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Project Title: Evaluation of Sulfur-Reducing Microorganisms for Organic Desulfurization DE-FG22-90PC90176

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ABSTRACT

Reductive biodesulfurization of organic functional groups is the goal of our project. Sulfate and elemental sulfur are well-known electron acceptors for anaerobic bacteria, but utilization of sulfur functionalities typical of coal has not been adequately researched. Dissimilatory reduction of sulfur would be the ideal mechanism for biodesulfurization because relatively large quantities of sulfur would be removed from coal in order to support a small biomass. According to our calculations, standard molar free energy changes are negative for oxidation of lactate with model sulfur compounds as electron acceptors.

Production of toluene indicates that reductive desulfurization of benzyl mercaptan (BMC), dibenzylsulfide (DBS), or -disulfide (DBDS) occurs in mixed cultures of strict anaerobes grown on lactate as electron donor. Cultures have been derived from swine waste, petroleum waste, sewage sludge, and pond sediment. Highest desulfurization rates occurred for BDSA2, a culture derived from pond sediment. Maximum rates of toluene production were 0.67 μg culture^{-1} day^{-1} on 62 mg l^{-1} DBDS and 2.3 μg culture^{-1} day^{-1} on 5 mg l^{-1} BMC. We theorize that 1,3-butadiene or biphenyl would be produced similarly by the reductive desulfurization of thiophene or dibenzothiophene (DBT), respectively; however, we have been unable to detect these compounds in any of our cultures.

Pure cultures of sulfate-reducing bacteria (SRB) produced some BMC but no toluene on DBDS. Other compounds, including DBT, DBT sulfone, thiophene, or phenyl sulfone were not attacked by SRB's.

The culture BDSA2 produced up to 0.5 mg l^{-1} sulfide on IBC-101 and 250 mg l^{-1} sulfide on Ugljevik lignite, both at 5% pulp density. The Forms-of-Sulfur Analysis did not indicate which sulfur fraction of IBC-101 served as the source of sulfide; the lignite has yet to be analyzed. A complication is that sulfide reassociates with finely ground coal, so that net sulfide production does not reflect actual desulfurization rates. Purging of sulfide will be required for a successful process.

(This project is funded by the Illinois Department of Energy and Natural Resources as part of its cost-shared program with the U. S. Department of Energy.)

EXECUTIVE SUMMARY

Sulfidogenesis, the release of H_2S from anaerobic systems such as the gut, sewage, and marine and freshwater sediments, is a common occurrence and the role of microorganisms in this process has long been recognized. Fermentation of organosulfur compounds is the principal source of sulfide in many cases. Although production of H_2S from biologically pertinent compounds such as cysteine has been studied extensively, production of H_2S from compounds representative of organosulfur functionalities in coal has received little attention. Kurita et al. (1971) isolated, from oil well sludge, a Gram-negative anaerobe that rapidly reduced sulfur in thiophene and crude petroleum products to H_2S. Köhler et al. (1984) reported that mixed cultures containing SRB's could reductively degrade DBS, DBDS, and DBT. Tilstra et al. (1990) reported that aryl and alkyl polysulfides could be reduced to H_2S by the hyperthermophile Pyrococcus furiosus. Our project has focused on obtaining similar cultures that could be used for organic desulfurization of coal.

In the previous funding period we sampled anaerobic environments that included pond sediment, swine waste, and petroleum waste. From each we obtained mixed cultures that produced H_2S from inorganic sulfate or the disulfide-containing amino acid, cystine, indicating that sulfur-reducing microorganisms were present. Subcultures proved capable of reductively degrading DBDS; toluene was detected and identified using GC-MS; BMC was a probable intermediate. Up to 39% degradation of DBDS occurred within 12 days at 25°C. Other substrates were DBS and dibenzyltrisulfide (DBTS). Production of H_2S occurred in initial enrichments on medium amended with thiophene or DBT; however, the source of sulfide could not be identified and this activity could not be sustained in subcultures. In preliminary coal desulfurization experiments, IBC-108, was incubated at 5% pulp density in selected cultures of DBDS-degraders for up to 4 weeks. Total and organic sulfur remained unchanged. We anticipated that detecting organic desulfurization would be difficult because we discovered that sulfide sorbs readily to finely ground coal and would reassociate shortly after removal. Our tasks for the present year are:

I. To obtain cultures that will reductively desulfurize thiophenic model compounds.

II. To continue to work toward optimizing the activity of the DBDS-reducing cultures.

III. To expand coal desulfurization work to include other coals such as IBC-101 and a lignite.

IV. To address the problem of sulfide sorption, by investigating the sorption capacity of coals in addition to IBC-108.

Our most important finding has been that coal can be desulfurized using anaerobic bacteria. BDSA2, which is a mixed culture derived from pond sediment, produced up to 0.5 mg l^{-1} sulfide on IBC-101 and 250 mg l^{-1} sulfide in preliminary experiments on Ugljevik lignite, both at 5% pulp density. That the coal desulfurization medium was essentially sulfur free proves that the coals served as the substrate for sulfide production. Although the goal of our project is organic desulfurization, it is still unclear as to which sulfur fraction serves as the source of sulfide. Because net desulfurization was low for IBC-101, the Forms-of-Sulfur-Analysis was not sensitive enough to detect differences between the desulfurized coal and the coal that had been processed in the uninoculated sterile control; further optimization is necessary. We are presently setting up more extensive experiments with Ugljevik lignite, which is a low rank coal that contains approximately 6.4% organic sulfur. Because of the higher organic sulfur content, we expect that any organic sulfur removal can be measured with better accuracy.

A complication in this work has been that sulfide reassociates with finely ground coal. Figure 1 shows sulfide remaining in a solution containing 50 mg 1-1 sulfide after addition of various coals at 2.5% pulp density. These data suggest that sulfide in the above desulfurization experiments represented only a portion of the total sulfide produced, with the remainder reassociating. An N_2-purged system would facilitate sulfide removal by preventing reassociation.

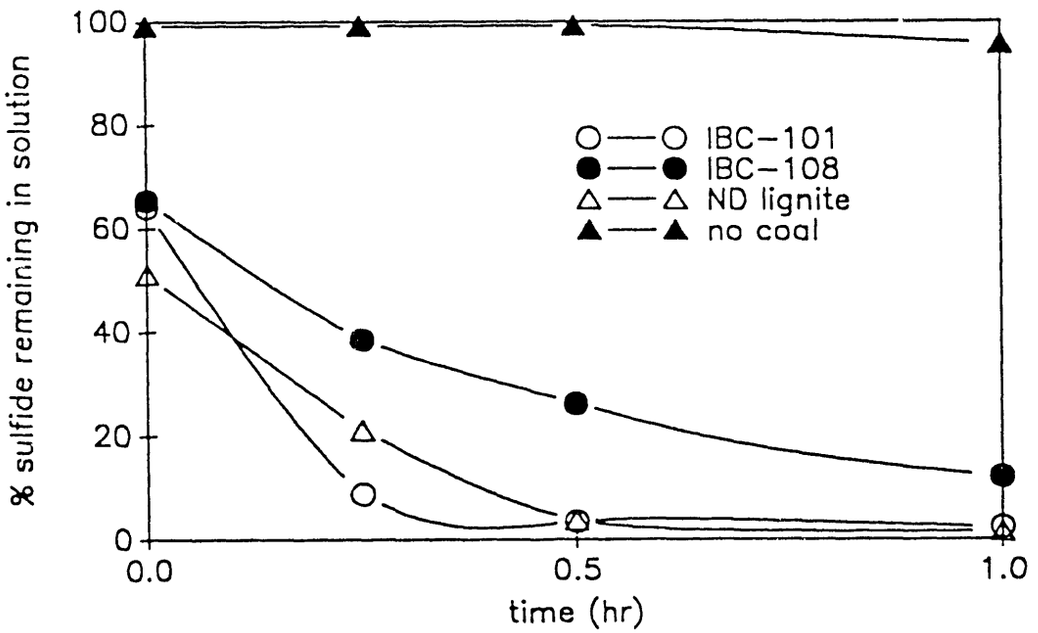

Fig. 1. Time course for sulfide sorption for 3 coals. Pulp density is 2.5% and initial sulfide concentration is 50 mg 1-1.

Production of toluene indicates that reductive desulfurization of BMC, DBS, or DBDS occurs in mixed cultures of strict anaerobes grown on lactate as electron donor. Cultures have been derived from swine waste, petroleum waste, sewage sludge, and pond sediment. Highest desulfurization rates occurred for BDSA2, a culture derived from pond sediment. This is the same culture that produced sulfide in coal desulfurization experiments. Maximum rates of toluene production were 0.67 μg culture^{-1} day^{-1} on 62 mg l^{-1} DBDS and 2.3 μg culture^{-1} day^{-1} on 5 mg l^{-1} BMC (Figure 2).

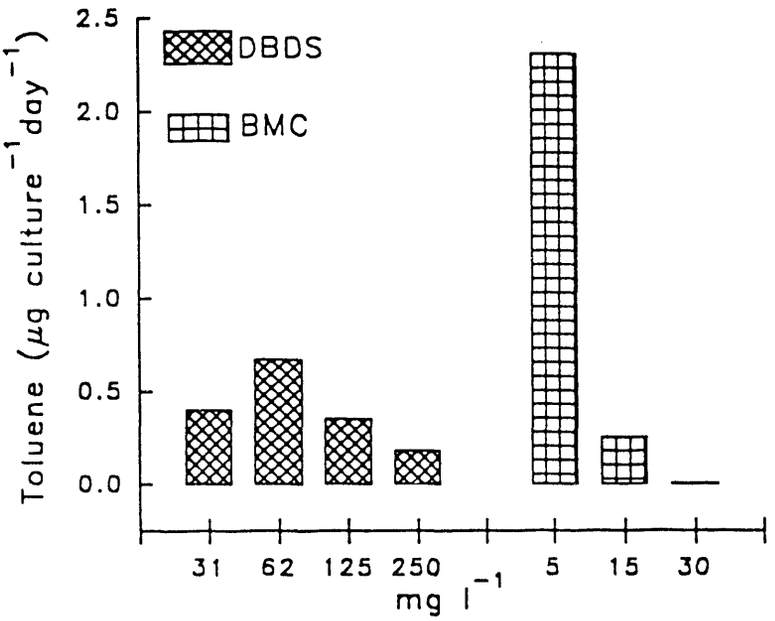

Fig. 2. Rates of toluene production by BDSA2 during exponential phase of growth on DBDS or BMC.

We theorize that 1,3-butadiene or biphenyl would be produced similarly by the reductive desulfurization of thiophene or dibenzothiophene (DBT), respectively; however, we have been unable to detect these compounds in any of our cultures. Our thermodynamic calculations suggest that thiophenic compounds are not energetically beneficial as electron acceptors, whereas reduction of sulfidic model compounds could possibly contribute to dissimilatory metabolism. Despite the reports cited above, we are drawing the conclusion that substantial reductive desulfurization of thiophenic functionalities will probably not be feasible.

Although there is evidence that thiophenic functionalities tend to predominate in higher ranking coals, spectroscopic studies have demonstrated that substantial quantities of sulfidic sulfur exist even in bituminous coal. One recent study sponsored by the CRSC even suggests

that disulfides may be the predominate form of organic sulfur in Illinois coals (Palmer et al., 1990). For this reason, it is likely that cleavage of sulfidic bonds by anaerobic microorganisms could lead to a reductive desulfurization method for bringing many coals into compliance, even though recalcitrant thiophenic functionalities remain.

Köhler, M., I.-L. Genz, B. Schicht, and V. Eckert. 1984. Mikrobielle Entschwefelung von Erdöl und schweren Erdöl fraktionen 4. Mitteilung: Anaerober Abbau organischer Schwefel-verbindungen des Erdöls. Zbl. Mikrobiol. 139:239-247.

Kurita, S. T., Endo, H. Nakamura, T. Yagi, and N. Tamiya. 1971. Decomposition of some sulfur compounds in petroleum by anaerobic bacteria. J. Gen. Appl. Microbiol. 17:185-198.

Palmer, S. R., J. C. Crelling, E. J. Hippo, and M. A. Kruge. 1990. Characterization of organic sulfur compounds in coals and single macerals. Final report to the Illinois Coal Development Board through the Center for Research on Sulfur in Coal, Carterville, IL.

Tilstra, L., G. J. Olson, G. Eng, and R. M. Kelly. 1990. Microbial degradation of polysulfides and insights into their possible occurrence in coal. In EPRI Proceedings of the first international symposium on the biological processing of coal, May 1-3, Orlando, FL.

OBJECTIVES

Because a substantial portion of the sulfur in Illinois coal is organic, microbial desulfurization of sulfidic and thiophenic functionalities could hold great potential for complementing pyritic sulfur removal. We are testing the hypothesis that organic sulfur can be reductively removed as H_2S through the activities of anaerobic microorganisms. Our objectives for this year include the following:

I. To obtain cultures that will reductively desulfurize thiophenic model compounds. In addition to crude oil enrichments begun last year, we sampled municipal sewage sludge.

II. To continue to work toward optimizing the activity of the DBDS-reducing cultures obtained during the previous year.

III. To expand coal desulfurization work to include other coals including IBC-101 and a North Dakota lignite, which might be more susceptible to the DBDS-reducing cultures due to its lower rank.

IV. To address the problem of sulfide sorption, by investigating the sorption capacity of coals in addition to IBC-108.

HISTORICAL PERSPECTIVE AND INTRODUCTION

Biological methods for organic sulfur removal are based on the premise that microorganisms can break carbon-sulfur bonds. Biooxidation of organic sulfur has been the focus of most research efforts to date.

Dibenzothiophene is the most popular model for sulfur functionalities occurring in fossil fuels, despite the fact that sulfides account for a significant percentage of the organic sulfur in coal (Attar, 1979). Biooxidation of DBT by Pseudomonas spp. (Kodama et al., 1973; Hou and Laskin, 1976; Monticello et al., 1985) and Beijerinckia spp. (Laborde and Gibson, 1977) results in various hydroxylated thiophenic end-products. The metabolic pathway involved is named the Kodama pathway. Pseudomonas spp. also produce hydroxylated products from benzothiophene, thiolane, 2-methylthiophene and 3-methylthiophene (Sagardia et al., 1975). In theory, the Kodama pathway could be used to transform sulfur containing components of the coal matrix to water soluble organic compounds that could be removed by leaching; however, the process would be accompanied by a high carbon loss.

A mutant pseudomonad, named CB1 (Isbister and Kobylinski, 1985), produces sulfate from DBT yet leaves the aromatic ring structure intact, following the "4S" metabolic pathway: DBT -> DBT sulfoxide -> DBT sulfone -> DBT sulfonate -> biphenyl or 2,2'-hydroxybiphenyl and sulfate. Krawiec (1988) using a fluorescent assay for 2,2'-hydroxybiphenyl isolated

naturally occurring cultures that utilize a modified "4S" pathway for assimilatory removal of sulfur from DBT. Afferden et al. (1990) reported that a Brevibacterium sp., which grows on DBT as a sole source of carbon and energy, uses a modified "4S" pathway producing sulfite, which is subsequently oxidized to sulfate. The "4S" pathway is highly desirable, because in most cases desulfurization is not accompanied by carbon loss. A strain of Escherichia coli that utilizes water-soluble derivatives of thiophene as sole sources of carbon and energy recently has been developed at Southern Illinois University (Abdulrashid and Clark, 1987; Clark and Klubek, 1987; Alam and Clark, 1988); in progress is a project directed at introduction of the genes responsible for the complete "4S" pathway into this strain (Clark et al., 1990).

Reports of successful coal desulfurization using aerobic cultures have been limited. A culture obtained by DBT enrichment produced a 20% decrease in the organic sulfur content of an Indian coal (Chandra et al., 1979). A thermophilic thiobacillus removed 50% of the organic sulfur from a Turkish lignite (Gokcay and Yuteri, 1983). The organism, CB1, removed up to 34% of the organic sulfur from a variety of coals (Isbister and Kobylinski, 1985). Cell preparations of S. acidocaldarius lowered organic sulfur by 44% and 25% respectively for a Pennsylvania coal and a petroleum pitch sample (Kargi and Robinson, 1986); however, other researchers report that Sulfolobus has little or no effect on the sulfur content of coals (Miller and Risatti, 1986: Olsen and Brinckman, 1986). Up to 91% organic sulfur removal was obtained using a sulfur assimilating isolate IGTS7 in sulfur limited culture (Kilbane, 1988), however, incubation time was a lengthy 212 days and there appeared to be no uninoculated control experiment. The lack of additional citations demonstrates that success in this area is considerably more tentative than in the well-researched area of pyrite removal using thiobacilli (reviewed by Kargi, 1984; Monticello and Finnerty, 1985; Olsen and Brinckman, 1986).

Desulfurization through bioreduction to H_2S is another option that should be explored. The release of H_2S from anaerobic systems such as the gut, sewage, and marine and freshwater sediments, is a common occurrence and the role of microorganisms in this process has long been recognized (e.g., Andrews, 1937; Zinder et al., 1977; Bremner and Steele, 1978; Spratt et al., 1987). Fermentation of organosulfur compounds is the principal source of sulfide in many cases (Barker, 1961; Voll et al., 1979; Forsberg, 1980; Smith and Klug, 1981). Kurita et al. (1971) reported the isolation of an anaerobic culture that reduced thiophenic sulfur to H_2S. The microorganism was isolated from oil well sludge, and was described as a Gram negative rod, which grew optimally at pH 7.2 to 7.8 and 38 C under N_2 or H_2. Sulfur functioned as an electron acceptor and evolution of H_2S occurred rapidly from thiophene and petroleum products. More recently, Köhler et al. (1984) reported that anaerobic mixed cultures containing sulfate-reducing bacteria could reductively

degrade DBS, DBDS, and DBT. Tilstra et al.(1990) reported that aryl and alkyl polysulfides could be reduced to H_2S by the hyperthermophilic anaerobe Pyrococcus furiosus. We have attempted to obtain similar cultures and have begun work that will assess the effectiveness of using these cultures to lower the organic sulfur content of coal.

MATERIALS AND METHODS

Medium. Basal medium contained, per liter, the following: lactic acid, 4 g; amino acid mixture (25 mg each of the 18 non-sulfur essential amino acids); $NaHCO_3$, 5.0 g; NaCl, 0.6 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.3 g; NH_4Cl, 0.24 g; $MgCl_2 \cdot 7H_2O$, 0.13 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $FeCl_2 \cdot 4H_2O$, 25 mg; resazurin, 1.0 mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; Na_2SeO_4, 0.5 mg; trace mineral and trace vitamin solutions (Balch et al., 1979). Titanium(III) citrate (0.15 to 0.3 mM) was added as a reducing agent (Zehnder and Wuhrmann, 1976). Basal medium, containing all ingredients except $NaHCO_3$ and Ti(III) citrate, was boiled under Ar for 15 min. After addition of $NaHCO_3$, medium was cooled under Ar and placed in a Coy anaerobic chamber, where 25 ml volumes were distributed in anaerobically equilibrated 160 ml serum bottles. Bottles were crimp-sealed with Teflon faced butyl rubber stoppers. After removal from the chamber, bottles were evacuated and pressurized to 5 psig N_2 three times. Medium was sterilized by autoclaving at 121°C for 20 min. A solution of Ti(III) citrate was sterilized separately and injected just prior to inoculation.

Enrichment cultures were prepared using basal medium amended with any one of various sulfur-containing model compounds, including the following: thiophene, DBT, BMC, or DBDS. These compounds were added after sterilization neat or in filter-sterilized concentrated acetone solutions. In preliminary experiments the concentration of these compounds was 0.1%, but in subsequent experiments concentrations of 0.05% or 0.025% were used.

Experimental cultures also employed the basal medium. In addition to the compounds listed above, DBS or DBTS also were tested as substrates. To determine the effectiveness of elemental sulfur as an inducer of sulfur-reducing enzymes, following sterilization, bottles containing medium were transferred to the anaerobic chamber where stoppers were removed and elemental sulfur (Aldrich, 99.999%) was added aseptically, 400 mg l^{-1}. Fresh, sterile stoppers were crimped on. After removal from the chamber, bottles were repressurized aseptically.

For experiments with sulfate-reducing bacteria, basal medium was amended with Na_2SO_4 at 1.0 g l^{-1}. The following model compounds were tested with and without sulfate as electron acceptors at concentrations of 0.01%: thiophene, DBT, DBT sulfone, or phenyl sulfone.

Microorganisms. Anaerobic environments sampled included the following: sediment from a pond in Parklands nature study area, McLean County; two swine waste holding pits at the Illinois State University Farm; two waste sites at the Parnell oil fields and three waste sites at the Wapella East oil fields in DeWitt County; and a methanogenic sewage digestor at the Bloomington-Normal Water Reclamation Center. Samples were collected in Ar purged containers and injected into enrichment media. After an incubation period at ambient temperature on a gyratory shaker, subcultures were prepared from the initial enrichments. Promising subcultures were stored at $4^{o}C$.

Experimental Design for Model Compound Degradation Studies. Basal medium amended with a model compound was inoculated (2%) with the culture to be tested. Cultures were incubated at ambient temperature ($23^{o}C$ to $25^{o}C$) on a gyratory shaker at 150 rpm. Headspace gases were monitored periodically by removing 100 μl aliquots with a Dynatech Pressure-Lok syringe. These gas samples were analyzed using GC-FID or GC-FPD, for non-sulfur or sulfur-containing compounds, respectively. At the conclusion of the incubation period, cultures were sacrificed. After acidification to pH 3 with HCl cultures were extracted with an equal volume of hexane or ether. Extractions were analyzed using GC-FID.

Experimental Design for Coal Desulfurization Experiments. Experiments employed basal medium. Ugljevik lignite or IBC-101, each 100% -200 mesh, was distributed in serum bottles prior to anaerobic equilibration in the Coy chamber. Pulp density was 5%. Following addition of 50 ml medium, serum bottles were sterilized as usual. For the experiment described with IBC-101, the following three cultures were employed: BDSA2, a non-methanogenic culture derived from pond sediment; BMX22 (also referred to as S23 in some previous reports), a methanogenic culture derived from swine waste; 5-WE, a methanogenic culture derived from petroleum waste. Cultures were grown up in medium containing DBDS at 125 mg l^{-1}, then coal slurries were inoculated with 0.5 ml culture. Coal desulfurization experiments were set up in quadruplicate in order to obtain enough coal for commercial analysis. Experiments were incubated for seven weeks at ambient temperature on a gyratory shaker at 150 rpm. At the conclusion of the incubation period, coal was harvested by filtration through Whatman #1 paper. The filtrate was tested for sulfide using the methylene blue sulfide assay. Then coal was rinsed with 1 N HCl and several volumes of water and air dried at room temperature for 48 h.

Coals. IBC-101 and IBC-108 were obtained from the Illinois Basin Coal Sample Program Bank at the Illinois State Geological Survey, Champaign. The North Dakota lignite was obtained from the University of North Dakota's Energy and Environmental Research Center, Grand Forks, with the aid of Curt Knudson. The Ugljevik lignite was obtained from Yugoslavia, with the aid of Chi-shing Wang, U.S. Bureau of Mines. IBC-108 was prepared from the damp filter cake by drying overnight at 60 C.

IBC-101 and the two samples of lignite were each ground under N_2 to 100% -200 mesh with a mortar and pestle. All coals were kept stored under N_2 in the Coy anaerobic chamber.

Sulfide Sorption Experiments. Solutions (40 ml) of $Na_2S \cdot 9H_2O$ (20 to 1000 mg l^{-1} sulfide) were placed in 160 ml serum bottles along with 1.00 g coal, IBC-101, IBC-108, or a North Dakota lignite. Bottles were crimp-sealed with Teflon septa. Controls received no coal. Bottles were shaken for 24 h at 24°C. At this time aliquots were removed, centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant analyzed for sulfide using the methylene blue assay. Controls also were centrifuged so that any sulfide loss not due to coal could be accounted for. Sulfide sorbed to coal was calculated as the difference between the original concentration and the final sulfide concentration, minus the sulfide loss observed for the control.

Analytical.

Gas Chromatography. Hexane or ether extractions were analyzed using a Varian 3300 gas chromatograph equipped with an on-column injector, 30 m x 0.53 mm SE-54 column and a flame ionization detector (FID) or flame photometric detector (FPD). Although various programs were used from time to time, a general program was developed that resolved all compounds of interest. Parameters included: injector, 180°C; column 40°C (5 min) to 90°C (1 min) @ 10 °C min^{-1} to 220°C (5 min) @ 5 °C min^{-1}, detector 220°C (FPD) or 250°C (FID); 6.0 ml min^{-1} He carrier. Methane or toluene vapor was measured using the same GC-FID configured with a flash injector; H_2S was also measured with the flash injector configured with the FPD. Parameters were the same as before, except that He flow was 3.5 ml min^{-1} through the flash injector. For liquid injections, standard curves were prepared using authentic compounds in the appropriate solvent. Methane standards were prepared by purging a serum bottle with pure methane, then sealing at atmospheric pressure; this stock was diluted in N_2 filled serum bottles. H_2S was prepared as needed from $Na_2S \cdot 9H_2O$ and 1 N HCl in N_2-filled serum bottles. Toluene vapor standards were prepared by injecting known quantities of toluene into serum bottles containing medium, then measuring toluene in the vapor phase at room temperature; pH did not affect this measurement. We attempted to measure BMC as a vapor, but pH fluctuations associated with microbial growth caused imprecision.

Methylene Blue Sulfide Analysis. Sulfide in solution was monitored using the colorimetric assay of Trüper and Schlegel (1964), which was modified for small volumes. A 1 ml volume of sample or standard was added to 2.0 ml Zn acetate (2%). After addition of 6 ml water, 1 ml dimethyl-p-phenylene diamine sulfate (0.2% in 20% H_2SO_4), and 50 µl $FeCl_3.6H_2O$ (10% in 2% H_2SO_4), the mixture was allowed to stand 10 min before reading absorbance at 670 nm on a Bausch and Lomb Spectronic 20 spectrophotometer

using a 1 cm light path. The standard was $Na_2S \cdot 9H_2O$.

Coal Analysis. Coals were analyzed commercially for Forms-of-Sulfur and Ultimate Analysis by Commercial Testing and Engineering, South Holland, IL.

RESULTS AND DISCUSSION

Calculation of Standard Free Energy Changes. Dissimilatory reduction of sulfur would be the ideal biodesulfurization mechanism because relatively large quantities of sulfur would be removed from coal in order to support a small biomass. Alternate schemes for removing organic sulfur, such as the assimilatory approach that is currently proposed by IGT (Kilbane, 1988; Srivastava and Kilbane, 1991), are conceptually ill-designed and will never be commercially feasible, due to the cost of maintaining an extraordinarily large biomass. Using information given in Benson et al. (1969) we calculated values for free energy of formation for thiophene, 1,3-butadiene, DBT, biphenyl, BMC, DBS, DBDS, DBTS and toluene, as ideal gases. These values, along with those of Thauer et al. (1977) were used to estimate standard molar free energy changes for oxidation of lactate with one of the model compounds as electron acceptor (Table 1). Stoichiometry was normalized to sulfur. All reactions would be energy yielding, however desulfurization of the sulfidic compounds could benefit cells in terms of energy conservation. Reductive desulfurization of thiophene borders on being energetically useless; however, reductive desulfurization of DBT could possibly result in energy conservation at low sulfide concentrations. It is important to emphasize that we have not yet proven that any of these reactions benefit real cultures. Free energy changes associated with desulfurization of model compounds cannot be directly applied to desulfurization of coal functionalities, but these values do not refute the possibility of a dissimilatory biodesulfurization process.

Table 1. Stoichiometry and standard free energy of reaction for oxidation of lactate with model compounds as electron acceptor.

reaction	$G^{o'}$ kcal(mol S)$^{-1}$
Dibenzylsulfide(DBS)^a + H_2O^b + Lactate^b --> Acetate^b + $CO_2{}^b$ + 2 Toluene^a + H_2S^b	-33.99
0.5 Dibenzyldisulfide(DBDS)^a + 0.75 H_2O + 0.75 Lactate --> 0.75 Acetate + 0.75 CO_2 + Toluene + H_2S	-19.31

Benzyl Mercaptan(BMC)[a] + 0.75 H_2O + 0.75 Lactate -->

0.75 Acetate + 0.75 CO_2 + Toluene + H_2S -17.24

0.33 Dibenzyltrisulfide(DBTS)[a] + 0.67 H_2O + 0.67 Lactate -->

0.67 Acetate + 0.67 CO_2 + 0.67 Toluene + H_2S -14.40

Dibenzothiophene(DBT)[a] + H_2O + Lactate -->

Acetate + CO_2 + Biphenyl[a] + H_2S -9.27

Thiophene[a] + H_2O + Lactate -->

Acetate + CO_2 + 1,3-Butadiene[a] + H_2S -1.36

[a] free energy of formation for ideal gas state calculated from values given in Benson, et al. 1969.

[b] free energy of formation given in Thauer, et al., 1977.

Reductive Degradation of Thiophenic Compounds. Because evidence suggests that thiophenic sulfur is the principal organic sulfur functionality (Nishioka et al, 1986; Spiro, et al., 1984; Winans et al., 1990), we believed that it was important to obtain a culture capable of reductive desulfurization of a thiophenic model compound. We were unable to detect degradation of thiophene or DBT in cultures obtained from pond sediment or from swine waste. We report here on three additional efforts aimed at obtaining cultures that reductively degrade thiophenic compounds. (1.) Samples were taken from beneath petroleum wastes. (2.) Samples were taken from a methanogenic sewage digestor. (3.) We isolated four pure cultures of SRB's from swine waste and used these plus Desulfovibrio desulfuricans (ATCC 25977) to test whether pure cultures of SRB's could attack an assortment of model compounds.

Experiments with Petroleum Waste. Detectable sulfidogenesis did not occur in petroleum enrichment cultures that contained thiophene or DBT as the principal source of sulfide. Only if sulfate or elemental sulfur were included in the initial enrichments could sulfide be detected. The DBT enrichment cultures from three sediment samples (3-WE, 4-WE, 5-WE) were used as inocula for experiments that compared degradation of DBT to degradation of DBDS. Elemental sulfur was added to some of these experimental cultures in an effort to induce enzymes active in sulfur reduction. When elemental sulfur was added there was substantial sulfide production (Figures 3 and 4). Net sulfide production was greater when

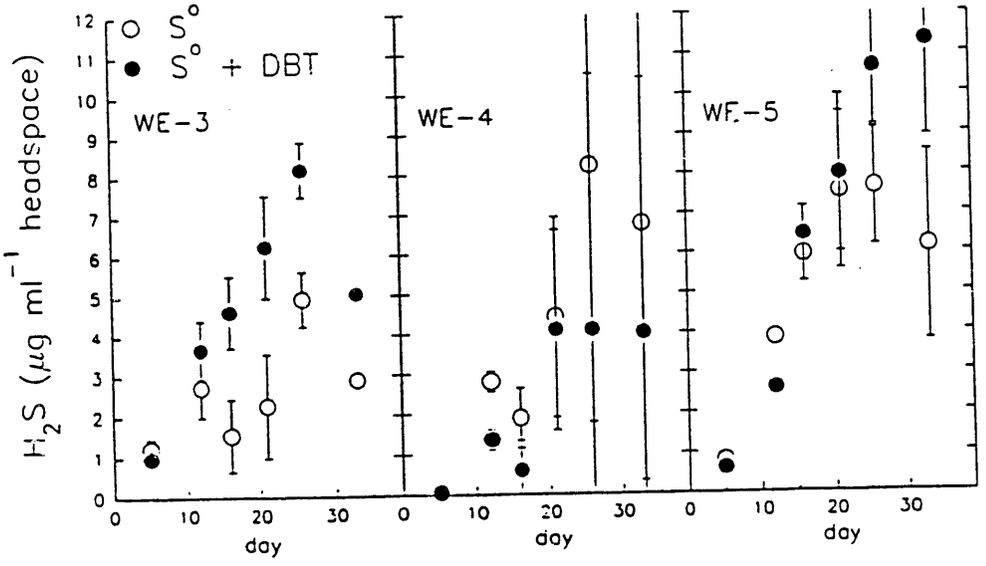

Fig. 3. Production of H_2S for 3 anaerobic mixed cultures in medium containing elemental sulfur (400 mg l^{-1}) or elemental sulfur plus DBT (250 mg l^{-1}). Production of H_2S for uninoculated sterile controls was negligible.

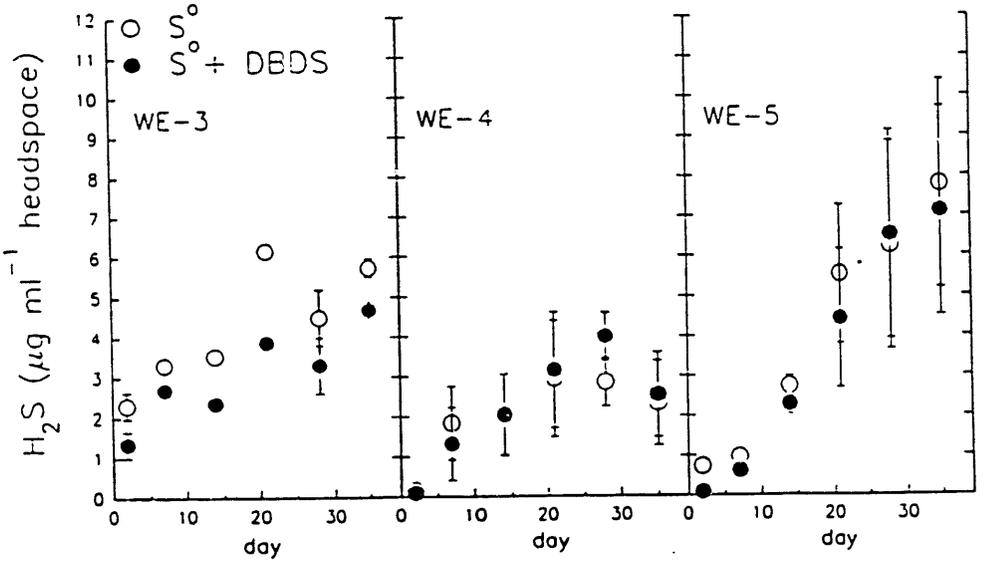

Fig. 4. Production of H_2S for 3 anaerobic mixed cultures in medium containing elemental sulfur (400 mg l^{-1}) or elemental sulfur plus DBDS (250 mg l^{-1}). Production of H_2S for uninoculated sterile controls was negligible.

DBT was present in 3-WE and 5-WE: for 4-WE, DBT had no significant effect on sulfidogenesis. Net sulfide production was unaffected by DBDS in 4-WE and 5-WE, but inhibited by DBDS in 3-WE. Despite the fact that DBT was associated with significantly greater net sulfide production, there was no indication that reductive desulfurization of DBT occurred in any culture. Concentrations of DBT in inoculated medium and sterile uninoculated controls were essentially the same (Table 2). Furthermore, concentrations of biphenyl, the most probable end-product of desulfurization, also were the same (Table 3). Traces of biphenyl were also present in DBT standards; we concluded that the source of biphenyl was either impurities in the DBT preparation, or thermal decomposition of DBT in the gas chromatograph. In any event, the ratio of biphenyl to DBT on a chromatogram was always similar; biphenyl was not of biogenic origin. We concluded that DBT had enhanced net sulfide production from elemental sulfur, possibly by inhibiting sulfide assimilation. DBT itself, however, was not a source of sulfide.

Table 2. Recovery of model sulfur compounds in hexane extractions of three mixed cultures, grown with and without elemental sulfur. Expressed as a % of the mean recovered for corresponding uninoculated controls. Mean of duplicate experiments.

	Dibenzothiophene		Dibenzyldisulfide	
	no sulfur	S^o	no sulfur	S^o
Cultures				
3-WE	96.3 ± 0.7	97.2[a]	86.8 ± 4.1*	86.1 ± 0.9*
4-WE	97.2 ± 4.6	102.9 ± 0.7	98.7 ± 5.0	98.1 ± 2.7
5-WE	97.7 ± 1.3	97.6 ± 0.7	89.5 ± 1.7*	96.1 ± 4.9
Controls	100 ± 2.0	100 ± 1.2	100 ± 3.4	100 ± 2.7

[a] one value only; * = significant loss

Table 3. Biphenyl concentrations (mg l^{-1}) in hexane extractions of cultures grown on DBT.

	no sulfur	S^o
Cultures		
3-WE	0.75 ± 0.04	0.88[a]
4-WE	0.73 ± 0.12	0.75 ± 0.01
5-WE	0.73 ± 0.10	0.73 ± 0.01
Controls	0.79 ± 0.04	0.80[a]

[a] one value only

Experiments with Methanogenic Sewage Sludge. As with the above experiments, detectable sulfidogenesis did not occur in sewage digestor enrichment cultures that contained thiophene or DBT as the principal source of sulfide. Enrichment cultures on thiophene were monitored for production of 1,3-butadiene by analyzing headspace samples using GC-FID; all cultures were negative. Enrichment cultures on DBT were monitored for the production of biphenyl by analyzing ether extractions using GC-FID; all cultures were negative. Although we were not searching for additional DBDS-reducing cultures, we noted that samples obtained from the methanogenic digestor were especially active in producing toluene from DBS, DBDS and BMC.

Experiments with Pure Cultures of Sulfate-Reducing Bacteria. With the aid of the new Coy anaerobic chamber, we isolated four pure cultures of SRB's from swine waste. Our isolates, along with a known culture, ATCC 29577, were tested for the ability to desulfurize thiophene, DBT, DBT sulfone, and phenyl sulfone in lactate medium, with and without sulfate. There was no indication of activity on these compounds. Production of H_2S was unaffected. No end products could be detected in ether extractions. No biphenyl was produced from DBT. No DBT or biphenyl was produced from DBT sulfone. No benzene, biphenyl, or phenyl sulfide was produced from phenyl sulfone. This limited sampling of bacterial cultures suggests that these compounds are not substrates for enzymes involved in dissimilatory sulfate reduction.

These data, along with those obtained last year, imply that there are no anaerobic cultures capable of substantial reductive degradation of thiophenic model compounds. Although these findings conflict with the results obtained by Kurita et al. (1971) for thiophene and by Köhler et al. (1984) for DBT they tend to agree with our thermodynamic calculations.

Optimization of Reductive Degradation of Sulfidic Model Compounds.

Experiments with Petroleum Waste Cultures. Although the petroleum waste cultures were inactive on DBT, DBDS was degraded. In the absence of elemental sulfur, degradation of DBDS occurred in 3-WE and 5-WE (Table 2); however, in the presence of sulfur, there was only a significant loss of DBDS in 3-WE. There was never a significant loss for 4-WE. Degradation products detected in hexane extractions included BMC and toluene, and possibly phenol and p-cresol (Table 4), which could be products of toluene degradation (Grbić-Galić and Vogel, 1987). Identities of these products were confirmed using GC-MS. Yields of toluene were comparatively small, but correlated with yields of BMC. Elemental sulfur inhibited production of BMC and toluene. Phenol and p-cresol were also present in cultures that did not contain DBDS. Only traces of these compounds were present in inocula, indicating that they were produced during the experiment and not carried over. We examined

hexane extractions for other compounds that might be products of DBDS degradation, but failed to detect the following: bibenzyl, stilbene (cis and trans), benzene, cyclohexanol, cyclohexanone.

Table 4. Concentrations (mg l^{-1}) of possible DBDS degradation products in hexane extractions of cultures. Values are the means for 2 experiments.

	Toluene	Phenol	p-Cresol	Benzyl Mercaptan
no DBDS, no sulfur				
3-WE	-[a]	0.37	1.43	-
4-WE	-	0.53	0.14	-
5-WE	-	0.80	1.33	-
controls	-	-	-	-
no DBDS + S^o				
3-WE	-	0.15	2.45	-
4-WE	-	0.45	0.45	-
5-WE	-	0.72	0.31	-
controls	-	-	-	-
DBDS, no sulfur				
3-WE	0.03	-	2.42	11.18
4-WE	-	0.93	-	3.03
5-WE	0.06	1.15	0.09	14.31
controls	-	-	-	0.13
DBDS + S^o				
3-WE	-	-	2.39	0.20
4-WE	-	0.45	0.06	0.26
5-WE	-	0.91	0.02	0.72
controls	-	-	-	-

[a] below detection limits of 0.002 for Toluene, and 0.01 for Phenol, p-Cresol, or Benzyl Mercaptan

As an incidental observation, methanogenesis was inhibited by DBDS in all cultures (Table 5). Effects of elemental sulfur on methanogenesis varied. For 3-WE and 4-WE elemental sulfur inhibited methanogenesis, however 5-WE consistently produced more methane with elemental sulfur. These results might be of interest to those involved in projects aimed at producing methane from high sulfur coals. It appears that sulfur functionalities influence methanogenesis.

Table 5. Methane ($\mu g\ ml^{-1}$) in headspace of cultures at 5 weeks.

	no DBDS		DBDS	
	no sulfur	S^o	no sulfur	S^o
3-WE	9.2 ± 9.6	1.4 ± 0.5	1.3 ± 0.7	0.4 ± 0.2
4-WE	1.4 ± 0.0	0.9 ± 0.8	0.6 ± 0.2	0.5 ± 0.4
5-WE	11.6 ± 9.6	38.6 ± 9.3	1.3 ± 1.1	1.4 ± 1.6

Optimization of Toluene Production for BDSA2. Although cultures derived from petroleum waste consistently produced toluene from DBDS, greatest yields of toluene occurred in the culture derived last year from pond sediment, BDSA2. We chose this culture for further experiments. Because, in our previous work, ether extractions of these cultures had contained no detectable bibenzyl or stilbene and production of BMC had always accompanied disappearance of DBDS, we had theorized that DBDS was degraded via a series of sequential reductions with BMC as an intermediate (Figure 5). The effect of DBDS concentration on toluene

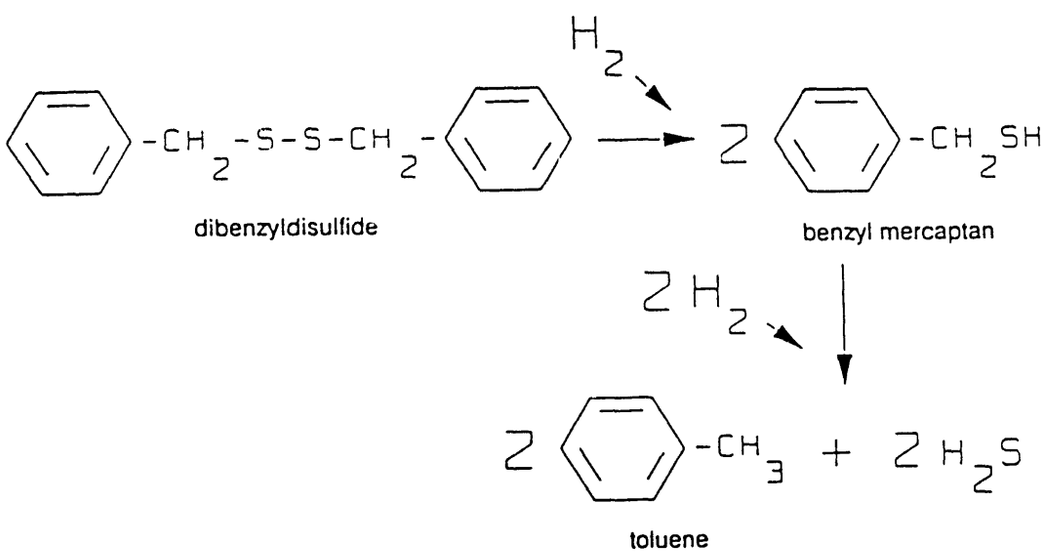

Fig. 5. Proposed pathway for reductive degradation of DBDS.

production was tested. Figure 6 shows that a concentration of 62 mg l^{-1} was optimum. Data presented in Figure 7 illustrate that BMC is the source of toluene. However, BMC appears to be highly toxic; optimum production of toluene occurred at the lowest concentration, 5 mg l^{-1}. This suggests that if the initial reduction of DBDS to BMC occurs too rapidly, high concentrations of BMC will inhibit the subsequent desulfurization step. One pitfall of working with model compounds is that a culture might be able to metabolize a model compound, but not the corresponding functionality in the coal matrix due to steric hindrance. However, the converse possibility exits that sulfur functionalities in the coal matrix might be more amenable to biodesulfurization than model compounds, because biological activity does not generate toxic compounds of low molecular weight, such as BMC. In the case of disulfides, the thiol intermediate would remain with the coal matrix, where it would not adversely influence cell metabolism.

Rates of desulfurization were calculated in terms of toluene produced per day during the exponential phase of growth (Figure 2 EXECUTIVE SUMMARY). Toluene was produced more rapidly on the BMC intermediate than on DBDS. Maximum rates of toluene production were 0.67 μg culture^{-1} day^{-1} on 62 mg l^{-1} DBDS and 2.3 μg culture^{-1} day^{-1} on 5 mg l^{-1} BMC.

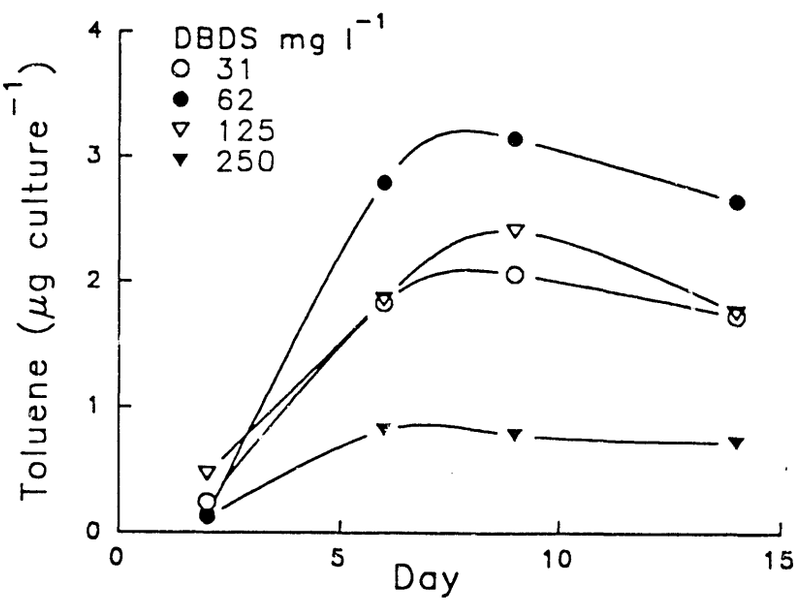

Fig. 6. Effect of DBDS concentration on production of toluene in cultures of BDSA2.

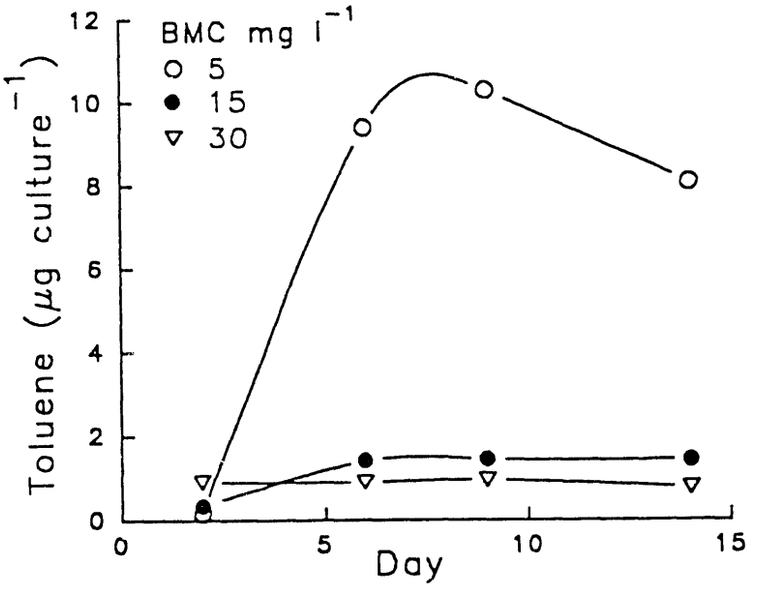

Fig. 7. Effect of BMC concentration on production of toluene in cultures of BDSA2.

Coal Desulfurization Experiments. The culture, BDSA2 produced up to 0.5 mg l^{-1} sulfide on IBC-101 (Table 6); none of the other cultures or sterile uninoculated controls contained measureable sulfide. Tables 7 through 9 give results for commercial analysis of IBC-101. Values for unprocessed coal as determined by Commercial Testing and Engineering (CTE) were different from values given by the IBC. Our sample of IBC-101 appeared to contain less sulfur; both the pyritic fraction and the organic fraction were lower than expected. Values for bioprocessed coal and coal from uninoculated sterile controls were compared with CTE values. The source of sulfide in cultures of BDSA2 could not be determined. Total sulfur was slightly lower for processed coal, but sterile controls were not

Table 6. Sulfide concentrations (mg l^{-1}) in effluent from IBC-101 desulfurization experiments, which employed three cultures and uninoculated sterile controls.

Experiment	1	2	3	4
Cultures				
BDSA2	0.28	0.08	0.17	0.51
BMX22	-*	-	-	-
5-WE	-	-	-	-
Sterile Controls	-	-	-	-

* < 0.05

Table 7. Forms of sulfur in IBC-101 before and after bioprocessing with three mixed anaerobic cultures. (% moisture free weight)

sample	pyritic	sulfatic	organic	total
	% (% orig.)	% (% orig.)	% (% orig.)	% (% orig.)
IBC values 2/91	1.20	0.06	3.12	4.37
BEFORE (orig.)	1.04 (100)	0.10 (100)	2.90 (100)	4.04 (100)
AFTER 7 WEEKS				
BDSA2	0.93 (89.4)	0.01 (10.0)	2.93 (101)	3.87 (95.8)
BMX22	1.05 (101)	0.01 (10.0)	2.90 (100)	3.96 (98.0)
5-WE	1.06 (102)	0.01 (10.0)	2.88 (99.3)	3.95 (97.8)
uninoculated control	0.95 (91.3)	0.01 (10.0)	2.92 (101)	3.88 (96.0)

Table 8. Elemental analysis of coal samples described in Table 7.

	C	H	O	N	S	% ash
IBC values 2/91	69.15	5.10	9.53	1.28	4.37	10.4
BEFORE	69.50	5.00	9.86	1.25	4.04	10.4
AFTER 7 WEEKS						
BDSA2	70.26	4.89	9.85	1.37	3.87	9.85
BMX22	70.05	4.91	9.75	1.34	3.96	9.99
5-WE	69.90	4.90	9.87	1.35	3.95	10.03
uninoculated control	70.16	4.91	9.93	1.30	3.88	9.93

Table 9. Elemental ratios for values given in Table 8.

	C/H	C/O	C/N	C/S
IBC values	13.56	7.26	54.0	15.87
BEFORE	13.90	7.05	55.6	17.20
AFTER 7 WEEKS				
BDSA2	14.37	7.13	51.3	18.16
BMX22	14.27	7.18	52.3	17.69
5-WE	14.27	7.08	51.8	17.70
uninoculated control	14.29	7.07	54.0	18.08

significantly different from inoculated experiments, indicating loss of sulfur was due to some aspect of processing that was not related to biological activity. Ash content was lower in processed coal, suggesting that minerals dissolved during preparation of the coal slurry. Sulfide sorption creates a problem in interpreting these results. Sulfide reassociates with finely ground coal, so that net sulfide production does not reflect actual desulfurization rates. The next section describes our experiments with sulfide sorption.

Experiments with Ugljevik Lignite. With the aid of Chi-Shing Wang, U.S. Bureau of Mines, we obtained a sample of Ugljevik lignite from Yugoslavia. This coal has been employed by others in previous experiments (Stoner et al., 1990). Because the organic sulfur content is very high, approximately 6.4%, decreases can be measured with accuracy. In preliminary desulfurization experiments, BDSA2 produced up to 250 mg l^{-1} sulfide on this coal at 5% pulp density. We will report further results in December in an amendment to this report. Ugljevik lignite was also used as the source of an additional culture, which has proven to be active in coal desulfurization. To obtain the culture, non-sterile lignite was added to sterile basal medium; within two weeks an extremely active sulfidogenic culture arose. We would like to do more work with this culture which was derived from coal itself; however, our project has been completed.

Sulfide Sorption Experiments. Figures 1 (EXECUTIVE SUMMARY) and 8 describe sulfide sorption. Ugljevik lignite has yet to be tested for sulfide sorption. All three coals had approximately the same maximum capacity for sulfide sorption. These values were 9.8, 10.9, and 14.5 mg g^{-1} for IBC-101, IBC-108, and the North Dakota lignite, respectively, as determined by the inverse of the slope of Langmuir reciprocal plots (Stevenson, 1982) (Figure 8). However, the sorption patterns differed at low concentrations, suggesting that sulfide uptake is mediated by various mechanisms in different coals. Figure 1 (EXECUTIVE SUMMARY) suggests that there is adequate time to purge sulfide as it is generated.

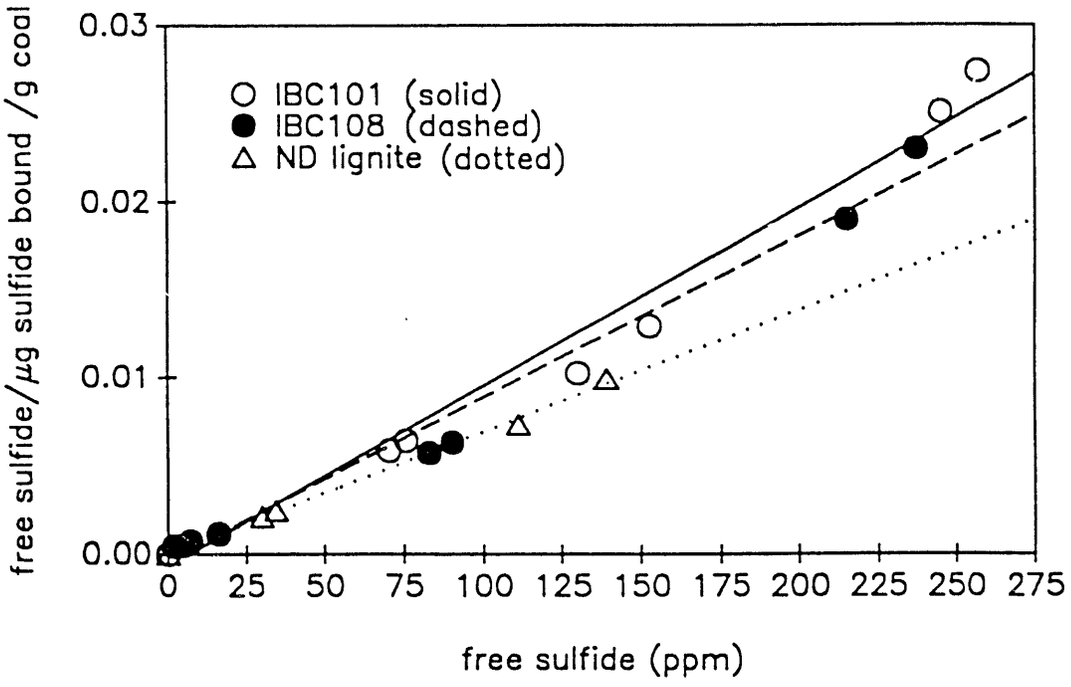

Fig. 8. Langmuir reciprocal plots describing sorbed sulfide as a function of free sulfide after 24 h equilibration time for 3 coals at 2.5% pulp density in solutions of $Na_2S \cdot 9H_2O$. 24°C.

CONCLUSIONS

1. Model compounds containing benzyl thiol, sulfide, disulfide, or trisulfide functionalities can be reductively desulfurized to toluene by mixed cultures of anaerobic bacteria.

2. Model compounds containing thiophenic sulfur do not appear to be amenable to anaerobic desulfurization. Neither disappearance of a model compound nor production of a desulfurized end-product can be demonstrated.

3. Anaerobic desulfurization of coal is possible. Production of H_2S on IBC-101 or Ugljevik lignite has been documented; however, the source of the sulfide has not been established.

4. Reassociation of sulfur that has been removed as sulfide is a potential problem. A sulfide-purged system should be considered.

RECOMMENDATIONS

We recommend that intensive efforts be directed toward developing better methods to quantify and characterize organic sulfur functionalities in coal. ASTM methods were developed for assessing the quality of coal in the ground, not for characterizing the subtle modifications that might

result from basic experimental research. Until treatment effects can be evaluated with greater sensitivity, we who are developing desulfurization processes are working in the dark. Under the present predicament it is not easy to project a course for success.

Finally, although there is evidence that thiophenic functionalities tend to predominate in higher ranking coals, spectroscopic studies have demonstrated that substantial quantities of sulfidic sulfur exist even in bituminous coal (Gorbaty et al., 1990; Kelemen et al., 1990). One recent study sponsored by the CRSC suggests that disulfides may predominate in Illinois coals (Palmer et al., 1990). For this reason, it is likely that cleavage of sulfidic bonds by anaerobic microorganisms could lead to a reductive desulfurization method for bringing many coals into compliance, even though recalcitrant thiophenic functionalities remain. We would recommend that research efforts be continued in this area.

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PROJECT MANAGEMENT REPORT
June 1, 1991 through August 31, 1991

Project Title: Evaluation of Sulfur-Reducing Microorganisms for Organic Desulfurization

Principal Investigator: Kathleen W. Miller
Biological Sciences Department
Illinois State University
Normal, IL 61761
(309) 438-3834 438-3669

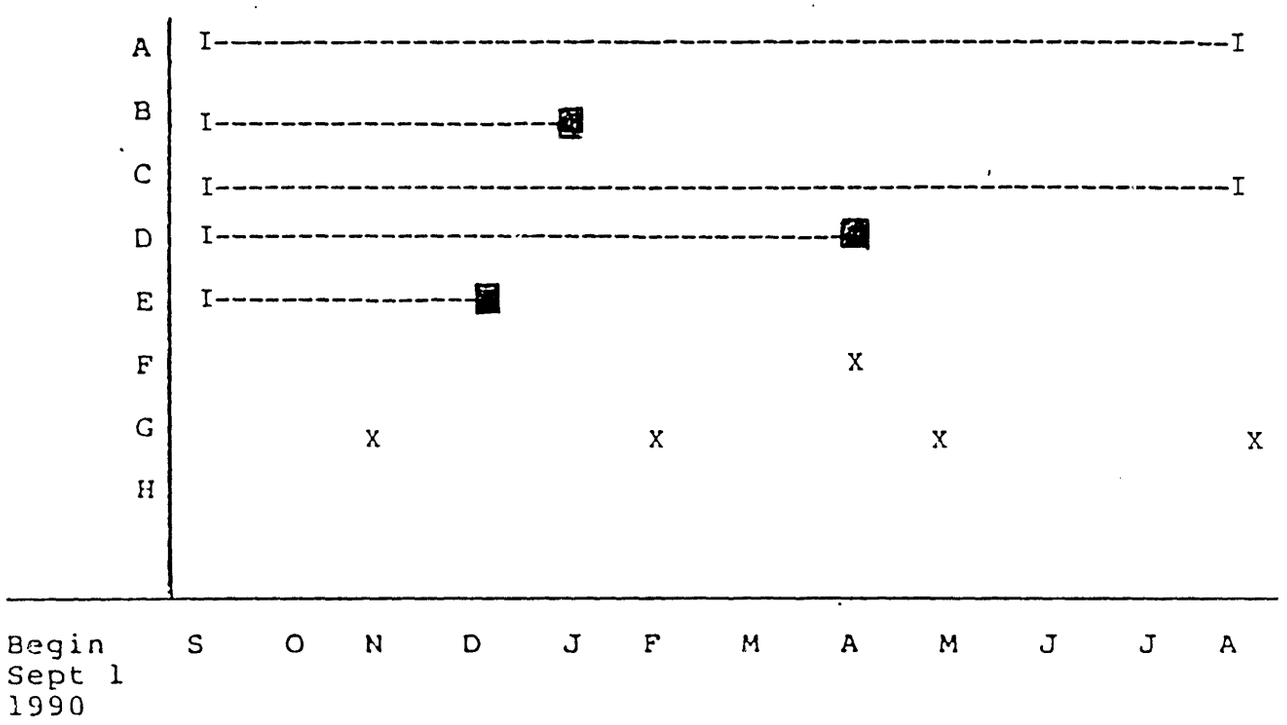
Project Monitor: Dan Banerjee, CRSC

We have received a no-cost extension through December 31, 1991 to expend our remaining funds, which total approximately $1800. At the conclusion of this extended period, I will submit an addenda to the Final Technical and Project Management Reports. These funds will be used primarily to finish research in progress and for publication page charges.

(This project is funded by the Illinois Department of Energy and Natural Resources as part of its cost-shared program with the U. S. Department of Energy.)

EVALUATION OF SULFUR-REDUCING MICROORGANISMS
FOR ORGANIC DESULFURIZATION

Kathleen W. Miller



A. Employ graduate student and undergraduate hourly workers
B. Sampling of additional anaerobic environments
C. Continue to optimize cultures; attempt to isolate and identify individual species
D. Obtain additional coals and perform desulfurization tests
E. Sulfide sorption studies
F. Evaluation of results so that experiments can be designed for 1991-92 proposal
G. Prepare Technical and Project Management reports

EXPENDITURES - EXHIBIT B

Projected and Estimated Actual Expenditures by Quarter

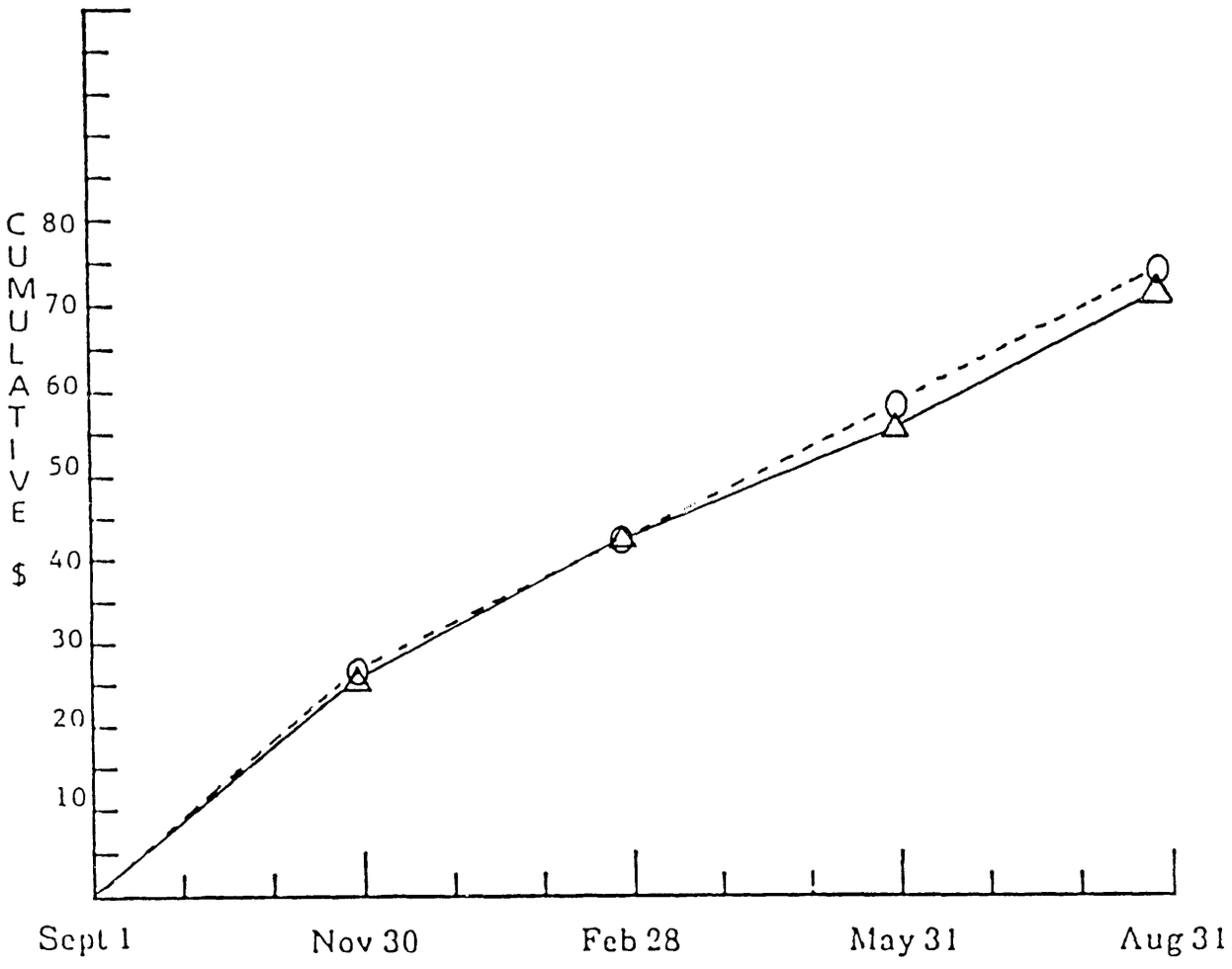
Quarter*	Types of Cost	Direct Labor	Materials and Supplies	Travel	Major Equipment	Other Direct Costs	Indirect Costs	Total
Sept. 1, 1990 to Nov. 30, 1990	Projected	11,588	1,500	0	10,275	1,141	2,450	26,954
	Estimated Actual	11,298	2,393	150	8,717	531	2,309	25,398
Sept. 1, 1990 to Feb. 28, 1991	Projected	23,176	3,000	700	10,275	2,283	3,943	43,377
	Estimated Actual	20,449	3,489	858	11,872	2,506	3,917	43,901
Sept. 1, 1990 to May 31, 1991	Projected	32,138	4,500	700	12,200	4,124	5,366	59,028
	Estimated Actual	29,184	4,430	858	12,149	3,226	4,984	54,832
Sept. 1, 1990 to Aug. 31, 1991	Projected	43,726	6,000	850	12,200	5,265	6,804	74,845
	Estimated Actual	42,112	6,010	858	12,116	5,206	6,630	72,932

* Cumulative by quarter

COSTS BY QUARTER - EXHIBIT C

EVALUATION OF SULFUR-REDUCING MICROORGANISMS
FOR ORGANIC DESULFURIZATION

Kathleen W. Miller



Months and Quarters

0 = Projected Expenditures ----------

Δ = Actual Expenditures ——————

Total CRSC Award $ 74,845

END

DATE FILMED

2/06/92