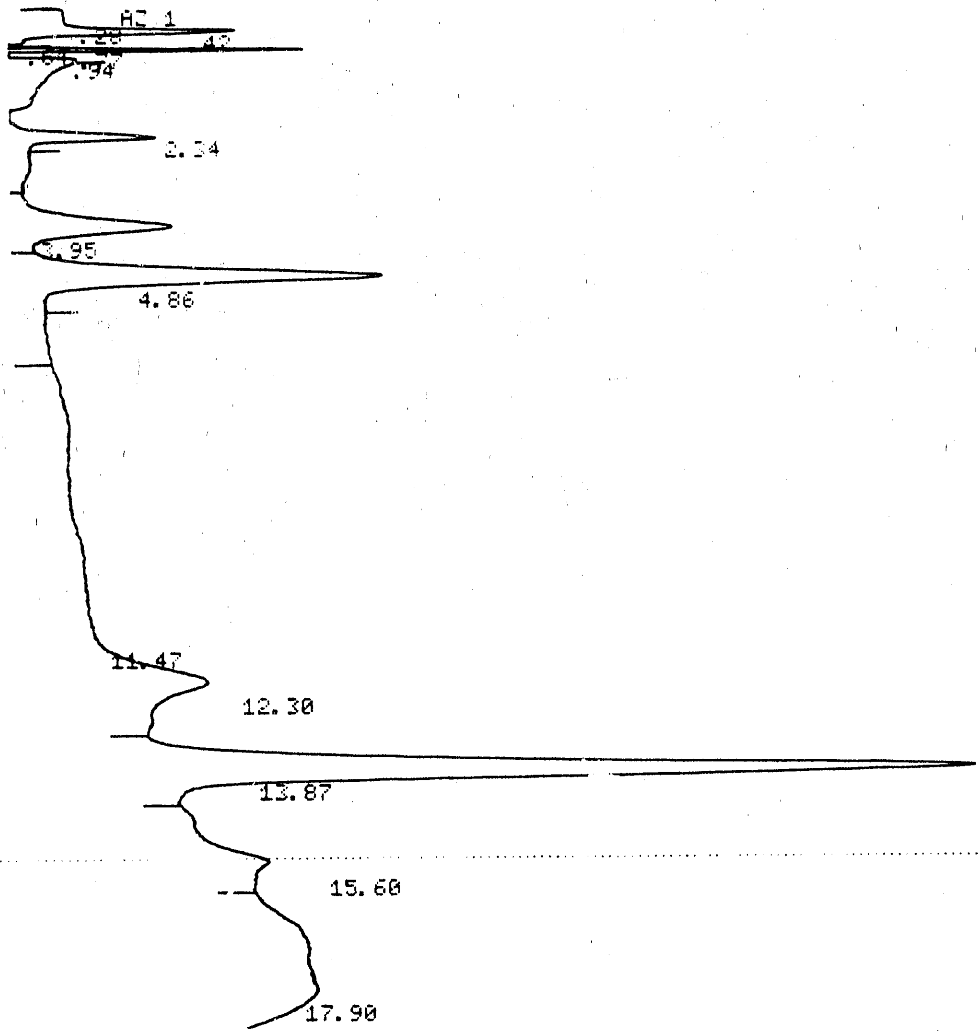


FILE 4. METHOD 0. RUN 37 INDEX 37

PEAK#	AREA%	RT	AREA BC
1	0.158	0.36	1735 02
2	0.17	0.46	1869 03
3	9.331	0.77	102712 02
4	7.812	1.02	85989 03
5	0.382	2.23	4201 02
6	3.799	2.4	41822 03
7	42.539	3.07	468271 02
8	6.931	4.08	76297 02
9	28.879	4.97	317900 03
TOTAL	100.		1100796

Fig. 2.2a: Standards of DBT "4S" oxidation products in methylene chloride. Methylene chloride, DBT sulfoxide, biphenol, and DBT sulfone elute at 2.4, 3.07, 4.08, and 4.97 minutes respectively.

Fig. 2.2b: Standards of DBT and its "4S" oxidation products in methylene chloride. DBT elutes at 13.87 minutes.



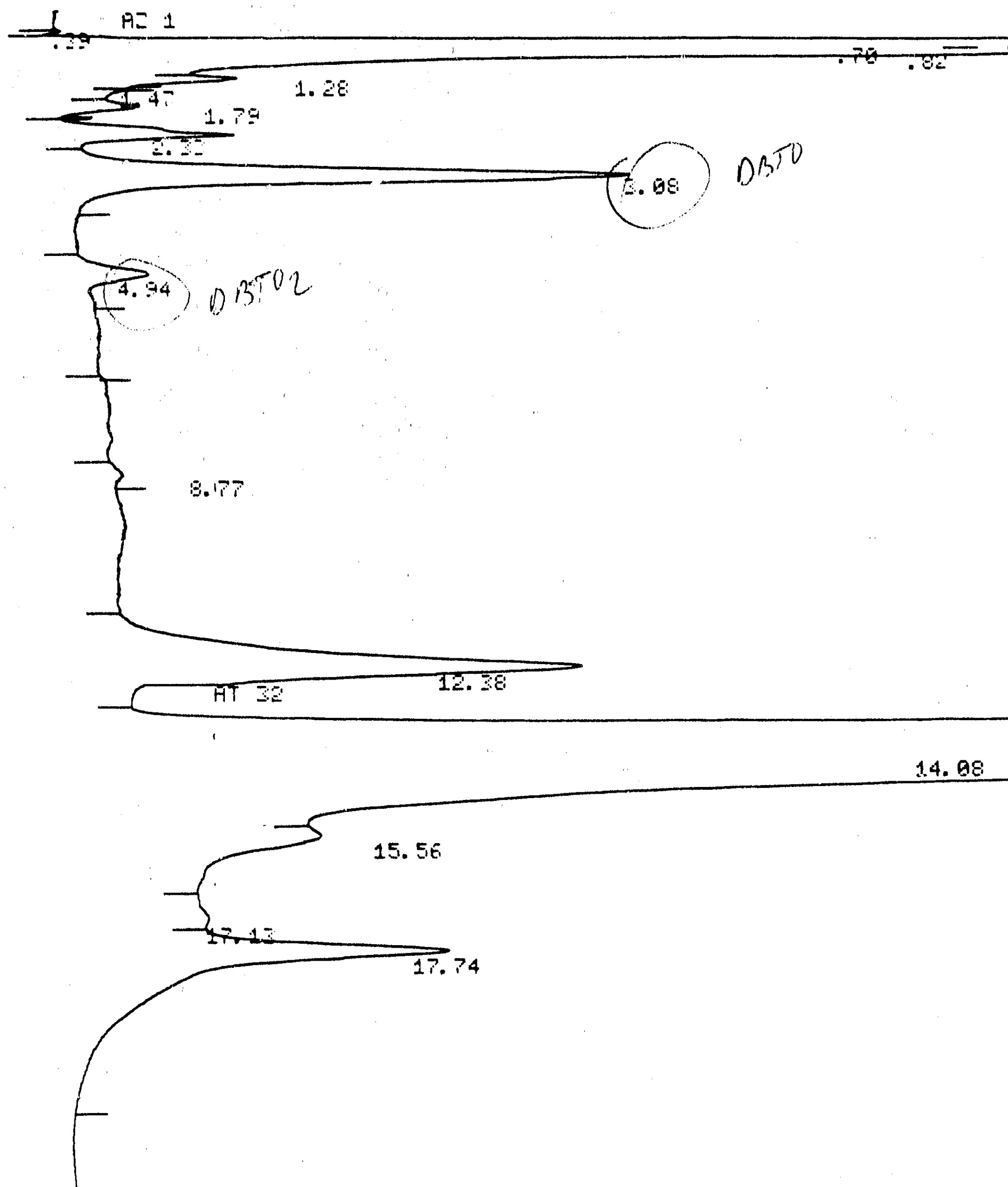
PEAK#	AREA%	RT	AREA	BC
1	0.232	0.28	8135	02
2	1.27	0.42	44585	03
3	1.765	0.77	61957	02
4	1.484	0.94	52089	03
5	1.16	2.34	40708	01
6	2.266	3.95	79531	02
7	5.191	4.86	182203	03
8	5.39	11.47	189212	02
9	8.928	12.3	313405	02
10	21.224	13.87	745024	02
11	13.761	15.6	483060	02
12	37.33	17.9	1310382	03
TOTAL	100.		3510291	

chromatogram. Other solvent systems are being tried as are modifications of the same program.

For analysis of samples by HPLC, GB-1 was grown with shaking in 100 ml volumes in media containing 0.05% and 0.01% DBT. For each time point, a whole bottle of cells and media was harvested. The bottle contents were acidified to pH 2.1 to 2.3 and then extracted with an equal volume of methylene chloride. The methylene chloride was evaporated to dryness and the residual material was resuspended in 2 to 3 ml of methylene chloride.

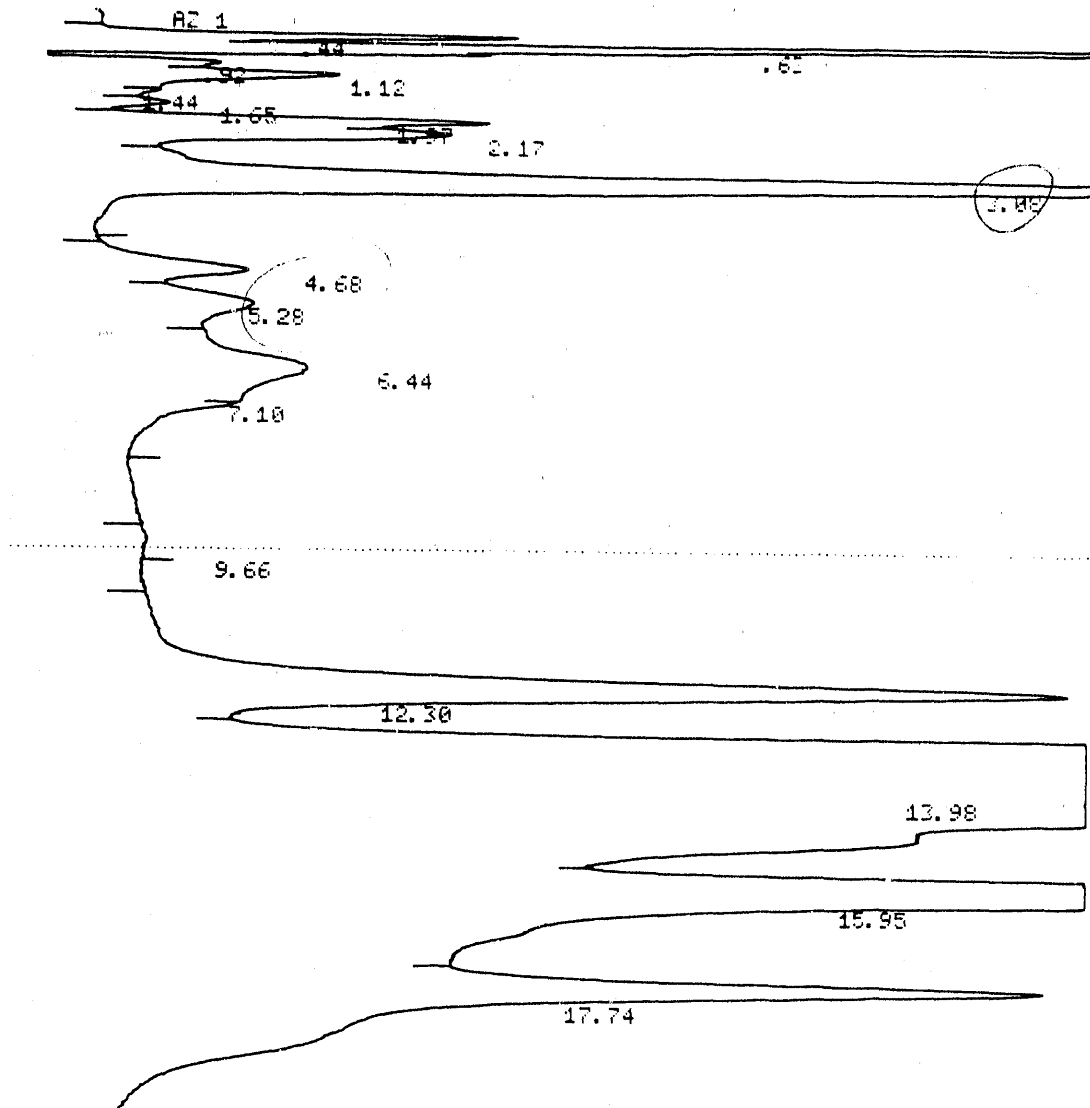
As seen in Figures 2.3 and 2.4, GB-1 grown in 0.05% DBT produces DBT sulfoxide and some DBT-sulfone. The apparent quantities of these compounds vary as a function of time of incubation. Figure 2.5 shows a similar chromatogram from a sample derived from a culture grown with 0.01% DBT from Figures 2.5 and 2.6, it is possible to see the decrease in DBT concentration as a function of time of incubation. Figure 2.7 shows a chromatogram of GB-2 which was grown with 0.05% DBT. The peaks obtained with GB-1 extracts are not observed in GB-2 extracts, indicating that they are indeed DBT oxidation products and not media artifacts.

Fig. 2.3: Sample of GB-1 grown in medium with 0.05% DBT and harvested at 65 hours.



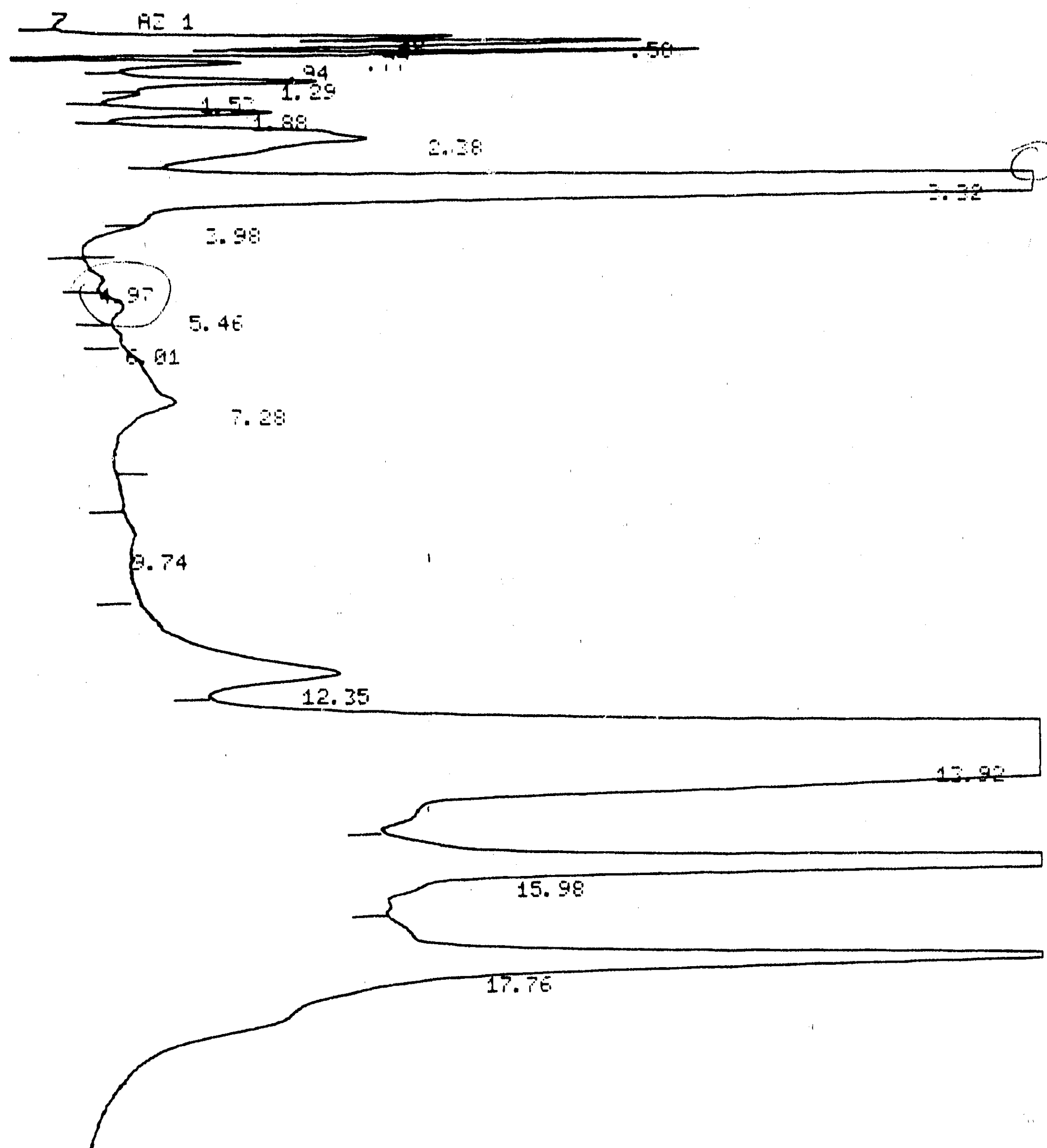
PEAK#	AREA%	RT	AREA	BC
1	0.002	0.39	1275	02
2	0.97	0.7	722204	02
3	1.605	0.82	1194166	08
4	0.018	1.28	13095	05
5	0.	1.47	68	05
6	0.021	1.79	15969	05
7	0.091	2.33	68097	02
8	0.455	3.08	338417	03
9	0.045	4.94	33286	01
10	0.006	8.77	4574	01
11	0.698	12.38	519116	02
12	93.203	14.08	69365094	02
13	1.04	15.56	773916	02
14	0.441	17.13	327942	02
15	1.406	17.74	1046584	03
TOTAL	100.		74423803	

Fig. 2.4: Sample of GB-1 grown in medium with 0.05% DBT and harvested at 113 hours.



PEAK#	AREA%	RT	AREA	BC
1	0.144	0.44	137958	02
2	0.521	0.63	500966	02
3	0.148	0.92	141992	02
4	0.369	1.12	354947	02
5	0.114	1.44	109824	02
6	0.166	1.65	159479	02
7	0.258	1.97	248003	02
8	0.326	2.17	312820	02
9	1.138	3.08	1093210	03
10	0.098	4.68	94585	02
11	0.165	5.28	158565	02
12	0.357	6.44	343257	02
13	0.071	7.1	68345	03
14	0.003	9.66	2604	01
15	0.836	12.3	803583	02
16	89.958	13.98	86427400	02
17	4.029	15.95	3871029	02
18	1.298	17.74	1246986	03
TOTAL	100.		96075553	

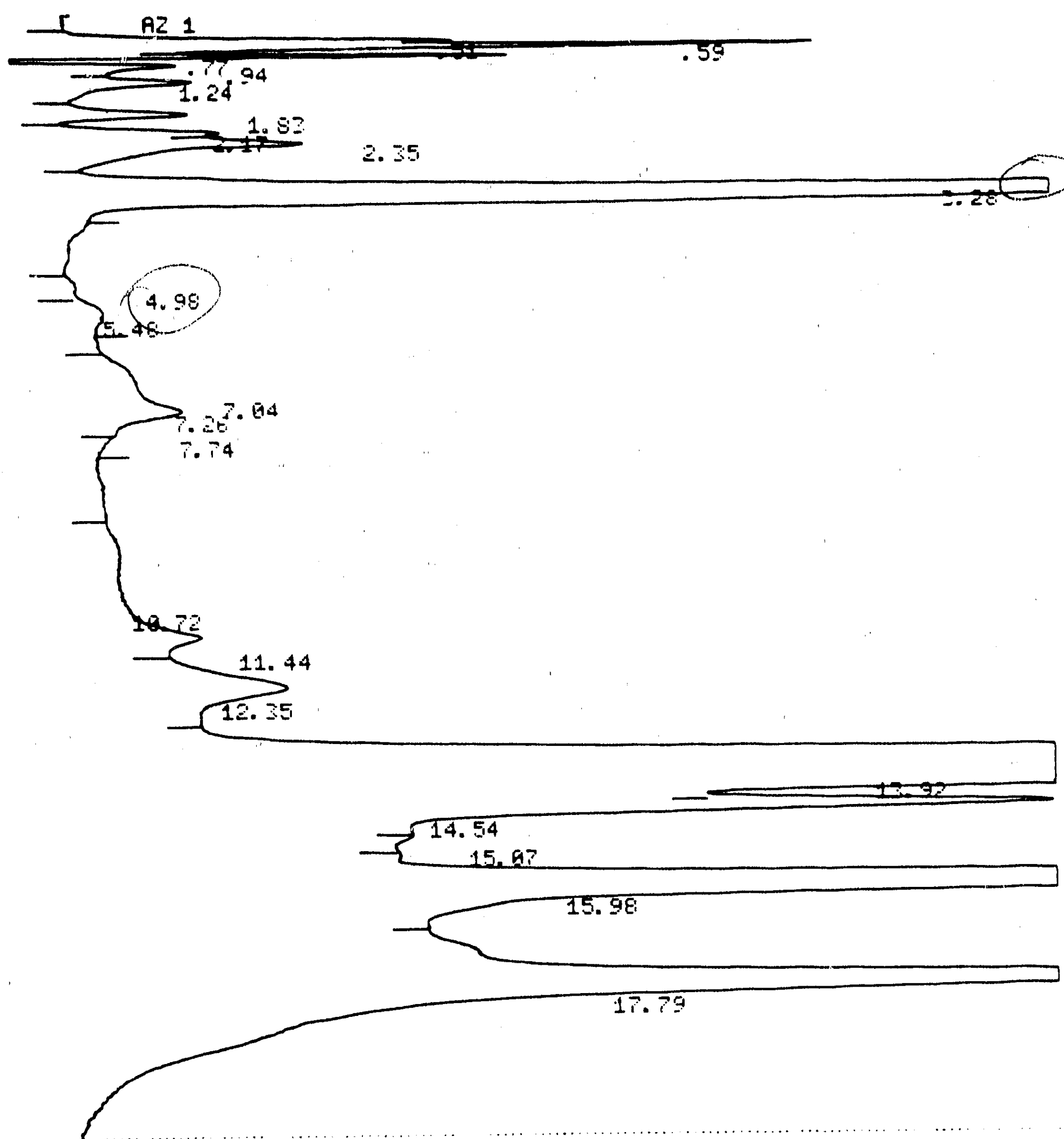
Fig. 2.5: Sample of GB-1 grown in medium with 0.01% DBT and harvested at 113 hours.



PEAK#	AREA%	RT	AREA	BC
1	0.2	0.48	91329	02
2	0.383	0.58	174615	02
3	0.256	0.77	116784	02
4	0.329	0.94	149954	02
5	0.497	1.29	226286	02
6	0.216	1.53	98436	02
7	0.393	1.88	179247	02
8	1.076	2.38	490402	02
9	5.319	3.32	2423327	02
10	0.082	3.98	37407	03
11	0.025	4.97	11202	02
12	0.06	5.46	27338	02
13	0.044	6.01	20045	02
14	0.293	7.28	133694	03
15	0.08	9.74	36329	02
16	0.743	12.35	338690	02
17	82.64	13.92	37652043	02
18	3.677	15.98	1675285	02
19	3.685	17.76	1678988	03
TOTAL	100.		45561401	

Figure 2.6

SAMPLE OF GB-1 GROWN IN 0.01% DBT AND
HARVESTED AT 161 HOURS

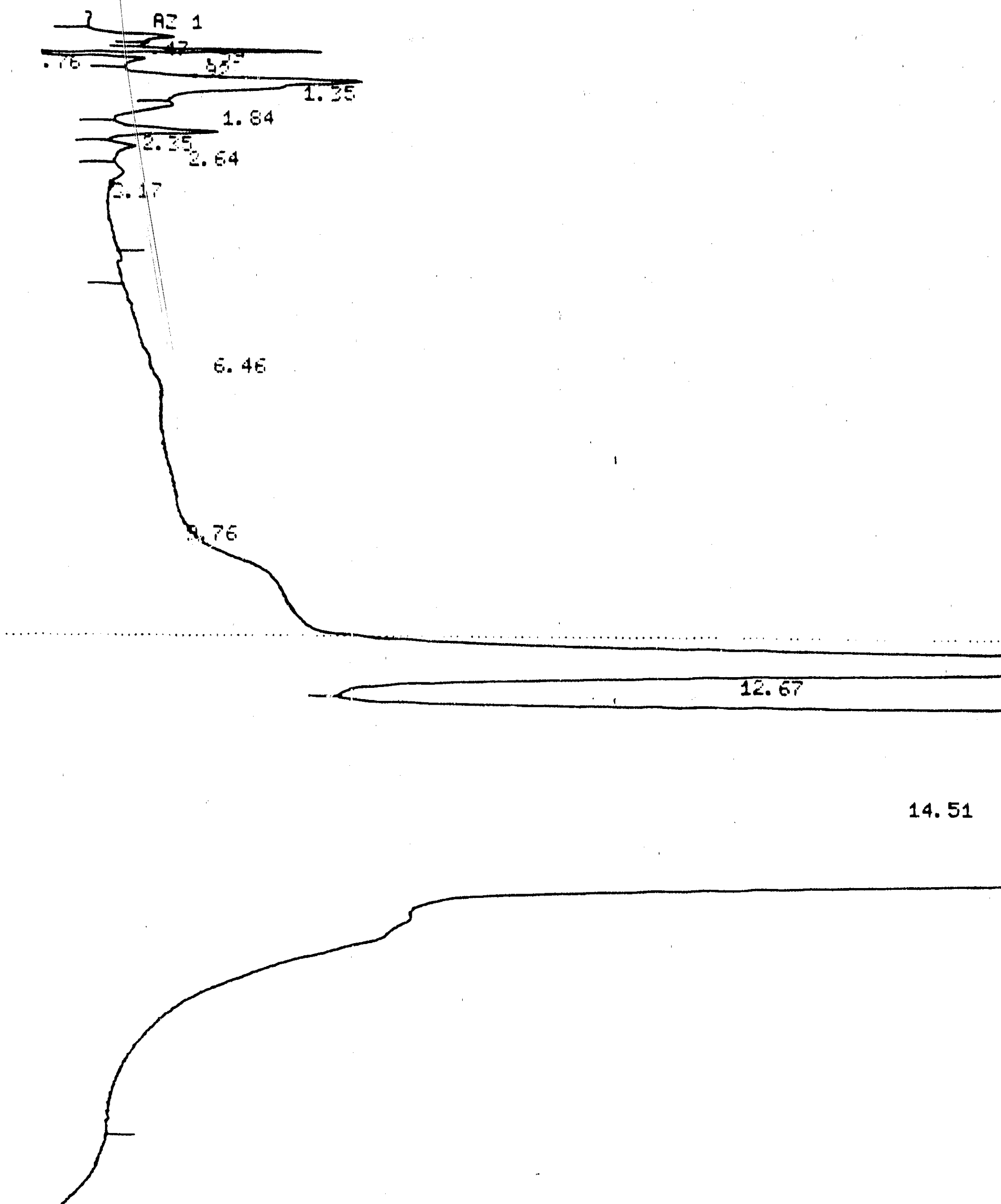


FILE 4. METHOD 0. RUN 46 INDEX 46

PEAK#	AREA%	RT	AREA	BC
1	0.429	0.51	96216	02
2	0.924	0.59	207141	02
3	0.428	0.77	95880	02
4	0.714	0.94	160109	02
5	1.317	1.24	295210	02
6	0.829	1.83	185819	02
7	0.497	2.17	111476	02
8	1.168	2.35	261868	02
9	5.137	3.28	1151416	03
10	0.017	4.98	3826	02
11	0.071	5.48	15948	03
12	0.195	7.04	43616	02
13	0.274	7.26	61320	02
14	0.021	7.74	4773	03
15	0.157	10.72	35210	02
16	0.501	11.44	112253	02
17	1.356	12.35	304005	02
18	62.062	13.92	13911057	02
19	2.837	14.54	635932	02
20	0.829	15.07	185731	02
21	10.959	15.98	2456329	02
22	9.277	17.79	2079489	03
TOTAL	100.		22414724	

Figure 2.7

SAMPLE OF GB-2b GROWN IN 0.05% DBT AND 0.1% SUCCINATE AND HARVESTED AT 120 HOURS



FILE 4. METHOD 0. RUN 54 INDEX 54

PEAK#	AREA%	RT	AREA	BC
1	0.027	0.47	54209	02
2	0.017	0.59	34722	02
3	0.035	0.76	70058	02
4	0.047	0.92	95446	02
5	0.19	1.35	381777	02
6	0.07	1.84	140557	02
7	0.063	2.35	126389	02
8	0.044	2.64	88768	02
9	0.074	3.17	149516	03
10	0.015	6.46	29621	02
11	0.151	9.76	304831	02
12	1.193	12.67	2403410	02
13	98.074	14.51	197559675	03
TOTAL	100.		201438979	

Section 3

ENZYMES - EXPERIMENTAL RESULTS AND DISCUSSION

3.1 Stability of Laccase in Ethylacetate or Acetonitrile

To examine the lifetime of laccase in the hydrated organic solvents, the following experimental procedure was followed:

(1) laccase stock solution was prepared as described in the previous quarterly report;

(2) aliquots of the stock solution were added to tubes of ethylacetate and acetonitrile, in the way done with DBT/EPS assays;

(3) one aliquot of each of the enzyme solutions in hydrated organic solvent was mixed with syringaldazine solution (in the matching solvent) and changes in absorbance in the range 200 to 800 nm were measured;

(4) the other enzyme solutions in hydrated organic solvents were stored at room temperature and at one day and at five days, aliquots were removed and assays with syringaldazine were repeated; and

(5) the results, changes in absorbance at selected wavelengths where changes were significant, are shown in Figures 3.1 and 3.2 which plot the absorbances at each time point and show the least squares fit for the first 30 minutes of the assay.

It can be seen that the activity of laccase vs. syringaldazine (as indicated by the slopes of the lines) is not significantly changed over the five day period.

3.2 Laccase Assay Vs. DBT Sulfone

Assay samples contained 0.2 mM DBT sulfone as the substrate, laccase as the enzyme (0.2 mg/ml) and either acetonitrile or ethyl acetate with 1% water (buffered to pH = 7) as the solvent. Blank samples (i.e., DBT sulfone with no laccase and laccase without DBT sulfone) were also prepared.

The samples were constantly stirred at room temperature for one week. Aliquots were removed at 0 hours, 24 hours, and 7 days. Upon removal, aliquots were filtered through a 0.2 µm pore size, Nylon 66, syringe filter unit (Rainin No. 38-159).

In an attempt to avoid the extensive time requirements for gas chromatography analysis, thin-layer chromatography was chosen as an alternate method. Plastic-backed silica gel 60 TLC plates (Merck No. 5735) with an indicator that fluoresces at 254 nm were used. By exposing the developed plate to UV light, a positive detection of a compound would be indicated by the lack of fluorescence. The solvent system used was chloroform:acetone (80:20). Samples were spotted on the plates with either 1 µl Microcaps® or with a 100 µl syringe. In the case of multiple drops of liquid per sample, the TLC plate was dried under a blower (room temperature) between applications of sample.

In the assay sample mentioned above, the purpose of the experiment was to determine whether laccase could convert DBT sulfone to O,O'-biphenol by the "4S" pathway or, for that matter, to any other compound. It was determined that 5 µl of 0.2 mM (initial concentration of DBT sulfone in the assay) "4S" standards could be detected in the described TLC system. If we assume a 1% conversion of DBT sulfone to biphenol, in order for the biphenol to be detectable, 500 µl of sample would have to be spotted. (500 µl of the biphenol standard could be detected by our TLC system.) However, no biphenol was detected in our assay samples ($t = 24$ hours and 7 days) in ethyl acetate and acetonitrile even when such large volumes were spotted. It is clear that the activity, if any, of laccase against DBT sulfone is negligible in the solvent systems tried.

3.3 Laccase Assay vs. Ethyl Phenyl Sulfide (EPS)

Assays similar to the DBT-sulfone:laccase assays (Section 3.2) were set-up using ethyl phenyl sulfide (EPS) as the substrate in either ethyl acetate or acetonitrile with 1% buffered water. The TLC plates and conditions used were identical to those described in Section 3.2. However, the detection limits of EPS and its "4S" components differ greatly from that of DBT and its components. It was found that the lower limit of detection

Figure 3.1

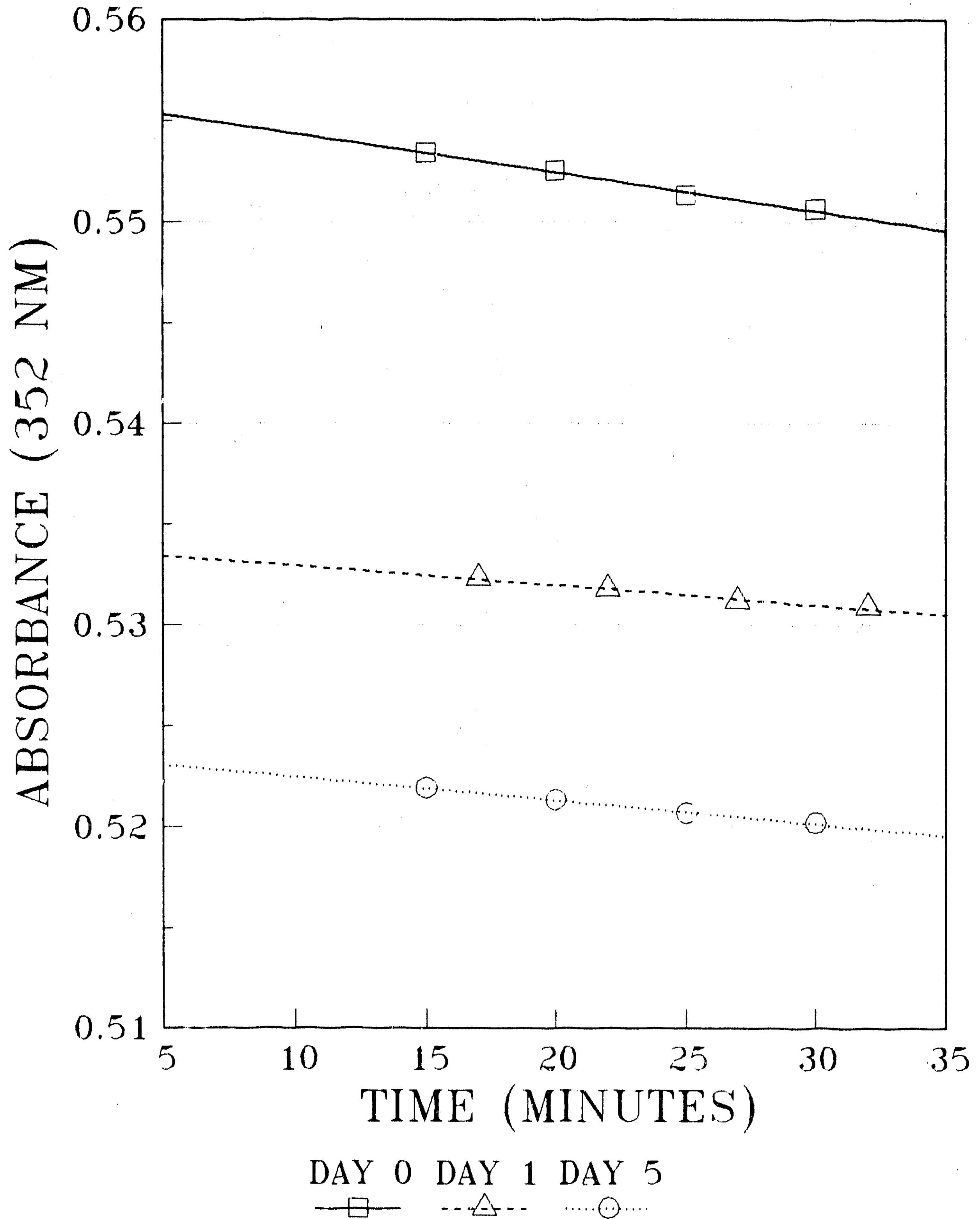
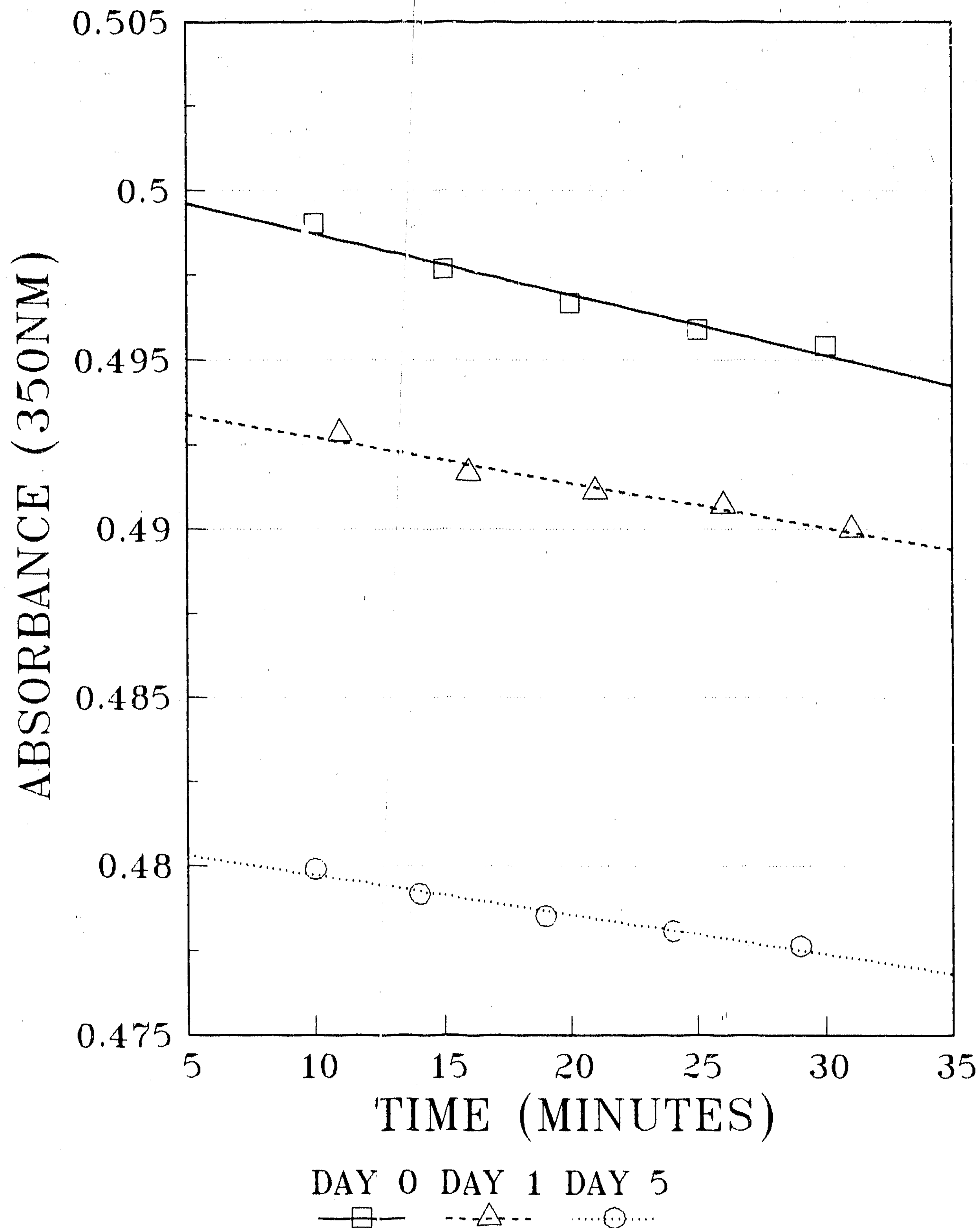


Figure 3.2



on the TLC plate was a 5 μl spot of a 20 mM solution of EPS. This gives a 0.001 mM lower detection limit compared to DBT and its "4S" oxidation products. If a 1% conversion of EPS (initial concentration = 0.2 mM) occurs, there would be 0.002 mM of the component(s). In order to see this small amount, 50 ml would have to be spotted. This is obviously beyond the limits of the experiment. GC analysis procedures are being investigated at this time to monitor assay results.

3.4 Laccase Assays Vs. DBT in Acetonitrile and Ethylacetate (Repeats)

The assays of laccase vs. DBT in hydrated acetonitrile and hydrated ethylacetate which were reported in the Third Quarterly Report were repeated. In that earlier report, we had shown a GC-mass spectroscopy analysis of the 24 hour aliquot from the laccase/DBT assay in acetonitrile. In this analysis, we observed the DBT peak and a small peak which ran earlier than biphenol. This second peak was identified from the Mass Spec Library as 1,1-thiobisbenzene. It was suggested to us by the DOE staff that this peak was actually biphenol.

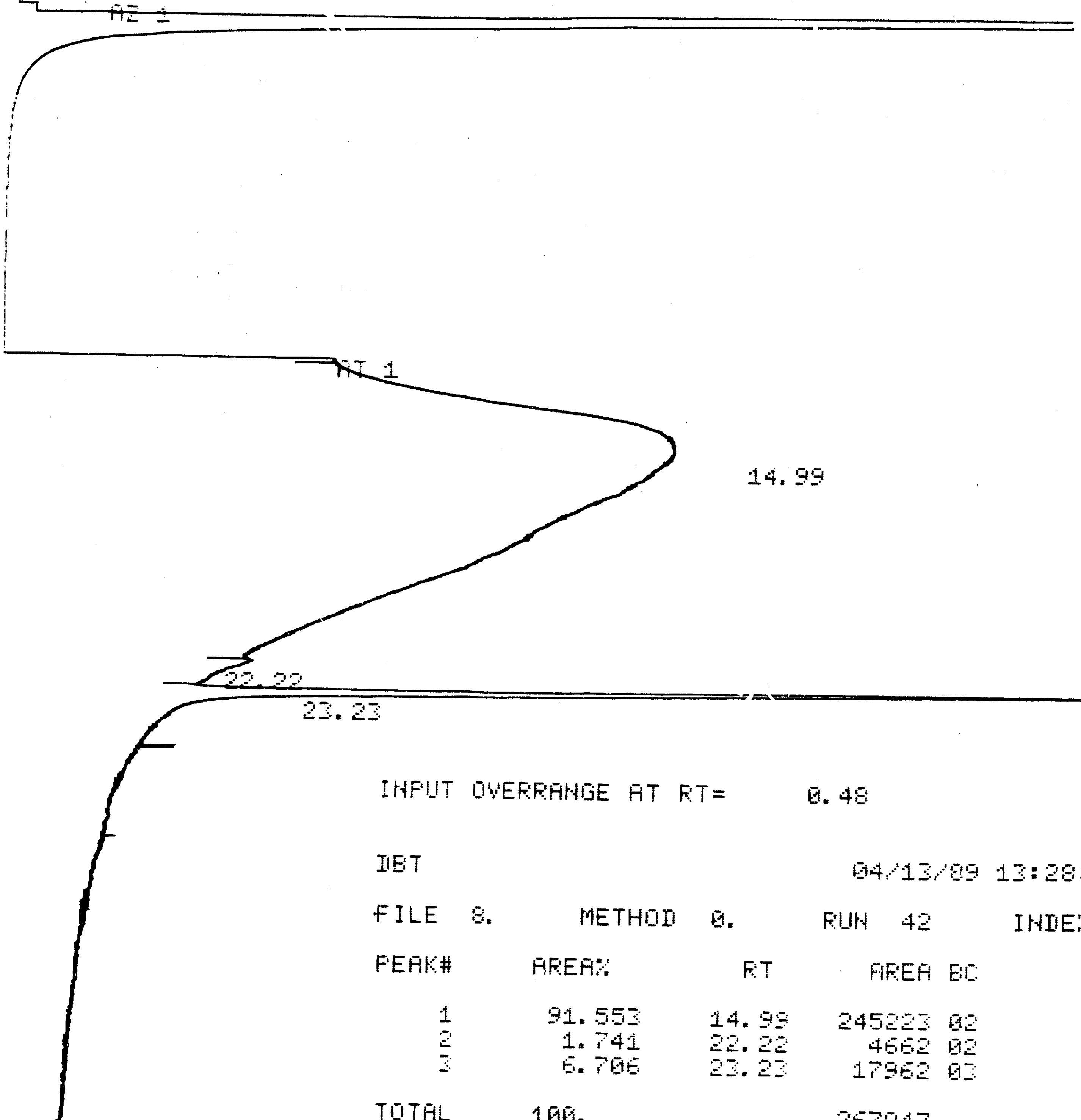
To evaluate this hypothesis, the comparable sample from therepeat assay was analyzed by GC and we observed a very small biophenol peak. The chromatogram and a "4S" standard run under the same conditions, are shown in Figure 3.3. Another 24 hour aliquot from this repeat experiment (laccase/DBT in acetonitrile) was analyzed by GC mass spectroscopy and neither biphenol nor the early peak seen in the previously reported sample was observed. This information is shown in Figure 3.4. These results are conflicting; because we have observed carryover problems with the GC, we are more inclined to believe the GC Mass Spec data.

3.5 Enzyme Kinetics - Reversibility Studies

In the Third Quarterly Report, kinetic studies of interactions of DBT, EPS, and their respective sulfur oxidation products with laccase, horseradish peroxidase, and sulfatase were reported. In this quarter, we have investigated the reversibility of the reactions observed.

Figure 3.3a

GAS CHROMATOGRAPHIC ANALYSIS OF A 24 HR.
ALIQUOT FROM LACCASE/DBT ASSAY
IN HYDRATED ACETONITRILE
(A - Sample C17-45-6F)

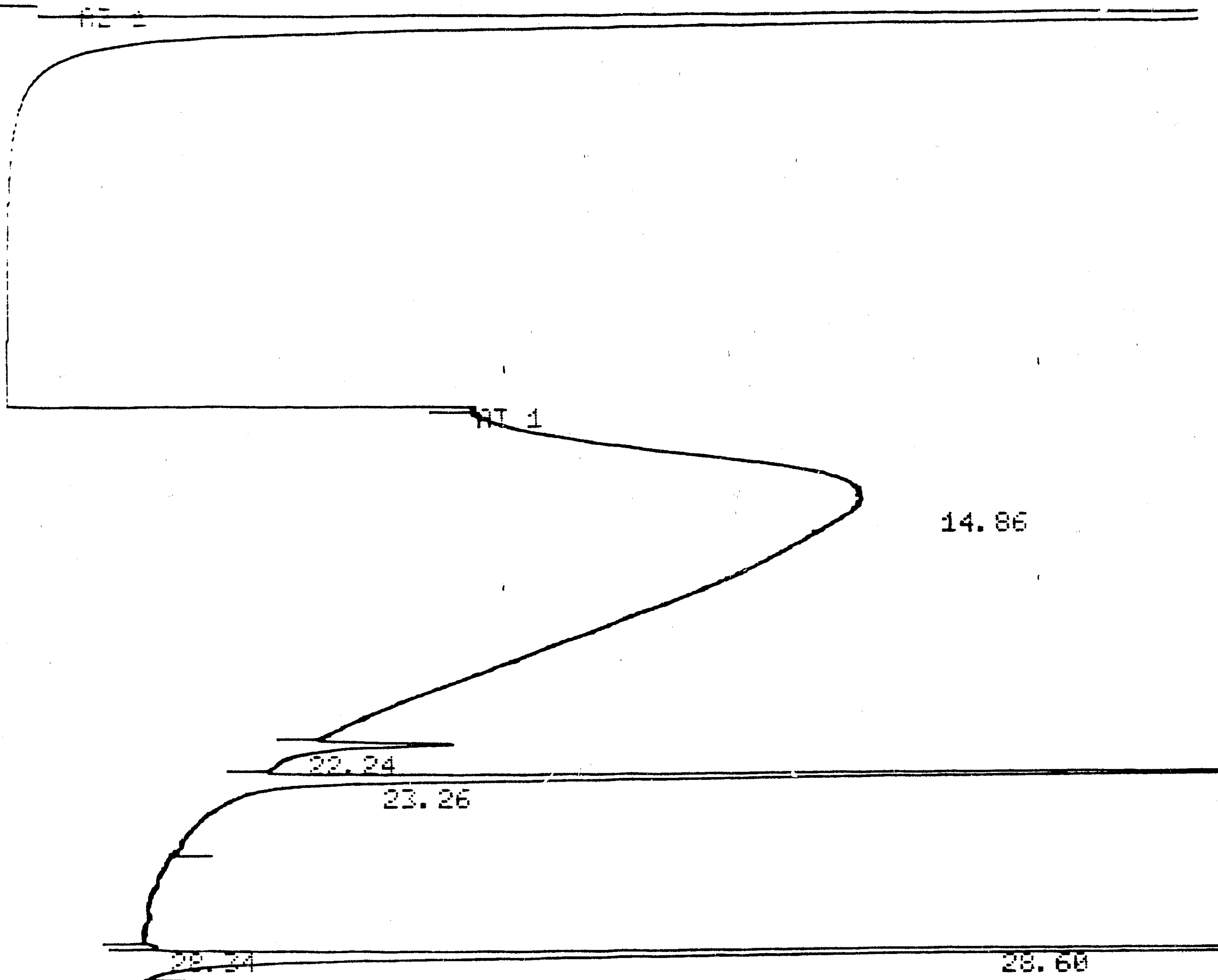


PEAK#	AREA%	RT	AREA BC
1	91.553	14.99	245223 02
2	1.741	22.22	4662 02
3	6.706	23.23	17962 03
TOTAL	100.		267847

Figure 3.3b

"4S" STANDARDS (0.2 mM Each)
1 ul injection

CHANNEL A INJECT 04/06/89 15:20:02



36.38

DBT 04/06/89 15:20:02

FILE 0. METHOD 0. RUN 9 INDEX

PEAK#	AREA%	RT	AREA	BC
1	81.457	14.86	257028	02
2	2.323	22.24	7329	02
3	6.961	23.26	21964	03
4	0.033	28.34	104	02
5	9.114	28.6	28757	03
6	0.113	36.38	357	01
TOTAL	100.		315539	

No. 2: biphenol
No. 3: DBT
No. 5: DBT sulfoxide/sulfone

Figure 3.4a

GC MASS SPECTROSCOPY ANALYSIS OF A 24 HR.
ALIQUOT FROM LACCASE/DBT ASSAY
IN HYDRATED ACETONITRILE
(A - Column Chromatogram)

```
MS data file header from : 4789

Sample: C14 43 6F               Operator: NANCY     Acq. Ctr. [illegible]
Misc   :
Sys. #:     1  MS model:  88  SW/HW rev.: 1A  ALS # :  0
  Method file: [illegible]      Tuning file: [illegible]   No. of extra records:  [illegible]
 Source temp.:  200   Analyzer temp.:  280     Transfer line temp. :   0

      Chromatographic temperatures :  35.   300.     0.     0.     0.
      Chromatographic times, min.  : 10.0    5.0    0.0    0.0    0.0
      Chromatographic rate, deg/min:  7.0    0.0    0.0     .1    0.0

>4789          C14 43 6F
  40.0| 800.0 ADC TIC
Upslope:     .20   Area Reject:10.00 %    Max Peaks:  1   Bunching:  1
Dnslope:    0.00   Results File VDIR82    Sorted by Time/Area  INT
```

Peak #	R.T. min.	first scan	max scan	last scan	peak height	raw area	corr. area	corr. % max.	% of total
1	29.88	[illegible]	936	939	8429	28100	28100	100.00	100.00

Sum of corrected areas: 28100.

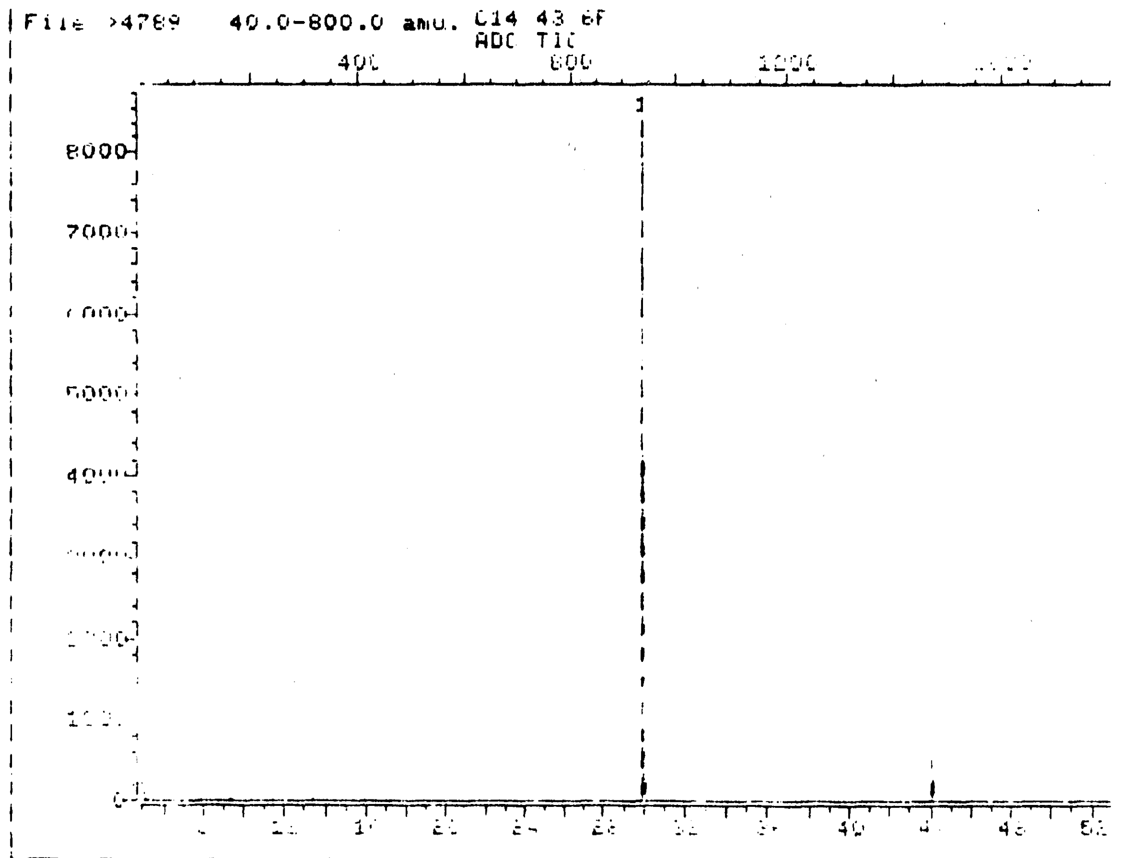
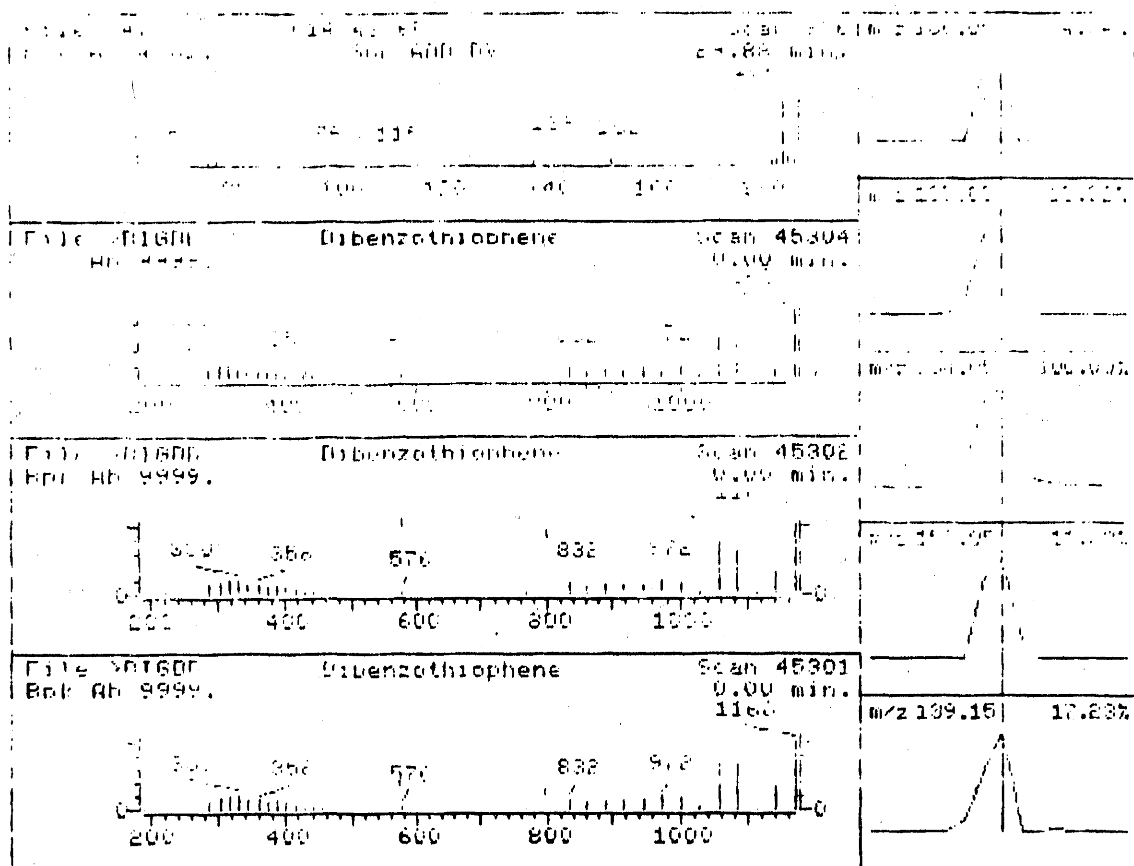


Figure 3.4b

MASS SPECTRUM ANALYSIS OF MAJOR PEAK



1. Dibenzothiophene 184 C12H8S
2. Dibenzothiophene 184 C12H8S
3. Dibenzothiophene 184 C12H8S
4. Dibenzothiophene 184 C12H8S
5. Dibenzothiophene 184 C12H8S

Sample file: [illegible]4789 Spectrum #: 93[illegible]
Search speed: 1 Tilting option: 5 No. of ion ranges searched: 67

	Prob.	CAS #	CON #	ROOT	F	DR	#FLG	TILT	[illegible]	CON	C_I	R_IV
1.	83*	132650	45304	"B1GDB	49	57	2	0	72	5	57	22
2.	79*	132650	45302	"B1GDB	59	40	2	1	91	[illegible]	46	33
3.	78*	132650	45301	"B1GDB	50	51	2	2	76	1	55	19
4.	76*	132650	45061	"B1GDB	31	82	3	0	100	1	55	13
5.	70*	132650	45303	"B1GDB	49	54	2	1	74	7	42	16

Reversibility of Organic Sulfur-Enzyme Interactions

In order to further evaluate enzyme inhibition by DBT, EPS, and oxidized products, we measured the reversibility of the binding of the compounds to the enzymes of interest. These experiments were done by simply varying the preincubation time for enzyme and test compound (before triggering the reaction by addition of standard substrate) recognizing that an increase in preincubation time will generally produce an apparently lower I-50 for irreversible ligands. The results are shown in Figure 3.5. Statistical analysis of the data by t-test demonstrated that all of the interactions studied are reversible.

The assay procedures for each enzyme (i.e., the defined substrates, buffers, and reagents that are conventionally used for determining specific activity of each enzyme) are described below. Aliquots collected are in storage awaiting analysis.

Horseradish Peroxidase Assay

HRP was assayed in the presence of hydrogen peroxide as cofactor and 4-aminoantipyrine in phenol as substrate/indicator in 25 mM Tris buffer, pH 6.0. The total reaction volume was 1.5 ml including organic solvent. Blanks were run without peroxide and without enzyme. Activity was measured at 510 nm. Enzyme was added to a complete reaction mixture at t = 0 and t = at 5 minutes, and the activity was read over a period of three minutes reaction time.

Laccase Assay

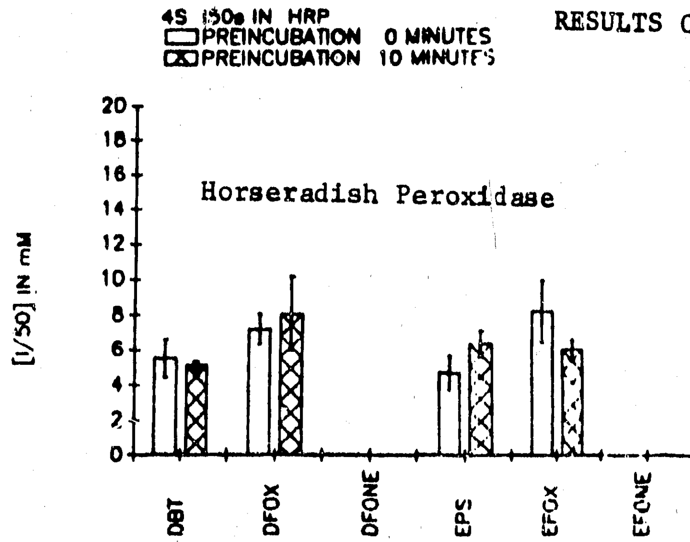
LAC activity was assayed with the substrate syringaldazine made up in organic solvent and the enzyme in a buffer of 0.1 M Na_2PO_4, pH 6.5. A blank was run without enzyme. Activity was measured at 530 nm. Enzyme was added at t = 0 and t = 8 minutes, and the activity was read over a period of three minutes reaction time.

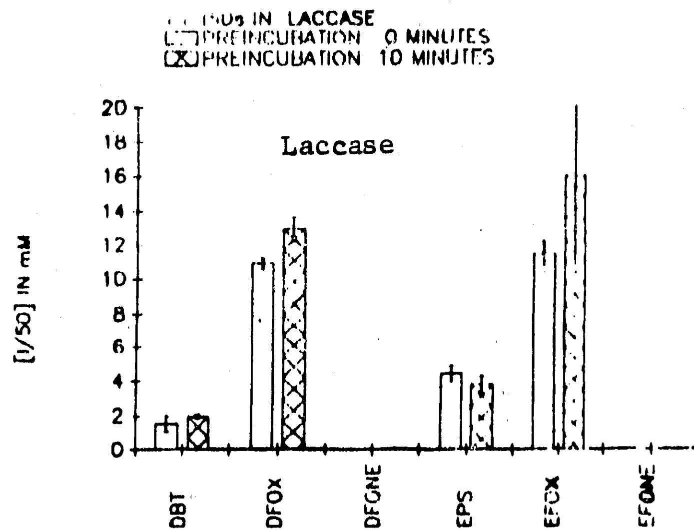
Sulfatase Assay

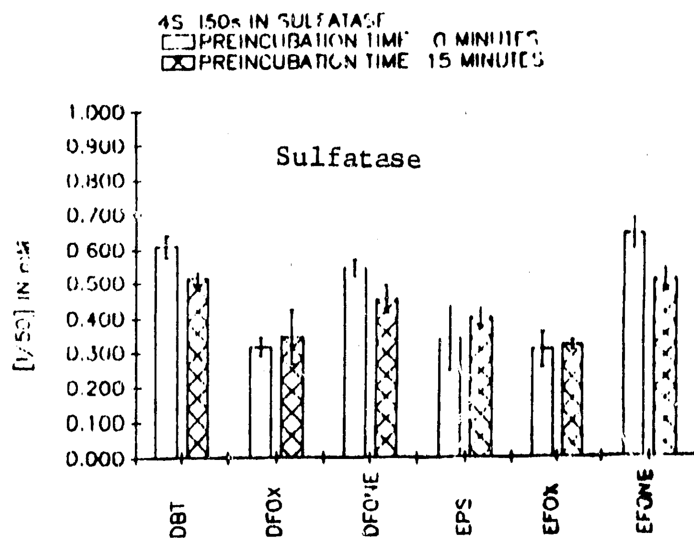
Sulfatase activity was found to be especially sensitive to temperature, so all assays were run at a constant temperature of 37°C. SULF

Figure 3.5

RESULTS OF BINDING REVERSIBILITY STUDIES







activity was assayed with p-nitrocatechol sulfate as substrate in a buffer of 0.2 M NaAc, pH 5.0. Parallel assays were set up to contain substrate, buffer, organic solvent, inhibitor, and enzyme or an equal volume of buffer. In the assays without enzyme (blanks), 5 ml of 1 N NaOH were added at t = 0 to stop the reaction. Blanks and enzyme assays were incubated in a water bath for 30 minutes. At t = 30 minutes, the enzyme assays were stopped with 5 ml of 1 N NaOH. The t = 0 blanks were zeroed at 515 nm, and the activity of the enzyme assays were read as single points.

For all three enzymes, dose-response curves were developed with at least five different substrate concentrations, and the data were evaluated by linear regression analysis of double-reciprocal (Lineweaver-Burk) plots of activity vs. substrate concentration. The following organic sulfur compounds were examined: DBT (Aldrich), DBT-sulfoxide (K&K Biochemicals), DBT-sulfone (Lancaster Bichemicals), EPS (Aldrich), ethylphenylsulfoxide (Lancaster Biochemicals), ethylphenylsulfone (Lancaster Biochemicals), and O,O-biphenol (Aldrich).

3.6 Multi-Enzyme Studies

A series of multi-enzyme studies following the schemes shown in Figure 3.6 were carried out. In summary, the experiments may be subdivided by substrate. For DBT as substrate, subseries A included: (1) the sequence HRP → LAC → SULF; (2) HRP → SULF; and (3) LAC → SULF. A similar subseries (B) utilizing EPS as substrate was carried out. In addition, (subseries C) DBT-sulfoxide was treated with the sequence HRP → LAC → SULF; and DBT sulfone (subseries D) was treated by the same sequence. Similar subseries with EPS sulfoxide (subseries E) and EPS-sulfone (subseries F) were evaluated.

Figure 3.6

MULTI-ENZYME STUDIES

SUBSERIES	SUBSTRATE	ENZYME SEQUENCE
A	DBT	HRP → LAC → SULF HRP → SULF LAC → SULF
B	EPS	HRP → LAC → SULF HRP → SULF LAC → SULF
C	DBT Sulfoxide	HRP → LAC → SULF
D	DBT Sulfone	HRP → LAC → SULF
E	EPS Sulfoxide	HRP → LAC → SULF
F	EPS Sulfone	HRP → LAC → SULF

Key: DBT = dibenzothiophene;
EPS = ethylphenylsulfide;
LAC = laccase;
HRP = horseradish peroxidase; and
SULF = sulfatase

Section 4

EVALUATION OF PROGRESS AND PLANS FOR THE NEXT QUARTER

4.1 Evaluation of Progress

One goal for the first four quarters was to evaluate the activity of selected enzymes against model compounds. Two types of activity were of interest: attack on aromatic rings (for compound solubilization) and sulfur oxidation. Very early in our work we were able to see that the enzymes laccase and horseradish peroxidase did attack the aromatic rings on DBT. We expended considerable effort seeking evidence of sulfur oxidation by these catalysts. This activity is somewhat difficult to determine for two reasons. One reason is that the separation of mixtures of hydrophobic DBT-derived compounds is complicated, and the other is that although we have standards for the sulfur oxidation only DBT-derived products, standards for ring-oxidation products and products in which both sulfur oxidation and ring-oxidation have occurred are not available. Periodically, we did see evidence of "4S" products, but we were plagued with analytical problems and have not been able to obtain conclusive identification of products. An important aspect of the process design was accomplished through kinetic work obtained in Dr. Marquis laboratory. She elucidated the extent of binding of the model compounds to the enzymes of interest.

We believe that the progress of the enzyme work in the first four quarters was very substantial. Although the course of the work did not flow exactly in the direction predicted, we did develop good methods of using the enzymes with DBT and our results will be of direct applicability to our own process development and to others. We have not completed the preliminary studies of the enzymes, particularly studies with EPS and EPS-oxidation derivatives and DBT sulfone as substrates.

The second goal of the first four quarters was to obtain DBT or EPS utilizing microorganisms from the hydrothermal vent soils collected by Dr. Jannasch's group. This work has been very successful, and one of the cultures obtained has been shown to be capable of removing sulfur from DBT.

Our progress in this area has exceeded expectations. It should be noted that to demonstrate this activity in the microorganisms, the microbial work was supported by DynaGen in excess of the amount of required contract cost sharing.

4.2 Plans for the Next Quarter

In the fifth quarter, we will turn our attention to work with coal. While methods of enzyme activity analysis are much easier when the substrate is a single compound, the ultimate program goal is to remove sulfur from coal, not from DBT or EPS. After we complete the analysis of our present enzyme work, we will design the procedures which are anticipated to work best for coal processing studies. The procedures to be elucidated include: choice of specific enzymes for sequential or simultaneous application; choice of media; and method of process validation. The next quarterly report will include a theoretical assessment of the various methods of analysis for sulfur removal from coal, as these methods relate to the enzymatic desulfurization studies. Assuming that the enzymes of choice include those produced by our microorganisms, enzyme isolation and purification will be an important aspect of the work in the fifth quarter.

END

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