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ENZYMATIC DESULFURIZATION OF COAL

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Fifth Quarterly Report

DynaGen Report No. 2473(R)
DynaGen Project No. DOE-12
DOE Contract No. DE-AC22-88PC88855

Submitted to:

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November 7, 1989

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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 SO_4H_2 . 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. Bazylnski (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.

1.3 Summary of Fifth Quarter Results

This report covers the period of June 16, 1989 to September 15, 1989.

During the fifth quarter, we completed more extensive testing of laccase, horseradish peroxidase, and sulfatase in hydrated organic solvents. We find that under the conditions used, the conversion of DBT to "4S" oxidation products is seen, although the yield is low. Other, as yet unidentified, oxidation products are also observed; these are most likely molecules in which carbon-carbon bond cleavage has occurred. While such compounds are "solubilization" intermediates, products targeted in our original work plan, they do not represent the most desirable products because of the loss of energy from carbon-carbon bonds. We have also begun work on the isolation of the microbial enzymes from GB-1 shown to produce "4S" products, however, we have not yet produced dried extract for testing in organic solvents.

Section 2

ENZYMES FROM MICROBES

2.1 Separation of Products of Microbial Degradation of DBT

To separate the products of microbial degradation of DBT, a silica gel column (1 cm x 20 cm) was prepared from a slurry of 6.65 g of silica gel G-60 (90-230 mesh ASTM) in 50 ml of methylene chloride. After the column had equilibrated, 1 ml of the concentrated culture extract was applied, the column was eluted with methylene chloride, and approximately 1 ml fractions were collected. After elution with about 40 ml of methylene chloride, the column was eluted with about 25 ml of ethanol. The fractions were analyzed by TLC first using chloroform:acetone [80:20] as the eluent and then some of the fractions were analyzed using hexane:methylene chloride [50:50] as the eluent (Wyza, 1989). Fractions with the same material were pooled and further observed by HPLC.

GB-1 was grown on 0.01% DBT for 7 days in 100 ml of artificial sea water medium with 0.05% yeast extract. The culture medium was then acidified, extracted in an equal volume of methylene chloride. The methylene chloride extract was evaporated and then taken up in 2 ml of methylene chloride. 1 ml of this extract was loaded onto the top of the silica gel column (as described above). The early methylene chloride fractions (Group A) from the silica gel column contained DBT, while several following fractions (Group B) contained a pink compound that appeared on the HPLC chromatogram at a retention time of 9 minutes in the method described below. These were followed by fractions which contained a compound with a retention time slightly longer than that of DBT (Group C). When the same silica gel column was further eluted (with 5 ml of ethanol), a red colored fraction was collected which exhibited several bands on TLC, including one corresponding to that of DBT-sulfoxide. This fraction, however, contained several other components that are as yet unidentified. The next two ethanol fractions contained a compound that gave a blue color with Gibb's reagent, indicative of the presence of phenolic compounds.

The HPLC conditions were a modification of the method of Wyza (1989): column, Waters C18 Resolve 5 micron spherical; solvent, tetrahydrofuran:acetonitrile:water [23:18:59]; flow rate, 1.5 ml/minute; injection volume, 2 microliters; detection at 242 nm using a Waters Lambda Max 481 LC spectrophotometer.

A fraction from Group B which was analyzed by HPLC was also analyzed by GC-mass spectroscopy. The two analyses are shown in Figure 2.1. As can be seen, the HPLC gives, in this instance, far greater information was obtained from the HPLC chromatogram than from the GC-MS.

2.2 Further Analyses of Cell-Free Extracts (CFE)

GB-1 cells were grown in artificial sea water medium with 0.05% yeast extract and no DBT. The inoculum was also from cells grown under the same conditions. We had previously determined that harvesting the cells after 65 to 72 hours yielded cell-free extracts, with maximum activity against DBT (Figure 2.2). Accordingly, cells were grown at 25°C with shaking and at the end of 70 hours, they were centrifuged at 10,000 rpm for 20 minutes. The cell pellet was discarded and part of the CFE was boiled for 5 minutes. The two fractions of boiled and unboiled supernatant (CFE) were incubated with 0.01% DBT for 1 week at 25°C with shaking. The CFE were extracted and concentrated and examined by HPLC. The heat-treated extract did not oxidize DBT, however, DBT-sulfoxide and DBT-sulfone were detected in the CFE that had not been heat treated. This is evidence that the reactions observed are biologically catalyzed.

CFE that had been obtained after allowing growth of GB-1 in DBT-free medium for 65 hours, 90 hours, and 114 hours were incubated with each of 0.01% DBT, 0.01% DBT-sulfoxide, and 0.01% DBT-sulfone. While all the three CFE oxidized DBT to DBT-sulfoxide and DBT-sulfone, no oxidation products were observed with DBT-sulfoxide and DBT-sulfone as substrates. No biphenol was detected.

The levels of DBT-sulfoxide and DBT-sulfone obtained as a result in the above of DBT oxidation by GB-1 experiments were much lower than in previous experiments. This may be attributed to the fact that pre-

Figure 2.1

**COMPARISON OF TWO METHODS
OF ANALYSIS OF THE SAME SAMPLE
(A Partially Purified Aliquot from GB-1 Media)**

Figure 2.2

**HPLC CHROMATOGRAM OF MEDIA EXTRACTED AFTER 8 DAYS INCUBATION OF
GB-1 CELL-FREE EXTRACT (COLLECTED AT 72 HR) WITH 0.01% DBT**

In the standard (Figure 1), DBT-sulfoxide elutes at 1.72 min, DBT-sulfone at 2.97 min, biphenol at 4.5 min, and DBT at 22.41 min.

viously the inocula had been from media containing DBT and with repeated transfers in media without DBT, the cells probably no longer produce as much DBT-oxidation enzyme(s) as they would in media containing DBT. In order to maximize enzyme production, it may be necessary to use an inoculum which was grown with DBT in the medium.

2.3 Growth of GB-1 on an Alternate Carbon Source

Although it had been established that GB-1 grows in medium with DBT as the sole sulfur source, in those experiments, DBT was the sole carbon source too. In order to see the effect of an additional carbon source, GB-1 was incubated in sulfur free medium with 0.01% DBT as sole sulfur source and 0.2% sodium succinate as carbon source; and as a control, with 0.01% DBT as carbon and sulfur source. A control of medium with DBT without cells was also included. As noted previously, GB-1 can grow on DBT as sole sulfur source; however, no oxidation products were detected when the cells were grown under such conditions. Supplementation of the sulfur free medium with succinate did seem to increase growth, but no "4S" oxidation products were observed. No "spontaneous" oxidation of DBT occurred in uninoculated flasks. Addition of 50 μM FeSO_4 to the medium did not improve DBT degradation by GB-1. In artificial sea water medium with yeast extract and DBT, DBT-sulfoxide and DBT-sulfone are produced by GB-1 from DBT.

2.4 Preliminary Isolation of Enzyme(s) from GB-1

As mentioned in Section 2.2, GB-1 CFE is capable of oxidizing DBT to DBT-sulfoxide and DBT-sulfone. Our goal is to isolate the enzyme(s) that bring about this oxidation. Accordingly, the initial objective was to obtain a partially-purified extract and check for activity against model compounds such as DBT and subsequently on coal. Toward this end, GB-1 was grown in 100 ml of artificial sea water medium without DBT. Cells were harvested at the end of 70 hours, centrifuged, and the supernatant was dialyzed against 10 mM potassium phosphate buffer, pH 6.8. The dialysis tubing used was a Spectropor membrane tubing with a molecular weight cut off of 6,000 to 8,000 from Spectrum Medical Industries, Inc. Prior to dialysis, an ethanolic solution of the protease inhibitor phenyl methyl sulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Dialysis was done in the

cold over 6 hours with a buffer change every 2 hours. The dialyzed medium was then lyophilized. For the lyophilization, the material was frozen. Due to the inefficiency of the lyophilization process, the material had thawed and was refrozen a few times before lyophilization was complete. A BioRad assay for determination of protein in the lyophilized material revealed very low protein content. We believe that considerable protein loss may have occurred in the dialysis step.

Lyophilization without dialysis was also not very successful regarding protein recovery. The repeated freezing and thawing would also be detrimental to the enzyme. Ultrafiltration as a means of concentration will be attempted next followed by column chromatography separation procedures.

Section 3

ASSAYS WITH COMMERCIAL ENZYMES

3.1 Experimental

3.1.1 Serial Enzyme Addition Studies

The sequential study was carried out according to the scheme illustrated in Figure 3.1. A 0.3 mM DBT solution was incubated with horseradish peroxidase (0.01 mg/ml) and hydrogen peroxide (176 nM) for 24 hours. At this time, laccase was added to a concentration of 0.01 mg/ml and after a further incubation of 24 hours, sulfatase was added to a concentration of 0.01 mg/ml. The experiment was concluded 24 hours after the sulfatase was added.

The resulting samples were analyzed by HPLC as described in Section 2.1, and the data are presented in Figures 3.2 and 3.3.

3.1.2 Evaluation of Two Buffers With Laccase

The activity of laccase against DBT was tested in four organic solvents: acetonitrile; ethyl acetate; dioxane; and dimethylformamide. Laccase was tested at a final concentration of 0.2 mg/ml in two buffers: 1 mM PIPES, pH 7.05; and 1 mM acetate buffer, pH 5.5. The two buffer concentrations used were 0.5% and 1% in organic solvent. The DBT concentration was 2 mM. Controls without laccase and without DBT were included for each solvent. The total volume per tube was 5 ml. The tubes were tightly capped, laid on their side, and placed in a shaker at 28°C. This allowed for adequate mixing and aeration. After 3 days, 50 µl from each tube was applied onto TLC plates (silica gel; Merck No. 5628) and run with chloroform:acetone [80:20] as eluent. The plates were examined by UV light. If a 1% conversion of DBT to its oxidation products had occurred, the application of 50 µl of sample would have detected it. No "4S" oxidation products were observed with any of the solvents at either pH and buffer concentration. Our previous data indicate that laccase is active even after

Figure 3.1

FLOW CHART FOR ASSAY INCORPORATING SERIAL ADDITION OF ENZYMES

Figure 3.2

Figure 3.3

**SERIAL ADDITION OF ENZYMES (HORSERADISH PEROXIDASE, LACCASE, SULFATASE) TO DBT IN DMF
DBT-SULFOXIDE AND DBT-SULFONE CONCENTRATIONS**

5 days. It was, therefore, apparent that laccase activity against DBT, if any, caused less than 1% conversion under the conditions tested.

3.1.3 Reevaluation of Activity of Laccase and Horseradish Peroxidase in Unbuffered Systems

The assays of laccase and horseradish peroxidase against DBT in selected hydrated solvents were repeated with DBT at 3.0 mM. The protocols were as follows: DBT stocks with 3.0 mM DBT were prepared. Aliquots of 9.5 ml solvent/DBT were mixed with 0.1 ml enzyme stock (which was 1 mg/ml). The vials were sealed and kept shaking vigorously. Samples were removed and filtered at 1 hour and 24 hours. Some brown color was observed in all the HRP vials. The samples were analyzed by HPLC according to the protocol previously described.

The results of this set of experiments are shown in Figures 3.4 to 3.7. The first two figures represent the concentrations of DBT, DBT-sulfoxide, DBT-sulfone, and biphenol in the reaction mixture at 1 hour and 24 hours for the laccase assays. It can be seen that the DBT change is not significant in acetonitrile and ethyl acetate. The rise in concentration in isopropyl ether may be due to solvent evaporation. Sulfoxide and sulfone appear at 24 hours, but in low concentrations.

The molarity of these compounds are calculated from the HPLC standard and the DBT concentration thus obtained for the HRP assays does not match our reported initial value of 3.0 mM. As all experimental pairs, e.g., laccase and HRP assays in same solvent, were from the same stock, we suspect that a contaminant in the HRP coelutes with the DBT in this system.

Figures 3.6 and 3.7 represent the concentrations of DBT, DBT-sulfoxide, DBT-sulfone, and biphenol in the horseradish peroxidase assays. It can be seen that in these experiments, the DBT concentration drops at 24 hours. Sulfoxide concentration rises, as does the concentration of sulfone. As with the laccase, the amount of oxidized product (4S) is less than 2% (in most instances less than 1%), consistent with our TLC results with laccase, in which 1% was the lower limit of detection.

Figure 3.4

24 HOUR LACCASE ASSAYS

ETOAC = ETHYLACETATE

ETOAC = ETHYLACETATE

IPRE = ISOPROPYLETHER

17

Figure 3.6

3.2 Discussion

The enzyme experiments are currently being repeated at much lower DBT concentrations in an effort to alter the equilibrium of the enzyme reaction in favor of stable product formation. Our earlier experience with measuring the kinetic constants for enzyme-ligand interactions (e.g., DBT and HRP) indicated very clearly that the inhibition constants for DBT and its sulfoxide are both the mM range. Thus, enzyme inhibition may be a problem under the conditions of these particular experiments. Furthermore, although sulfone production is very low, we have not observed any sulfone as a contaminant of the DBT starting material, so any sulfone measured is an indication of enzyme-mediated sulfur oxidation.

The data also suggest that, as predicted, DBT sulfoxide is more readily oxidized enzymatically than the fully reduced parent compound. Experiments are in progress to more fully define the relative susceptibility of DBT and its sulfoxidized derivatives to HRP and LAC activity.

Section 4

PLANS AND PROGRESS ASSESSMENT

4.1 Evaluation of Progress

We have made good headway on the utilization of three enzymes in hydrated organic solvents for coal desulfurization. The yields are still very low, however, we believe that the additional work discussed in Section 4.2 will be beneficial in increasing the yield of DBT oxidation products. We are also optimistic about the potential of the enzymes from GB-1 microbes to facilitate the sulfur oxidation process; the oxidation reactions have been shown to proceed well in buffered aqueous medium. As discussed with the Project Manager at the Contractors' Meeting in Pittsburgh in August, work on coal will be delayed because results with model compounds are more readily evaluated.

4.2 Future Plans

The goal of the second year of the program is to further optimize the utilization of sulfur oxidizing enzymes in hydrated solvents. Our work will include both continued utilization of the microbial enzymes from GB-1, which we hope to isolate in a crude, lyophilized, cell-free extract, and the further refinement of the HRP, laccase, and sulfatase series. We will be looking at control of the reactions through increased oxygen availability, reduced substrate concentration, and product removal processes. These parameters will also be important in assessing the ultimate feasibility of the bioprocessing method of coal desulfurization.

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