AIIM

Association for Information and Image Management

1100 Wayne Avenue, Suite 1100
Silver Spring, Maryland 20910

301/587-8202

Centimeter

Inches

MANUFACTURED TO AIIM STANDARDS
BY APPLIED IMAGE, INC.

1 of 3

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

FINAL REPORT

Published: August 1993

DISCLAIMER

Prepared by

Dr. Bill B. Elmore, Louisiana Tech University
Department of Chemical Engineering
Ruston, LA 71272

Prepared for

The United States Department of Energy
Pittsburgh Energy Technology Center
Contract No. DE-AC22-88PC88854

ABSTRACT

As an alternative to post-combustion desulfurization of coal and pre-combustion desulfurization using physicochemical techniques, the microbial desulfurization of coal may be accomplished through the use of microbial cultures that, in an application of various microbial species, may remove both the pyritic and organic fractions of sulfur found in coal. Organisms have been isolated that readily depyritize coal but often at prohibitively low rates of desulfurization. Microbes have also been isolated that may potentially remove the organic-sulfur fraction present in coal (showing promise when acting on organic sulfur model compounds such as dibenzothiophene).

The isolation and study of microorganisms demonstrating a potential for removing organic sulfur from coal has been undertaken in this project. Additionally, the organisms and mechanisms by which coal is microbially depyritized has been investigated.

Three cultures were isolated that grew on dibenzothiophene (DBT), a model organic-sulfur compound, as the sole sulfur source. These cultures (UMX3, UMX9, and IGTS8) also grew on coal samples as the sole sulfur source (from which greater than 95% (w/w) of the pyritic sulfur had been previously removed).

Numerous techniques for pretreating and "cotreating" coal for depyritization were also evaluated for the ability to improve the rate or extent of microbial depyritization. These include prewashing the coal with various solvents and adding surfactants to the culture broth. Studies showed that in low slurry concentrations (e.g. 2% w/v), organic inhibition of the depyritization rate existed as a result of organic by-products. In contrast, in more concentrated slurries (20% w/v) the accumulation of leachable organics from the coal may have been the strongest limiting phenomenon to depyritization.

Using a bituminous coal containing 0.61% (w/w) pyrite washed with organic solvents at low slurry concentrations (2% w/v), the extent of depyritization was increased approximately 25% in two weeks as compared to controls. At slurry concentrations of 20% w/v, a tetrachloroethylene treatment of the coal followed by depyritization with *Thiobacillus ferrooxidans* increased both the rate and extent of depyritization by approximately 10%.

DISCLAIMER

TABLE OF CONTENTS

Page

I. INTRODUCTION 1
- A. Task 1-Project Planning 2
- B. Task 2-Coal Procurement and Preparation 2
- C. Task 3-Analytical Procedures for Total Organic Sulfur 3
- D. Task 4-Organic Sulfur Removal 5
- E. Task 5-Project Management and Reporting 12

II. LITERATURE CITED 13

III. ATTACHMENT A 17
- A. Appendix I--August 1, 1988 to March 15, 1989 18
- B. Appendix II--March 15, 1989 to March 15, 1990 40
- C. Appendix III--March 15, 1990 to April 20, 1992 69

IV. ATTACHMENT B 129

Subcontract Report Summary for Task 4 as conducted by Dr. Bailey Ward at the University of Mississippi

A. Appendix I—Biological Samples, DBT Flourescence Spray-Plate Assay, Culture Media, GC Methods, ASTM Sulfate Assay, Lehigh University Cultures, Solvent Extraction Methods, Chromatogram of Organic Sulfur Standards

B. Appendix II—Summary of DBT Spray-Plate Assays, Lehigh Cultures and Kodama Products, GC/MS, Sulfur-stress Assay

C. Appendix III—Solvent Extraction Efficiency, Thin-layer Chromatography, GC/MS Analysis

D. Appendix IV—Bioactivity on DBT-Sulfone, Spray-Plate Assay, Shake-flask Assays, Cell-Slurry Assays, ASTM Sulfate Assays

E. Appendix V—The UMX Consortium, The Sulfur-Stress Assay, Ion Chromatographic Sulfate Assay, Tests on Depyritized Coal, Focus on UMX3 and UMX9, Cell-free Extracts

F. Appendix VI—Assays on UMX3 and UMX9, Assays on IGTS-8, Taxonomic Studies, Tests of UMX3 and UMX9 on Coal

G. Appendix VII—Materials and Methods

H. Appendix VIII—Proceedings Manuscript, "Microbial Extraction of Sulfur from Model Coal Organosulfur Compounds", Purdy, Ward and Lepo, Third Symposium on Biotechnology of Coal and Coal-Derived Substances, 23-24 September, 1991, Saalbau, Essen.

INTRODUCTION

Research on microbial desulfurization has been active at Louisiana Tech since the late seventies. Several papers and theses have resulted (see references 3 and 14) as well as the sponsoring of a national symposium on "Biological and Chemical Removal of Sulfur and Trace Elements in Coal and Lignite" (44) in November of 1982. In 1982, three microorganisms with thiophenic sulfur oxidizing characteristics were isolated (Arrowood [3]). One organism, designated as OC7-A, was separated from an oil sludge pond in Oil City, Louisiana. OC7-A was found to reduce the organic sulfur content of some coals by 25% in preliminary shaker flask tests in a 24 to 48 hour period at neutral pH and 25 °C. Organisms have also been isolated and tested that oxidize pyritic sulfur (Franklin [14]). A *Thiobacillus ferrooxidans* strain separated from an acid mine drainage in W. Virginia reduced pyritic sulfur by 95% in 10 to 14 day treatments.

Expertise was therefore considered present at Louisiana Tech to launch a major effort in the biodesulfurization area. Concentration in this current project was focused on organic sulfur removal and initially involved further application of the OC7-A microorganism. The problems of reliably quantifying organic sulfur were also addressed at Louisiana Tech. Research was conducted with Scanning Electron Microscopy combined with Energy Dispersive X-Ray Spectroscopy (EDS-SEM). Analyses of National Bureau of Standards samples were used to develop an analytical method for determining organic sulfur directly. With this method, resident atoms are excited from the application of an electron beam. As these atoms subsequently decay, the wavelengths for the various atoms are monitored.

The research also involved a search for and isolation of new bacterial species exhibiting organic sulfur oxidizing abilities. Selected organisms were examined for the presence of DNA plasmids (extrachromosomal rings of DNA that replicate autonomously in bacteria) responsible for producing organic sulfur oxidation. Upon identification, it was planned that selected plasmids would be introduced into organisms capable of thriving at economically conducive conditions.

Final evaluation of OC7-A and other microorganisms were to be evaluated for their application in batch or continuous bench-scale coal treatment systems.

The proposed research was divided into five tasks: (1) project planning, (2) coal procurement and preparation, (3) development of analytical techniques for determining organic sulfur content of coal directly, (4) isolation and deployment of microbes for removing organic sulfur (including plasmid studies) and (5) project management. Each task is addressed independently in the following discussion. Individual introductions and literature citations are provided for major tasks. Explanations of the proposed objectives and procedures for the major tasks are outlined through a series of subtasks.

Task 1. Project planning.

This task was completed by Dr. Joseph Fernandes. The final, corrected copy of the Project Work Plan was submitted on December 13, 1988.

Task 2. Coal Procurement and Preparation.

Subtask 2-1: Grinding, Sieving, and Storage

The objective of this task was to grind and sieve all the coal to be utilized in this investigation.

The coal was ground to various mesh sizes in a ball-mill grinder. The ground coal was separated into mesh sizes ranging from 50 to 270. After grinding a single batch of coal (coal was received in 20 pound batches), the coal to be used in the various tests was riffled to ensure homogeneity of all samples. The coal was then stored (prior to and following microbial treatment) in plastic bags in an inert environment of argon or nitrogen until further use. Analysis and characterization of the coal followed grinding and biotreatment according to the ASTM methods prescribed.

Subtask 2-2: Microbial Pyrite and Sulfate Removal

This project was concerned primarily with addressing the problems of microbial organic sulfur removal. The coal was therefore further processed after grinding and sieving to remove a majority of the pyritic and sulfate forms of sulfur. This simplified the sulfur analyses and allowed a focus on eliminating the organic sulfur remaining.

The pyritic sulfur was initially removed utilizing *Thiobacillus* species in a process developed by Franklin (14) at Louisiana Tech. As the project progressed, stock cultures of *Thiobacillus* species were supplemented by cultures of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* obtained from the Americal Type Culture Collection (ATCC). A large batch process was used to depyritize the coal to be used throughout the duration of organic sulfur removal studies of the project. The coal was well mixed and stored dry, under inert gas (argon) to prevent further biological oxidation. A pyritic sulfur removal of greater than 90% was achieved in most instances.

Task 3. Analytical Procedures for Total Organic Sulfur

Background

The removal of organically bound sulfur from coal prior to combustion offers many technical challenges. One of the most difficult is the development of an analytical procedure for organically bound sulfur that is: specific, accurate, precise, and fast. The method of reference is the American Society of Testing Materials (ASTM) procedure D-2492, Standard Test Methods for the Forms of Sulfur in Coal (2). This is an established procedure that is well accepted. It was originally published in 1966. It involves wet chemical procedures and determines the organic sulfur indirectly. The difference between the sum of the pyritic and sulfate sulfur and the total sulfur is attributed to organically bound sulfur.

This method provides a common reference point for other analytical methods. However, it is not a perfect procedure. It has been shown to yield a lower value for pyritic sulfur than other more direct analyses (24). Mossbauer spectroscopy provides a non-destructive assay of the pyritic sulfur in coal. This method uses a radioactive isotope of iron (Fe-57) and provides a specific value. The concentrations measured by this method are generally higher than those from ASTM D-2492.

Scanning Electron Microscopy combined with Energy Dispersive X-Ray Spectroscopy (EDS-SEM) has been used to provide a direct determination of organic sulfur in raw and chemically treated coals (39). In this method, coal regions free of mineral inclusions were identified. A few cubic micrometers of this material were excited with the electron beam as the wavelengths for sulfur, iron, calcium, silicon, and aluminum were monitored. This enabled the exclusion of any data point that included any finely divided mineral matter. The reported results agree with the ASTM values for most samples if the counting statistics are considered.

The sulfur content of two Canadian coals were analyzed by several recently developed analytical techniques including EDS-SEM (1). However, the authors did not attempt to provide quantitative values for the organic sulfur by this method. They also conducted Fourier Transform Infra-Red spectroscopy with photoacoustics and electrophoresis in their study. This analysis found considerable differences between the coals even though their proximate analyses were similar. They attributed the differences to the incorporated clay and pyrite variations.

Instrumental neutron activation analysis (INAA) has been used in our laboratories to determine the trace element concentration in lignite (40) and in many other laboratories for the analysis of coals (24). INAA is an extremely sensitive method that provides data for many elements. The sulfur values are obviously total values. This method requires a large neutron flux and specialized handling equipment. Data reduction is accomplished in most cases by using the comparison method in this procedure. National Bureau of Standards (NBS) Standard Reference Materials (SRM) are included with each set of samples during the exposure times. Their known values are used to calculate the concentrations of the unknown samples.

Objectives

There have been many volumes of analytical procedures written for coal. It is an important fuel that has been used for centuries. We know many things about it. The one thing that all coal investigators seem to agree on is that coal composition is a variable. This fact requires that any study that attempts to measure the reduction of organic sulfur concentration by various procedures must begin with a sufficient quantity of coal that has been homogenized and analyzed as thoroughly as possible.

Subtask 3-1: Characterization of Starting Material

Sufficient quantities of coal that had been ground into desired particle sizes (but prior to any microbial treatment) were obtained from Task 2. This material was stored in large plastic bags under an inert gas environment. Parameter variation resulting from storage was evaluated by an ultimate and proximate analysis characterizing each portion. ASTM D-2492 was followed to determine the forms of sulfur in the starting material. A sufficient number of analyses were conducted to insure that the deviations between samples and lots were within the ASTM limits for precision and repeatability.

Subtask 3-2: Quality Assurance and Testing

An overall QC/QA plan covering all phases of the project was submitted and approved after award of the contract.

Quality control for sulfur determination was maintained by running the same procedures on NBS SRM 1632a and 1635. At least 1 out of each 25 samples was randomly selected as a standard. The data generated was used to construct and maintain quality control charts that were stored on the laboratory computer. If any standard samples fell out of the control limits, the analyses of that period were repeated and the cause of the error was corrected.

Subtask 3-3: Analytical Procedures

Pyritic and sulfatic sulfur were determined via ASTM D-2492, organic sulfur by ASTM D-3177, and total sulfur by the Eschka method using a LECO Total Sulfur analyzer.

Subtask 3-4: Microscopic Analysis

Initially, EDS-SEM analyses of NBS-SRM 1632a were conducted to determine the number of spots that must be excited and averaged in order to provide direct data that has no more variation than the ASTM procedure. The SEM was also used to photograph the macerals of the coal before and after the treatment procedures.

The distribution of pyritic and organic sulfur of a sample was mapped before and after biological treatment. An infrared analysis of the starting material was performed using a new sampling procedure that was developed at Louisiana Tech to minimize the strong absorption bands that interfere in the carbon sulfur bond stretching region.

Task 4. Organic Sulfur Removal

Background

Organic sulfur makes up 1/2 to 1/3 of the sulfur found in domestic coal. Model compounds representative of coal organic sulfur species are diphenylsulfide, benzothiophene, dibenzothiophene, and thianthrene. Although mercaptans, aliphatic sulfides, and disulfides are present in coal, the hetero-aromatic sulfur compounds are found in predominating concentrations (43). The heterocyclic sulfur compounds are recalcitrant to most chemical desulfurization processes and represent the species expected to be most resistant to microbial attack.

Expensive chemical desulfurization processes are not as limited by the internal inaccessibility of organic sulfur as are the biological processes. Even at the small particle sizes effective in the biological removal of inorganic sulfur, much of the organic sulfur is inaccessible to the microbial enzymes responsible for the oxidation of the model organic sulfur species. Only the sulfur compounds residing at the surface of the particle would be vulnerable to enzymatic attack.

The metabolism of condensed thiophene compounds in the environment is not well known. Few microorganisms have been characterized as dibenzothiophene (DBT) oxidizers, but those that do are strongly oxidative. Most of the research conducted to date has been oriented toward the potential application of the DBT degrading microorganisms in removing organic sulfur from petroleum, with some reports on coal biodesulfurization. DBT degradation has been demonstrated to be a product of cometabolism; and for some microorganisms, DBT may serve as the sole carbon, energy, or sulfur source. The rate of its metabolism is lowered when lighter, less carbon-condensed aromatic compounds are available to the bacteria. Bailey et al. (5) showed that when the benzene series of compounds are present, the disappearance of DBT is inhibited.

Microbial desulfurization processes have been reported since 1935 when Maliyantz described the bacterial desulfurization of petroleum with the concomitant accumulation of hydrogen sulfide. In 1950 and 1951, Strawinsky patented procedures for microbial desulfurization. In order to reduce carbon loss, i.e. petroleum BTU loss, the former patent involved a two step oxidation and reduction procedure. *Pseudomonas, Alcaligenes,* and *Bacillus* species initially converted the sulfur compounds in petroleum to oxidized inorganic forms. *Desulfovibrio* sp., of the sulfate-reducing bacteria, reduced the oxidized sulfur to sulfide. *Desulfovibrio desulfuricans* was used in 1953 in a reductive procedure patented by Zobell. Kirshenbaum patented a new procedure in 1951 utilizing *Thiobacillus thiooxidans, Thiobacillus thioparus,* and *Thiophyso volutans* to convert sulfur in petroleum to water soluble inorganic forms, especially sulfates. The attempted microbial desulfurization with *Arthrobacter* sp. by Isenburg in 1961 showed little success (44).

Yamada et al. (43) reported on the isolation and identification of DBT-utilizing bacteria. Ideally, they sought bacteria that would oxidize the DBT without the loss of hydrocarbon. Their intent was to apply the bacterial system in petroleum desulfurization. Six DBT active strains were isolated, including *Pseudomonas fragii.* Two new taxonomic strains were named: *Pseudomonas abikonensis* and *Pseudomonas jianii.* DBT utilization was evident in the culture medium when brightly colored ring-fission products were formed. Two water soluble degradation products were also found. One was an aromatic compound with sulfur, phenolic, and enolic hydroxyl groups; and the second was an aromatic with an enolic hydroxyl group and a mercaptan group.

Nakatani et al. (35) described culture conditions optimal for DBT oxidation by *Pseudomonas abikonensis* and *Pseudomonas jianii.* The mineral-salts meat extract base was supplemented with DBT dissolved in light oil. Dissolving the DBT in light oil enhanced the oxidation process. The DBT degradation products had absorption maxima at 395 and 470 nm. Since a correlation was observed between optical density and aqueous sulfur content, soluble sulfur concentration was determined spectrophotometrically. Meat extract as a nitrogen source was optimal at a concentration of 0.4%, with DBT at 10%, a pH of 7.3, and a temperature of 28 °C. Vigorous aeration was favorable for DBT conversion. A 40% conversion ratio was attained within a period of 3 days. Optical density, representing growth, peaked on the second day.

Kodama et al. (27) reported on the isolation and identification of oxidation products by *Pseudomonas jianii.* Five water soluble organic sulfur compounds were detected as degradation products of DBT. Three of these compounds were identified: 3-hydroxy-2-formyl-benzothiophene, dibenzothiophene-5-oxide, and 3-oxo-2(3'hydroxy-thionaphthenyl-(2)-methylene) dihydrothionaphthene. Ultraviolet spectra, infrared spectra, and nuclear magnetic resonance data were used in the identification of the isolated products.

Kodama et al. (26) reported further identification of the DBT oxidation products and proposed an oxidation pathway for DBT. The newly identified compounds were trans-4(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoic acid and the second as the hemiacetal form of the first.

Kodama (25) reported that DBT was oxidized by resting cells of *Pseudomonas jianii* in the presence of lactate or glycerol as energy sources. This finding agrees with the assessment of DBT oxidation as a phenomenon of co-metabolism. Induction of the DBT oxidizing enzymes occurred in the presence of compounds containing benzene rings having no side chains, such as DBT, naphthalene, and anthracene. Kodama concluded that DBT was practically unusable as a carbon source for energy and without a co-substrate, like lactate or glycerol, could not induce specific enzymes.

Sagardia et al. (36) reported that *Pseudomonas aeruginosa* PRG-1 degrades benzothiophene (BT) and related compounds. Oxidation of the BT was measured spectrophotometrically by the disappearance of the 225 nm peak. The oxidation pathway was not elucidated, but it was suggested to be similar to that reported by Kodama. The organism was isolated from oil-contaminated soil. BT as a sole nutritive source would not support growth. Yeast extract was required for BT oxidation to occur. As in Yamada's DBT research, the BT was dissolved in light oil and added to a basal-salts yeast extract medium. The oil-aqueous system reduced the toxic effects of the substrate.

Hou and Laskin (16) reported on the oxidation of DBT by *Pseudomonas aeruginosa* ERC-8. Degradation products appeared as red pigments within the bacterial cells. The products identified were 4(2-(3-hydroxy)-thionaphthenyl)-2-hydroxy-3-butenoic acid. The second compound was a tetradecane ester of the first compound with an hydroxylated hydrocarbon moiety. DBT was not degraded unless n-paraffin was supplied in the enrichment screening medium. The paraffin apparently acted as the energy source. The DBT degradative enzyme action of *Pseudomonas aeruginosa* ERC-8 attacked only the benzene ring and did not alter the sulfur moiety.

Laborde and Gibson (31) reported on the metabolism of DBT by the mutant *Beijerinckia* B8/36 in the presence of succinate. The two initial water soluble degradation products that accumulated before ring-fission products appeared were: (+)-cis-1,2-dihydroxy-1,2-dihydrodibenzo-thiophene and dibenzothiophene-5-oxide. The organisms were cultured at 30 °C in 10-liter quantities of a mineral-salts medium supplemented with 0.2% succinate and 0.05% DBT. Air was supplied at 12 liters per minute. The culture was stirred at 600 rpm. Yellow-ring fission products were observed but not identified (30).

Chandra et al. (9) reported on the microbial removal of organic sulfur from coal. Following the screening technique reported by Yamada et al. (43) an unidentified mixed bacterial culture was isolated exhibiting DBT utilization where DBT was the sole carbon source. A coal sample was ground to 240 mesh and suspended in a mineral-salts beef extract medium (pH 7.5) at a concentration of 6.6%. The coal suspension was inoculated with 5 ml of a culture containing 10E9 cells/ml and incubated on a rotary shaker for 10 days at 30 °C. Sterile coal was shown to lose 17.9% organic sulfur following bacterial treatment and unsterile coal lost 14.9%. Activity of the culture on coal previously treated to remove the inorganic sulfur was not reported.

Finnerty (13) reported on the degradation of DBT by a mixed-culture bacterial system. The sulfur-containing aromatic compounds DBT, BT, thioxanthine, thianthrene, and benzene disulfide were oxidized but would not serve as a sole source of energy, carbon, or sulfur. The culture converted 97% of the DBT to water-soluble compounds within 24 hours. The degradation products were identified as 1,2-dihydroxy-dibenzothiophene, 3-hydroxy-2-formyl-benzothiophene, trans-4(2-(3-hydroxy)-thionaphthenyl)-2-oxo-3-butenoic acid and an unidentified product. It was found that DBT was oxidized by *Pseudomonas putida*, which would grow on naphthalene as a sole carbon and energy source. Observing that the degradation products of DBT from different bacterial strains were similar, and in some cases the same, Finnerty was led to examine and determine whether the genes coding for DBT oxidation were located on plasmids. It was discovered that the plasmid containing the genes for naphthalene oxidation also contained the genes for DBT oxidation. No taxonomic identification of the mixed-culture system was reported.

Jerusik (21) working with Finnerty, reported the tentative identification of the microorganisms in the mixed culture responsible for oxidation of DBT. The Gram-negative rod was tentatively designated as an *Acetobacter* sp. and the Gram-positive coccus as a member of the Micrococcaceae. The possible environmental impact of the DBT degradation products was studied using nonspecialized and specialized bacteria. Little toxic effect was observed when *Shigella flexneri*, *Pseudomonas aeruginosa*, or *Escherichia coli* were cultured in a liquid medium supplemented with DBT degradation products at 90% concentration. No change in the lag or logarithmic phases was observed, although the stationary cell mass did decrease. E. coli continued to grow in the presence of the cell products after attaining the stationary phase, suggestive of diauxic growth. The exact nature of the observed stimulation was not determined.

Eckart et al. (12) reported on the isolation of bacteria exhibiting desulfurizing activity on crude Romashkino oil. The bacterial strains were isolated from oil polluted water, soil, and remnants in oil tanks. DBT was used as the model organic sulfur species in the screening media. All strains were aerobic, strongly oxidative, and tentatively identified as species of Pseudomonas. The most active strains were capable of removing 50 to 55% of the sulfur within 5 days. The incubation was carried out in discontinuously running laboratory fermentors. There was no mention of applying the cultures to desulfurize coal.

Kurita et al. (29) reported the isolation of a bacterial culture that reductively degraded thiophene, producing hydrogen sulfide. The anaerobic culture was isolated from oil well sludges or crude oil reservoir bottoms. The thiophene decomposing bacterial cells were Gram-negative rods that grew optimally at pH 7.2-7.8 at 38 °C in a nitrogen or hydrogen environment. Polypeptone was required for growth. Hydrogen sulfide production was enhanced by the addition of $FeSO_4$. Cell-free extracts of the culture catalyzed the production of H_2S from thiophene when supplied with methyl viologen in hydrogen gas. The culture did not utilize thiophene as a sole source of carbon, sulfur, or energy. Cripps (11) reported the isolation of a *Flavobacterium* sp. capable of using thiophene-2-carboxylate as a sole source of carbon, energy, and sulfur for growth. The sulfur atom of the thiophene nucleus was oxidized to sulfate and accumulated in the culture medium.

Arrowood (3) described a bacterial isolate designated OC7-A, a naturally occurring soil microorganism which degraded DBT. OC7-A, found in oil samples collected around an oil well slush pond near Oil City, Louisiana, was tentatively identified as a species of Arthrobacter. Desulfurization by OC7-A occurred in nature at 25-28 °C and pH of 7.0. It required relatively low cost nutrient additives of secondary carbon sources. Coal samples with a total sulfur content of 5.1% were treated with OC7-A in 1% and 5% slurries. A calculated percent organic sulfur lost from coal was reported as 25.78 in 48 hours. The levels of organic sulfur reduction varied with the slurry concentration and the added carbon source. Succinate as an added carbon source showed the highest level of organic sulfur reduction. As a model organic sulfur compound, Arrowood also studied desulfurization of DBT by OC7-A using Hutner's vitamin free-mineral base; 18% organic sulfur reduction was found. An AE mineral-salts solution gave even better results. In determining whether co-metabolism would enhance organic sulfur degradation, a secondary carbon source was added. Napthalene was found to be inhibitory while yeast extract showed a reduction of 18.67-24.43%.

The ambient conditions and the effectiveness of organic sulfur removal from coal by the organisms reported by Arrowood, especially the naturally occurring OC7-A, show promise for an economically feasible large-scale process of coal cleaning.

Isbister and Kobylinski (20) found what they called a unique desulfurizing microorganism. This organism, designated CB1, is a DBT degrading organism that was developed by mutagenically altering the DNA of the naturally occurring parent organism. The parent organism degraded DBT minimally. CB1 was initially grown in a basic mineral-salts medium. CB1 reduced organic sulfur in a bench model coal reactor at pH 7.0 over a temperature range of 25-35 °C for 48 hours. The treatment process was then moved to a continuous pilot plant capable of treating 1000 to 2500 pounds of coal per day. Using ^{14}C-DBT and ^{35}S-DBT, the action of CB1 in DBT was traced. CB1 was found to be different than other microorganisms that oxidize DBT, because it released 2,2 di-hydroxybiphenyl instead of breaking the carbon ring to yield 3-hyroxy-2-formylbenzothiophene. Cleaned coals containing approximately 90% organic sulfur were used in the initial research to bypass problems associated with organic sulfur analysis. The total sulfur content of these coals was determined using the Fisher Total Sulfur Analyzer before and after treatment. The organic sulfur content was reduced 18-28% when treated with CB1. When run of the mine coals were used with CB1 treatment, the organic sulfur removed varied from 25% to 34%. The variations in the data may be attributed to differences in particle sizes (desulfurization increases as particle size decreases), surface characteristics of the coal (desulfurization is inhibited by surface oxidation), or differing amounts of thiophene sulfur in the coals. Using a direct method of organic sulfur determination, which involved energy-dispersive x-ray microanalysis combined with scanning election microscopy (EDS-SEM), pulverized coal of 140 mesh was tested for organic sulfur content before and after treatment with CB1. The reduction in organic sulfur was 32.9% to 34%. Initial cost estimates for enough medium to produce the microorganisms capable of treating 1 ton of coal was $117. Further studies showed that at a much lower level of microorganism dose and using a less expensive carbon source, the medium cost estimate was reduced to $2.34/ton. The overall cost estimate, depending on whether the final product was dried or used with 30% moisture, was calculated to be $25.50 or $21.09 per ton of product coal. Of this cost, $11.39 was estimated as the cost/ton for organic sulfur removal.

Murphy et al. (34) used the microorganism *Sulfolobus acidocaldarius* to reduce sulfur from coal. They adapted the organism to grow on high sulfur coal without the addition of nutrients. They believe that a 50-60% reduction in organic sulfur is possible in 3-6 days when the coal used was 200 mesh, temperature 55-80 °C, and pH 1-2. The estimated cost was given at $10-14/ton or about $12. *S. acidocaldarius* must have oxygen to be viable so air must be pumped or stirred in. *S. acidocaldarius* also utilizes the CO_2 in air as its carbon source. Surfactants were added with varying results, from increased to decreased efficiencies.

Kargi and Robinson (23) also used *Sulfolobus acidocaldarius* to oxidize sulfur in treated coal. They found that *S. acidocaldarius* used Fe_2^+ and reduced sulfur as energy sources. They used coal of 100-150 mesh at a temperature of 75 °C and pH 2.5. Initially a double strength mineral-salts medium was used. Yeast extract, $FeCl_3$, and other nutrients were added as needed. When the yeast extract was added, both the rate and the amount of sulfur reduction decreased. When the initial coal was 11% total sulfur, 15.6 mgS/(L h) was removed. When the total sulfur was 4%, 3.1 mgS/(L h) was removed. On the average, there was a total sulfur removal of 40%. Total sulfur was determined using the Eschka method. Inorganic sulfur was determined using standard methods of ASTM. Organic sulfur levels were found by subtracting the inorganic sulfur content from the total sulfur content.

Monticello et al. (33) conducted a study that linked plasmids to the oxidation of DBT. They used plasmids of two strains of *Pseudomonas* that oxidized DBT. Each of the plasmids has a molecular weight of approximately 55 x 10^6. The products of the DBT oxidation appeared to be identical to products of other DBT reducing microorganisms, substantiating their findings.

Vaseen (41) reported that *Thiobacillus ferrooxidans* may be used to reduce organic sulfur from coal under certain conditions. It requires a temperature of 20-35 °C and a pH of 1.2-2.5. This pH is very corrosive so moving parts must be minimized. For effective sulfur reduction, the coal must be pulverized to a uniformly-reduced particle size. Oxygen is a requirement of *T. ferrooxidans*, it may be sparged or added through agitation. *Thiobacillus ferrooxidans* also gets needed carbon from the CO_2 in the air. Vaseen believed that with a bioreactor he had designed, 100,000 metric tons of coal with 2.3% total sulfur could be processed in a year.

Kublek and Clark (28) reported that mutagens of *Escherichia coli* degraded thiophene and several other organic and inorganic sulfur compounds. These mutants can hydrolize DBT on a minimal medium of yeast extract, peptone, and 0.5% glucose at pH 7.0. Mutant generations of E. coli were tested in an effort to produce an efficient thiophene degrader.

Objectives

In the past twenty years, genetic engineering and recombinant DNA work with bacteria has created the potential for altering microorganisms to perform new and different metabolic reactions. One area that has more recently received attention is the use of bacterial plasmids, extrachromosomal entities, in mediating the parent cells to metabolize difficult, recalcitrant substrates. One of these substrates is the sulfur-containing heterocyclic organic compounds found in coal, coal tars, and crude oil. Plasmid-mediated degradation of dibenzothiophene (DBT) by *Pseudomonas* species has been reported by several workers as referenced in the above section on Background of organic sulfur removal. Plasmid-mediated degradation also has promise for application to several species of bacteria that live in the environments of coal mining operation areas, coal mines, and in acid-mine drainage from such areas.

Based on the information to date, the following objectives and approaches were pursued to accomplish the removal or reduction of organic sulfur from coal by biodesulfurization with selected microorganisms in precombustion treatments.

Subtask 4-1: Desulfurization with OC7-A

Objectives and methodology in Subtask 4-1 were to utilize the bacterial isolate reported by Arrowood (3), OC7-A, to reduce the organic sulfur in DBT and coal, in a series of laboratory experiments designed to enhance the levels of organic sulfur removal found in Arrowood's work. Substrate modification and co-metabolic additives were screened to determine the best combinations for maximum organic sulfur reduction. (Note: While this objective existed in the original proposal, difficulties in observing any organic sulfur removal from coal with this culture arose shortly after the commencement of the project. Work with OC7-A then ceased and isolation and identification efforts shifted to other cultures).

Subtask 4-2: Search for New Biodesulfurizing Microorganisms

Objectives and methodology in Subtask 4-2 were to screen soils, coals, waters, and acid-mine drainage samples in a search for new microorganisms capable of removing organic sulfur from coal/water slurries. Shake-culture techniques were used to screen large numbers of samples to detect the microbial populations containing organic sulfur oxidizers. Techniques employing enrichment and isolation procedures reported by other researchers (3, 33) were used to search for microorganisms that remove organic sulfur from coals. Emphasis was given to the autotrophic bacteria in order to reduce the need for expensive substrates, since this group of bacteria utilize CO_2 or simple organic compounds as carbon sources. A part of this subtask was also concerned with techniques for maintenance of the active bacterial organic sulfur oxidizers once they had shown potential for use in coal biodesulfurization.

Subtask 4-3: Plasmid-mediated Techniques

The main objective and methodology of Subtask 4-3 involved the use of plasmid mediation of the most promising and most active organic sulfur oxidizers. By using the procedures reported by Kado and Liu (22) and Monticello et al. (33), a search for plasmids coded for organic sulfur oxidation was made. Some effort was planned for determining whether cell-free extracts could be used in biodesulfurization as effectively as whole cell preparations. In order to determine the role of plasmids in organic sulfur removal, a program of screening the selected isolates for extrachromosomal DNA was planned to determine which parent cells were most productive of plasmids. The final phase of Subtask 4-3 was concerned with regulation of organic sulfur oxidation by the plasmid-mediated strains for maximum performance in biodesulfurization of selected coal samples prior to attempts at continuous bench-scale treatment of coal.

The report on this Task as received from the subcontractor, the University of Mississippi, is presented as Attachment B to this Final Report.

Task 5. Project Management and Reporting

This task consisted of coordination of all tasks, procurement of supplies and equipment, maintenance of ledger of expenses, record keeping of results, preparation of reports and publications.

It should be noted that shortly after the initiation of the project, Dr. Joseph Fernandes left Louisiana Tech University. The project management then shifted to Dr. Brace Boyden who was also in the Department of Chemical Engineering. In February, 1990, Dr. Boyden also left the university and was replaced by Dr. Gary Zumwalt in the Department of Petroleum Engineering and Geosciences. Dr. Zumwalt served as the Project Manager and Principal Investigator until the arrival of Dr. Bill Elmore in May, 1990--a newly hired Assistant Professor of Chemical Engineering. Dr. Elmore then served as PM and PI throughout the remainder of the 36-month project period and the subsequent 12-month, no-cost, contract extension.

The following account of the work accomplished in this project generally follows a task-by-task chronology of events proceeding in a task-by-task analysis. Task 2 (Coal Procurement and Preparation) was expanded in scope (with DOE Project Manager approval) under the direction of Dr. Elmore during the 12-month, no-cost extension obtained in the latter stages of the project. While originally intended as a preparation step only for the subsequent study of microbially removing the organic sulfur fraction, the microbial pyrite and sulfate removal (Subtask 2-2) became the subject of research focus with the intention of enhancing the pyritic removal process. Analytical procedures (Task 3) were conducted at Louisiana Tech and are also presented. The microbial studies for isolating and identifying microorganisms capable of removing organic sulfur were conducted jointly by Louisiana Tech and the University of Mississippi.

LITERATURE CITED

1. Angle, C., W. Leung, R. Mikula, and H. Hamza, "Sulfur Characterization of Eastern Canadian Coal for Optimization of Beneficiation Process". In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Processing and Utilization of High Sulfur Coals, Elsevier, New York, 68-88, 1985.

2. Annual book of ASTM standards. Part 26-Gaseous Fuels, Coal and Coke. ASTM D 2492-79.

3. Arrowood, Michael J., "Isolation of DBT-Oxidizing Microorganisms for Application in Coal Desulfurization", (Master's thesis, Louisiana Tech University), Ruston, Louisiana, 1983.

4. Attia, Y. A., and M. A. Elzeky, "Biosurface Modification in the Separation of Pyrite from Coal by Froth Flotation". In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Processing and Utilizing High Sulfur Coals, Elsevier, New York, 673-682, 1985.

5. Bailey, N. J. L., A. M. Jobson, and M. A. Rogers, "Bacterial Degradation of Crude Oil: Comparison of Field and Experimental Data", **Chemical Geology 11**:203-221, 1973.

6. Beier, E., "Removal of Pyrite from Coal Using Bacteria", In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Coal, Elsevier, New York, 653-672, 1985.

7. Birks, L., **Electron Probe Microanalysis**, 2nd edition, Wiley-Interscience, New York, 1971.

8. Carrington, A., and A. McLachlan, "Introduction to Magnetic Resonance", Harper & Row, New York.

9. Chandra, D., P. Roy, A. K. Mishra, J. N. Chakrabarti, and B. Sengupta, "Microbial Removal of Organic Sulfur from Coal", **Fuel 58**:549-550, 1979.

10. Chiou, K., and O. Manuel, "Chalcogen Elements in Coal and Their Redistribution in Combustion Products". In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Processing and Utilization of High Sulfur Coals, Elsevier, New York, 1985.

11. Cripps, R. E., "The Microbial Metabolism of Thiophene-2-carboxylate", **Biochem. J. 134**:353-366, 1973.

12. Eckart, V., W. Heike, J. Bauch, and H. Gentzsch, "Mikrobielle Entschwefelung von Erdol und schweren Erdolfraktionen 1. Untersuchugen zur Mikrobiellen aeroben Entschwefelung von Romaschkinoroherdol". ("Mineral Desulfurization of Petroleum and Heavy Petrolum Fractions 1. Studies on Microbial Aerobic Desulfurization of Romashkino-crude Oil) **Zbl. Bakt. II. Abt. 135**:674-681, 1980.

13. Finnerty, W. R., "Microbial Desulfurization and Denitrogenation", Symposium on Microbial Processes for Metals Accumulation and Sulfur/nitrogen Removal from Fuels, Second Chemical Congress of the North American Continent, San Francisco, California, 1980.

14. Franklin, T. J., "Search for Inorganic Sulfur-oxidizing Microorganisms for Application in Coal Desulfurization, (Master's thesis, Louisiana Tech University), Ruston, Louisiana, 1983.

15. Genuit, W., J. Boon, and O. Faix, "Characterization of Beech-milled Wood Lignin by Pyrolysis-gas Chromatography-photoionization Mass Spectrometer", **Anal. Chem. 59**:508-513, 1987.

16. Hou, C. T., and A. I. Laskin, "Microbial Conversion of Dibenzothiophene", **Develop. Industr. Microbiol. 17**:351-362, 1976.

17. Hubner, T. F., E. Ras, and N. W. F. Kossen, "Design and Scale-up of a Reactor for the Microbial Desulphurization of Coal: A Kinetic Model for Bacterial Growth and Pyrite Oxidation", Laboratory of Bioengineering Department, Dept. of Chemical Technology, Dept. Univ. of Technology, Julianalaan. 67, 2628 B.C. Delft, The Netherlands.

18. Hubner, T. F., N. W. F. Kossen, P. Bos, and J. G. Kuenen, "Design and Scale-up of a Reactor for Microbial Desulphurization of Coal: A Regime Analysis", Laboratory of Bioengineering, and Laboratory of Microbiology; Delft Univ. of Technology, Delft, The Netherlands.

19. Hubner, T. F., N. W. F. Kossen, P. Bos, and J. G. Kuenen, "Modelling Design and Scale-up of a Reactor for Microbial Desulphurization of Coal", First Symposium on Biohydrometallurgy, Cagliari, Italy, 1983.

20. Isbister, J. D., "Microbial Desulfurization of Coal". In Y. A. Attia (editor), **Processing and Utilization of High Sulfur Coal**. Proceedings of the First International Conference on Processing and Utilizing High Sulfur Coal, Elsevier, New York, 1985.

21. Jerusik, R. J., "The Microbial Metabolism of Sulfur-containing Hydrocarbons", (Ph.D. Dissertation. University of Georgia), Athens, GA, 1979.

22. Kado, C., and S. Liu., "Rapid Procedure for Detection and Isolation of Large and

23. Kargi, F., and J. M. Robinson, "Microbial Desulfurization of Coal by Thermophilic Microorganism *Sulfolobus acidocaldarius*", **Biotech. and Bioeng. 26**:2115-2121, 1982.

24. Karr, C., "Analytical Methods for Coal and Coal Products", vols. I, II, and III. Academic Press, Inc., New York, 1978.

25. Kodama, K., "Induction of Dibenzothiophene Oxidation by *Pseudomonas jianii*", **Agric. Biol. Chem. 41**:1193-1196, 1977.

26. Kodama, K., K. Umehara, K. Shimzu, S. Nakatani, Y. Minoda, and K. Yamada, "Identification of Microbial Products from Dibenzothiophene and Its Proposed Oxidation Pathway", **Agric. Biol. Chem. 37**:45-50, 1973.

27. Kodama, K., S. Nakatani, K. Umehara, K. Shimizu, Y. Minoda, and K. Yamada, "Microbial Conversion of Petro-sulfur Compounds, Part III. Isolation and Identification of Products from Dibenzothiophene", **Agric. Biol. Chem. 34**:1320-1324, 1970.

28. Kublek, B., and D. Clark., "Microbial Removal of Organic Sulfur from Coal (Degradation of Sulfur Containing Heterocyclic Compounds), Final report, DOS/FE/60339-T21 DE85-018429, July 1, 1984-June 30, 1985.

29. Kurita, S., T. Endo, H. Nakamura, T. Yagi, and N. Tamiya, "Decomposition of Some Organic Sulfur Compounds in Petroleum by Anaerobic Bacteria", **J. Gen. Appl. Microbiol. 17**:185-198, 1971.

30. Laborde, A. L., "Initial Reactions in the Microbial Metabolism of Dibenzothiophene and Naphthalene", (Ph.D. Dissertation, The University of Texas at Austin), Austin, TX, 1979.

31. Laborde, A. L., and D. T. Gibson, "Metabolism of Dibenzothiophene by a *Beijerinckia* species", **Appl. Environ. Microbiol. 17**:185-198, 1977.

32. Maijgren, B., W. Hubner, K. Norrgard, and S. Sunduall, "Determination of Organic Sulfur in Chemically-cleaned Coals by Scanning Electron Microscopy and Energy Dispersive X-ray Spectrometer", **Fuel 62**:1076-1078, 1983.

33. Monticello, D. J., D. Bakker, and W. R. Finnerty, "Plasmid-mediated Degradation of Dibenzothiophene by *Pseudomonas* species", **Appl. Environ. Microbiol. 49**:750-760, 1985.

34. Murphy, Julie, *et al*, "Coal Desulfurization by Microbial Processing". In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Processing and Utilizing High Sulfur Coal. Elsevier, New York, 1985.

35. Nakatani, S., T. Akasaki, K. Kodama, Y. Minoda, and K. Yamada, "Microbial Conversion of Petro-sulfur Compounds Part II. Culture Conditions of Dibenzothiophene-utilizing Bacteria", Agric. Biol. Chem. 32:1205-1211, 1968.

36. Sagardia, F., J. J. Rigau, A. Martinez-Lahoz, F. Fuentes, C. Lopez, and W. Flores, "Degradation of Benzothiophene and Related Compounds by a Soil *Pseudomonas* in an Oil-aqueous Environment", **Appl. Microbiol. 29**:722-725, 1975.

37. Spencer, D., Research and Development Status Report, EPRI Journal. 37-47, 1984.

38. Straszheim, W., R. Greer, and R. Markuszewski, "Direct Determination of Organic Sulfur in Raw and Chemically Desulphurized Coals", **Fuel 62**:1070-1075, 1983.

39. Thompson, R., "Trace Element Concentrations in Louisiana Lignite". Symposium on Biological and Chemical Removal of Sulfur and Trace Elements in Coal and Lignite, Louisiana Tech University, Ruston, Louisiana, 1982.

40. Van Dyck, P., S. Torok, and R. Van Grieken. "Enhancement Effect in X-ray Fluorescence Analysis of Environmental Samples of Medium Thickness", **Anal. Chem. 58**:1761-1766, 1986.

41. Vaseen, V. A., "Commercial Microbial Desulfurization of Coal", In Y. A. Attia (ed.), **Processing and Utilization of High Sulfur Coal.** Proceedings of the First International Conference on Processing and Utilizing High Sulfur Coal. Elsevier, New York, 1985.

42. Wrathall, J. A., and E. E. Peterson, "Desulfurization of Coal Model Compounds and Coal Liquids", University of California, Lawrence Berkeley Lab., Pub. No. LBL-8576.

43. Yamada, K., Y. Minoda, K. Kodama, S. Nakatani, and T. Akasaki, "Microbial Conversion of Petro-sulfur Compounds Part I. Isolation and Identification of Dibenzothiophene-utilizing Bacteria", **Agric. Biol. Chem. 32**:840-845, 1968.

44. Proceedings of Symposium on "Biological and Chemical Removal of Sulfur and Trace Elements in Coal and Lignite," November 11-12, 1982, Louisiana Tech University, Ruston, Louisiana.

ATTACHMENT A

Attachment A contains a chronological description of Task 2--Coal Procurement and Preparation and Task 3--Analytical Procedures for Total Organic Sulfur in three Appendices. The contents of the Appendices are as follows:

Appendix I covers the project period from August 1, 1988 to March 15, 1989. Task 2 was conducted primarily as a "service" task for preparing coal for organic sulfur removal studies. Initial work consisted of setting up detailed procedures for coal pretreatment (e.g. microbial depyritization) and storage. EDS-SEM equipment troubles slowed establishment of Task 3. A transition occurred in the Project Management and Principal Investigator positions from Dr. Joseph Fernandes through Dr. Brace Boyden to Dr. Gary Zumwalt.

Appendix II covers the project period from March 15, 1989 to March 15, 1990. During this stage of the project, Tasks 2 and 3 were completed as per the original contract requirements. Project Management and Principal Investigator responsibilities again changed in May, 1990 from Dr. Gary Zumwalt to Dr. Bill Elmore--a new faculty member entering the Chemical Engineering Department. A complete presentation of the work for analytically measuring organic sulfur by EDS-SEM is provided in the thesis, **"Advanced Scanning Electron Microscopy and Energy Dispersive Spectroscopy Techniques for Sulfur Analysis in Coal", Sandeep Sayal, M.S.Ch.E, Louisiana Tech University, May, 1991.** This thesis is available through the Louisiana Tech University library.

Appendix III covers the project period from March 15, 1990 to April 20, 1992. While Tasks 2 and 3 had been completed, an ongoing literature survey revealed the need for additional research into the depyritization process, the findings of which are contained in the thesis, **"Studies and Methods for Increasing the Desulfurization Activity of *Thiobacillus ferrooxidans* on Bituminous Coal", David Krueger, M.S.Ch.E., Louisiana Tech University, August, 1993.** This thesis is also available through the Louisiana Tech University library.

ATTACHMENT A
APPENDIX I
Activity from Project Initiation (August 1, 1988) to March 15, 1989

BIODESULFURIZATION TECHNIQUES: APPLICATION OF SELECTED MICROORGANISMS FOR ORGANIC SULFUR REMOVAL FROM COALS

Project Report from August 1, 1988, to September 16, 1988

Task 1: Project Planning

The draft of the work plan was prepared and submitted to TPO on August 10, 1988. Input from TPO into the draft plan is included in the attached copy.

Task 2: Coal Procurement and Preparation

A sample of Illinois No. 6 coal (IBCSP #1, 1101600) has been received from Illinois Geological Survey. Origination information has been requested from IBCSP.

Subtask 2-1: Grinding, Sieving, and Storage

Ball-mill grinder (ceramic with stones, Paul O. Abbe, Inc. #31343) and Ro-Tap Testing sieve shaker (#11028) have been set up and cleaned.

System for Coal Storage in a Nitrogen Environment

To prevent oxidation reactions in the coal samples, we induce a stasis condition by placing them in a nitrogen atmosphere.

The system consists of a dessication chamber, a vacuum pump, a nitrogen source, three valves, and a pressure gauge as shown in Figure 1. [A "T" connector is threaded into a connection on the side of the dessicator. Connected to the branches of the "T" are a valve and tubing leading to a vacuum pump, and a pressure gauge and valve with tubing leading to a nitrogen cylinder and regulator. Also in place between the valves is a pressure release valve.]

Procedure for Storage

The coal samples are placed in loosely capped vials in the chamber and the lid is sealed airtight. Air is removed by opening the vacuum valve and running the vacuum pump for five minutes. The valve is closed and the nitrogen valve is opened to fill the chamber with 1.5 psi of nitrogen. This is repeated once to flush out remaining oxygen, if any.

After one hour at 1.5 psi the nitrogen is released and the lid is removed. The vial caps are quickly tightened and the chamber is resealed. The air is again removed and replaced with 1.5 psi of nitrogen.

Subtask 2-2: Microbial Pyrite and Sulfate Removal

Samples of acid mine waters were collected from ponds near the Andelex Resources coal mine at Madisonville, Kentucky. Shaker flasks are being set up to determine the operating conditions of either pure or mixed cultures of Thiobacillas ferrooxidans, such as medium type and strength, pH, and leaching time, to remove at least 90% of the pyrite sulfur from coal.

Task 3: Analytical Procedures for Total Organic Sulfur

Subtask 3-1: Characterization

ASTM procedures of moisture, ash, total sulfur, pyritic sulfur, sulfate sulfur, heating value and volatiles are being set up. A set of sulfur/coal calibration samples have been received. Accuracy and reproducibility of the above procedures will be determined by using the standards.

Subtask 3-2: Quality Assurance/Quality Control

QC/QA plan prepared by Dr. John Rowley is attached for approval.

Subtask 3-4: Microscopic Analyses

The first month was dedicated to acquiring a complete set of controls and calibrating equipment. A set of sulphur/coal calibration samples ranging from 1.05% sulphur to 4.39% sulphur was acquired from LECO. Mineral calibration samples for iron sulfide have been ordered. A Zeiss vitrinite reflectance microscope was received from Mobil Oil Corporation. This microscope was set up and the process of calibrating it has begun. The scanning electron microscope was cleaned and rebuilt, and the energy dispersive x-ray spectrophotometer was calibrated.

Task 4: Organic Sulfur Removal

Subtask 4-1: Desulfurization with OC7A

Strain OC7A was delivered by Joseph B. Fernandes (Project Manager) to Dr. Ward as a suspension of cellular material kept for several years in a frozen state (at Louisiana Tech University). Routine surface plating revealed that several apparently different organisms were present in the culture. Each of the co-contaminants were isolated and studies were begun on each to determine if indeed it had OC7A, and if it (or one or more of the other strains) still would degrade DBT as described by Arrowood.

Subtask 4-2: Isolation and Screening Tests for 4S Pathway Bacteria Versus DBT

About two dozen apparently unique bacterial isolates were derived from two sources of weathered coals and from one source of weathered motor oil from a dump. Serial dilutions of derived isolates yielded plate cultures containing scattered, isolated colonies. Developed colonies were sprayed with a 1% (w/v) ethanolic solution of DBT which, after evaporation of the solvent, left a thin coating of crystalline DBT over the colonies and the agar surface. Controls consisted of colonies sprayed with ethanol only or unsprayed cultures. After incubation at 30°C for 4-6 hours, 10-12 hours, 24 hours, 48 hours, and 120 hours, the plates were tested for long-wavelength UV fluorescence to indicate formation of biphenyl from DBT via the 4S Pathway. Several DBT-coated cultures showed fluorescence from the colonies, but so did the controls of each of these false positive isolates. No tested isolate exhibited fluorescence that could be attributed to DBT degradation. A series of isolations and screening of new organisms derived from different source samples collected from a variety of weathered coals or petroleum deposits has just begun. Testing of new methods of screening isolates for 4S activity has also begun.

The process of establishing the ASTM D 516 method for determination of sulfate ions has been started. Standard reagents were prepared and fidelity of calibration curves are being tested using a Spectronic 70 spectrophotometer.

Dr. Ward worked full time on the project during the first two weeks of August (the planned beginning date for the project to be August 1, 1988) with his salary paid from another account, which he plans to reimburse from the subcontract account when it is activated (The University of Mississippi academic year contracts began during mid-August). The delay in funding of the subcontract has created problems with obtaining personnel to work on the project. Dr. Ward was able to employ two gradute students part time during September by "borrowing" from other accounts, which he will need to reimburse.

Dr. Ward had planned for the project to be funded earlier in the year, allowing him to work on the project full time during the summer months to get the research off to a solid start. He received no release time from his academic year duties, thus he now cannot devote as much time to the project as he would wish. He had planned to have employed a full-time postdoctoral research associate to begin in August or September, so that the work could continue without interruption. However, because of the delay in funding, the timing was such that he could not make a monetary commitment to a prospective postdoctoral associate, who was forced to accept another position. He advertised the postdoctoral research associate position during August by direct national mailing to a few hundred universities. Every applicant was expected to complete degree requirements by the end of this (fall, 1988) enrollment period. He has offered the position to one of the applicants, and hopes that he can have her on board no later than January 1989.

Task 5: Project Management and Reporting

The project manager collected samples of acid mine water in Kentucky and attended the Contractor's Review Meeting in Pittsburgh from August 8 to August 11, 1988. Status reports of other researchers in the area of removal of organic sulfur and individual discussions with peers were useful for a good start of the project. Enroute to Tyson's Corner, Vienna, the project manager stopped on August 11 and August 12, 1988, at Morgantown to meet peers attending the Consortium for Coal Liquefaction Science. Participated in the coal workshop "Bioprocessing of Coals" at Tyson's Corner from August 15 to August 18, 1988. Enroute to Ruston, Louisiana, collected soil and weathered coal samples in Virginia and Western Kentucky. Also stopped at Oxford, Mississippi, to deliver the samples to Dr. Ward and to review the contents of the meetings.

Dr. Boyden and Dr. Zumwalt began their tasks on September 1, 1988. One research assistant was hired on September 1, 1988, and is working on Task 3 with Dr. Boyden. Another research assistant (her wages provided by Louisiana Tech University as matching) began working on the project September 8. Two student workers started work on Task 2 on September 8 and another began on September 19. Dr. John Rowley started the work on QC/QA and his working draft is included in this report.

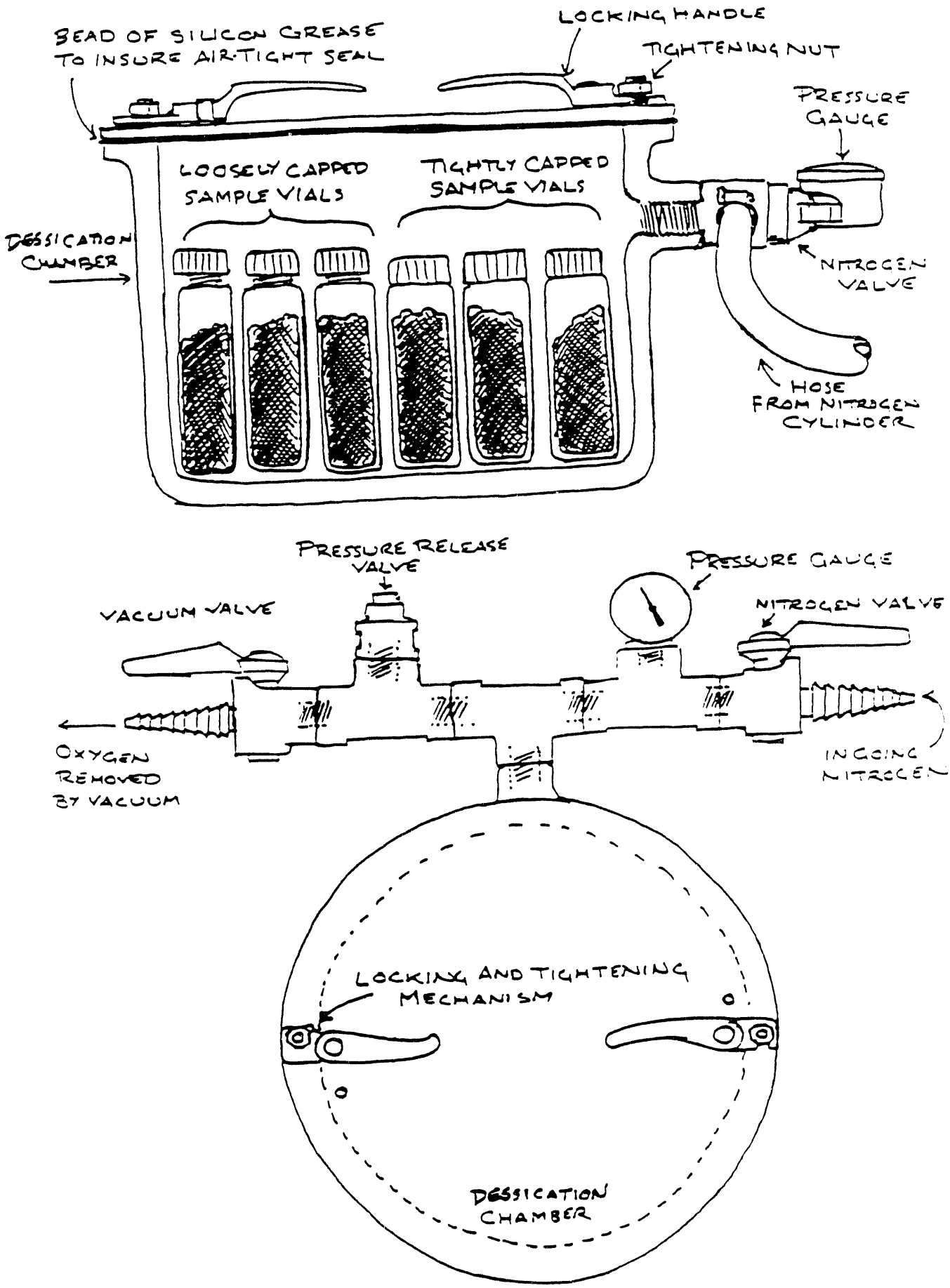


FIGURE ONE: SYSTEM FOR COAL STORAGE IN A NITROGEN ENVIRONMENT

Project Report from 9/15/88-12/15/88

1 PROJECT PLANNING

A corrected Project Plan with hours demarcated as per the categories of labor, task, and month for each year of the project was forwarded December 13, 1988. This Task should therefore be completed.

2 COAL PROCUREMENT AND PREPARATION

2.1 Grinding, sieving, and Storage

More Illinois #6 coal is being ground (200 mesh) and stored under nitrogen. Grinding will continue until several pounds are stored for use throughout the duration of the project.

2.2 Microbial Pyrite and Sulfate Removal

The removal of pyrite is continuing. Several shaker flasks, inoculated with Thiobacillus ferrooxidans in 10POSC medium (staggered in two-week cycles) and Illinois #6, are currently in operation. Preliminary results have indicated more effective removal with shaker flasks than with the aerated, conical 3-gallon reactor.

3 ANALYTICAL PROCEDURES FOR TOTAL ORGANIC SULFUR

3.1 Characterization

All procedures for determination of moisture, ash, volatile matter, total sulfur, sulfate and pyrite concentration, and heating value via ASTM methods have been checked with coal standards for accuracy. When Subtask 2.1 and 2.2 are completed, the coal will be homogenized and characterized. Re-characterization of the stored coal will be performed periodically to evaluate the effects of prolonged storage. Coal from Subtask 2.1 & 2.2 (before and after microbial de-pyritization) are currently being checked for sulfur content (ASTM methods) to evaluate pyritic removal procedures.

3.2 Quality Assurance/Control

The QA/QC master file (compilation of analytical procedures is about complete and upon completion will be forwarded to Dr Rowley for evaluation.

3.3 Other Analytical Procedures

Coal from Subtask 2.1 & 2.2 (before and after microbial de-pyritization, respectively) are currently being checked for sulfur content (ASTM procedures) for evaluation of the pyrite removal process (on an as needed basis).

3.4 Microscopic Analysis

3.4.1 EDS-SEM

After promising advancement in last month's report, the EDS-SEM experienced a series of equipment failures. The diffusion pump (required to achieve the high vacuum) initially failed. This was fixed and put back into service. There now is an electronic "glitch" with respect to the X-ray sensor. Remediation of this problem is currently underway.

Ms. Nory Robbins (Coal Bacteriologist, USGS, Reston, Virginia) was contacted and will send samples of micro-distributed FeS_2 for EDS/SEM analyses. She does not believe that organic sulfur in coal exists to the extent reported, but rather the sulfur is bound/incorporated as micro-pyrite.

1 PROJECT PLANNING

This Task was completed as of 12/13/88.

2 COAL PROCUREMENT AND PREPARATION

2.1 Grinding, Sieving, and Storage

Ten pounds of Illinois #6 coal are ground, homogenized and stored under nitrogen. Arrangements are being made to procure quantities of Kentucky #11 and Pittsburgh #8 coals. Sulfur contents are such in the Illinois #6 coal (see below) that higher sulfur contents are desired.

2.2 Microbial Pyrite and Sulfate Removal

Treatment of the Illinois #6 coal for pyrite and sulfate removal is proceeding. Some delay was encountered due to inactive (i.e. old, lysed, few viable cells) cultures of Thiobacillus ferrooxidans and Thiobacillus thiooxidans. New cultures were obtained from Dr. Stevens at the University of Mississippi as well as from ATCC.

The first shipment of pyrite and sulfate free coal (approximately 200 grams) to Dr. Ward at U.M. Should occur within the next two weeks. Design and construction is currently underway to increase our treatment capacity to remove pyritic and sulfate sulfur. Immediate plans will utilize 20 liter containers anchored to a reciprocal shaker bed. Each container is filled with 15 liters of medium, 150 grams of coal, and 1.5 liters of inoculum of the aforementioned organisms. Plans for an even larger reactor system are being developed (see Attachment I) and should be realized within two weeks of this report. Desulfurization of the current coal batch (designated #1001) is being monitored using a Spectronic 20 spectrophotometer to analyze for sulfate release.

3 ANALYTICAL PROCEDURES FOR TOTAL ORGANIC SULFUR

3.1 Characterization

A typical proximate analyses of the Illinois #6 under nitrogen storage before pyrite and sulfate removal is as follows:

Total Sulfur:	3.39 wt%	+/- 0.05 wt%
Pyrite Sulfur:	1.59 wt%	+/- 0.04 wt%
Sulfate Sulfur:	0.25 wt%	+/- 0.02 wt%
Organic Sulfur:	1.55 wt%	+/- 0.05 wt%
Moisture:	8.98 wt%	+/- 0.09 wt%
Volatiles:	37.8 wt%	+/- 0.6 wt%
Ash:	9.26 wt%	+/- 0.13 wt%

These analyses could change slightly as initial tests were conducted on coal before it was completely homogenized. Caloric value of the coal, particle size distribution, and ultimate analyses will be determined in the near future.

3.2 Quality Assurance and Control

All of the analytical procedures have been forwarded to Dr. Rowley for review. Attachments of two approved procedures (as examples) are included as Attachment II and III. A numbering scheme for each analytical procedure has been devised and these procedures are being kept in a central file.

3.3

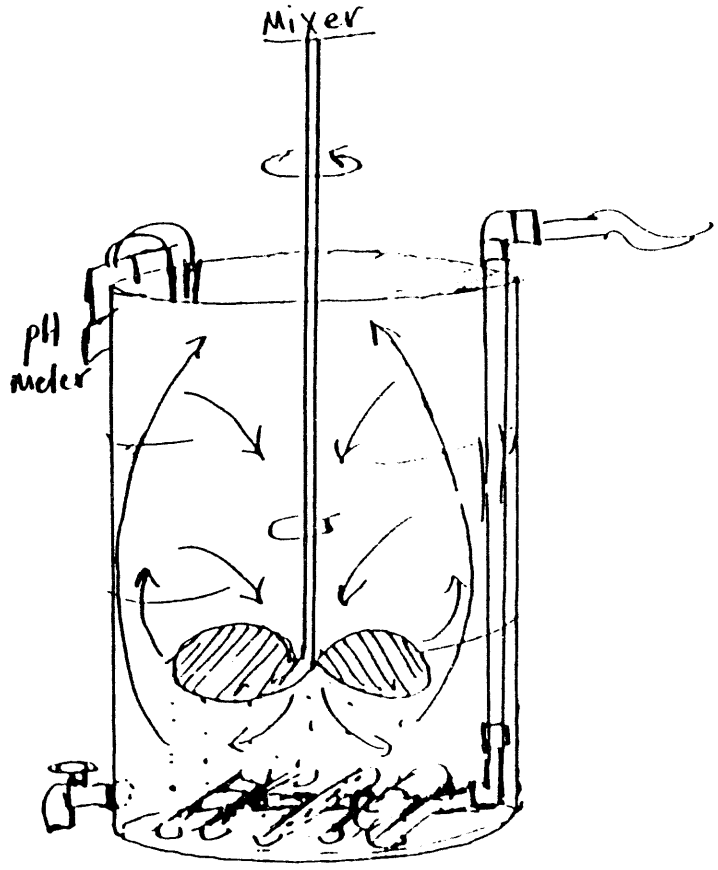
3.4 Microscopic Analysis

3.4.1 EDS-SEM

Work in this area has been delayed to equipment breakdowns. However, most of the problems have been corrected and more activity on this Subtask should be forthcoming in the future.

With side arm aeration

★ BEST



55 gal
polypropylene tank

Chemostat

Reactor for the aeration/mixing and suspension of coal slurry —

[De-pyritization]

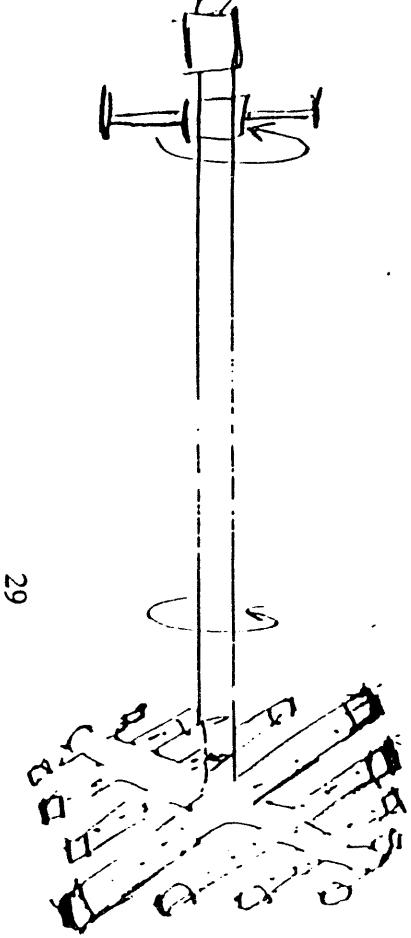
Constant Temp

Also continual pH monitoring

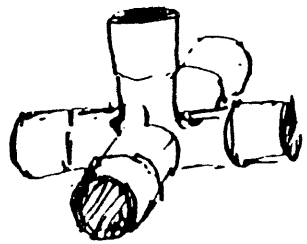
* Also the side arm can be perforated, but the compressor would have to provide enough pressure to produce even aeration.

air

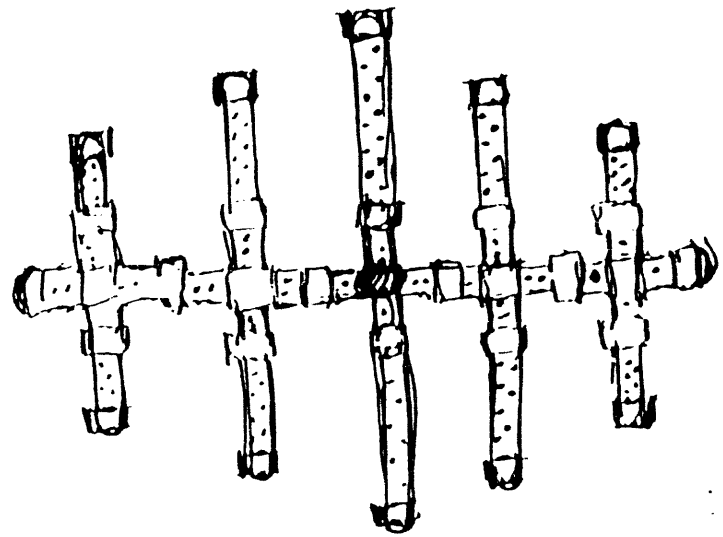
compressor



Most Feasible – stationary aeration w/ mixer



4-tee PVC



pipe out of page

(This will be circular)

perforated PVC pipe – fits in bottom of drum

some clip pipe

* If find – possibility of connecting to mixer and using for both mixing and air

Attachment II

QC/QA PROGRAM
LOUISIANA TECH UNIVERSITY

DOE COAL BIODESULFURIZATION TECHNIQUES:
APPLICATION OF SELECTED MICROORGANISMS FOR ORGANIC SULFUR REMOVAL FROM COAL

DE-AC22-88PC3854

TECHNICAL PROCEDURE REVIEW FORM

PROCEDURE DESIGNATION TP-001, Rev.0: Procedure for Adiabatic Calorimeter

REVIEW DATE November 29, 1988

REVIEWER John C. Rowley

COMMENTS

DISPOSITION

APPROVED ✓ DISAPPROVED _____

APPROVED CONDITIONALLY _____

CONDITIONS TO BE MET FOR APPROVAL

Signature of reviewer John C. Rowley

Date 11/29/88

Note: See typo and suggested number / designator system.

Attachment II (Continued)

PROCEDURE FOR ADIABATIC CALORIMETER

Read and understand ASTM Procedure D 1015-77.

Turn on water supply under counter.

Turn on water heater, water cooler, distilled water tank, master controller, and calorimeter.

Set buret stopcock to direct water through buret. Be sure hot and cold water lights cycle even when the tank temperature stabilizes. (5 seconds/cycle see page 6 of Manual No. 156).

Loading Bomb

Place preignited stainless steel crucible on balance and tare weight.

Place sample pellet in crucible and note weight.

Install 10 cm of firing wire on the bomb head electrodes as shown on page 6 of operations manual.

Place crucible in ring with the wire in contact with sample.

Pipet 1 ml of distilled water into bottom of bomb.

Put top on bomb, hand tighten ring and close valve.

Attach oxygen line to bomb and pressurize to 20 atm.

Release pressure in line with toggle and remove line.

Place bomb in corresponding bucket and fill bucket with 2000 ml of distilled water from buret.

Open calorimeter by raising thermometer (pull black ball) and releasing (pull black ball and lift) cover and slide to right.

Place filled bucket in opening and attach wires to electrodes. Close cover and lower thermometers.

Standardization

Load bomb with benzoic acid pellet and place in calorimeter. Set toggle switches on Master Controller to Ref., Stand., and BTU/lb.

Press reset, then start and respond to lights.

CAL. ID. requires a number for the bomb and bucket combination--1 or 2.

SAMPLE ID is a number for this run.

Attachment II (Continued)

SAMPLE WT. is the weight in grams of benzoic acid.

Master Controller will now conduct the test and give a preliminary report.

After the report is printed, remove the bomb and slowly release the pressure. Open the bomb and rinse the inside with methyl red solution into a beaker.

Titrate this solution till the red color disappears.

Multiply the ml. used by 10 to get acid correction.

Measure the length of unburned wire on the card. This is the wire correction.

Press Sample ID, its numeral, and Enter.

Enter the acid and wire correction and receive the final report which contains the energy equivalent.

Average ten energy equivalent runs for each bomb and bucket combination. The standard deviation of each series must not be greater than 6.5 BTU/°C (see ASTM D2015 TABLE X1).

Check standardization at least once a month if the new standard value exceeds old value by ± 6 BTU/°C, see ASTM D2015 Sec. 9.

Input the average of the energy equivalent for each bomb and bucket combination by pressing *, 0, Enter; the number you selected for the bomb and bucket combination, Enter; and the corresponding energy equivalent.

Running a Sample

Use the pellet press to make a coal pellet from 1 gram of coal, and load the bomb as previously described.

Set the toggle switches to Ref., Det. and BTU/lb.

Press reset and start.

Respond to lights - CAL. ID and SAMPLE WEIGHT.

After preliminary report is printed, open bomb, titrate content and measure wire.

Press SAMPLE ID, enter ID NO. and respond to lights with fuse correction, % sulfur of sample, and acid correction. Final report will then print.

When finished for the day, turn off heater, bucket filling system, calorimeter and water supply.

Leave Master Controller on but set 3-position switch to Man. and press start to go to standby status.

Attachment II (Continued)

Duplicate results should not be considered suspect unless they differ by more than 50 BTU/lb, dry basis.

Attachment III

QC/QA PROGRAM
LOUISIANA TECH UNIVERSITY

DOE COAL BIODESULFURIZATION TECHNIQUES:
APPLICATION OF SELECTED MICROORGANISMS FOR ORGANIC
SULFUR REMOVAL FROM COAL

DE-AC22-88PC3854

TECHNICAL PROCEDURE REVIEW FORM

PROCEDURE DESIGNATION TP-002, Rev.0: Microbial Pyrite and Sulfate Removal

REVIEW DATE November 29, 1988

REVIEWER John C. Rowley

COMMENTS _____

DISPOSITION

APPROVED ✓ DISAPPROVED _____

APPROVED CONDITIONALLY _____

CONDITIONS TO BE MET FOR APPROVAL _____

Signature of reviewer John C. Rowley

Date 11/29/88

Note: See typo and suggested number identifier.

MICROBIAL PYRITE AND SULFATE REMOVAL

Samples of acid mine water and pond mud from Madisonville, Kentucky, were set up in shake flaskes of ISP medium to screen for iron-oxidizing and inorganic sulfur utilizers. Incubation was at room temperature on a reciprocal shaker at 100 shakings/min until the medium showed an orange-brown color. Plates of ISP agar were streaked from the flaskes showing the pressure of iron-oxidizing and inorganic sulfur utilizers to determine the bacterial types. Plates of ISP agar were also streaked directly from the acid mine water samples and the pond mud used to detect inorganic sulfur utilizers.

METHOD II
PROCESSING TREATED COAL SAMPLES --
WATER WASH SHAKE + CENTRIFUGATION METHOD

1. Shake samples vigorously as in #1, Method I.
2. Centrifuge the coal-water suspension at 1000 X g for 5 min. (500 X may also work.)
3. Aspirate or decant the supernatant.
4. Resuspend the pellet in water and filter as in #7, Method I.
5. Transfer sample as in #8, Method I for drying.
6. Transfer sample for analysis as in #9, Method I.

ENVIRONMENTAL SAMPLE INVENTORY
Louisiana Tech

Sample Number	Type	Site Taken From
1	Soil	Highway 1 at 1/2 mile south of Vivian at a tank battery storage area (Oil City)
2	Soil	1/2 mile north of highway 2 on highway 1 at new tank battary storage area (Oil City)
3	Soil	Same as above but from old battery storage area (Oil City)
4	Soil	Sludge pit on highway 2, NE of Vivian, 1 mile before Caddo Lake (Oil City)
5	Liquid	Oil pit on the same site as samples 2 and 3 (Oil City)
6	Liquid	Same site as sample 1
7	Liquid	Same site as sample 2
8	Liquid	Same site as sample 3 (Oil City)
9	Liquid	Same site as sample 4
10	Liquid	Same site as sample 5
11	Weathered lignite	Dolet Hill in Mansfield, La.
12	Weathered lignite and soil	Dolet Hill in Mansfield, La.
13	Pristine lignite	Dolet Hill mine, F-2 range, Mansfield, La.
14	Soil	Oil seepage in California (California State University, Northside)
15	Soil	From 2.6 pH pond, Kentucky

Sample Number	Type	Site Taken From
16	Weathered coal --- Andelex	Near pond in sample 15
17	Liquid	pH 2.6 pond, Kentucky
18	Liquid	pH 2.34 pond, Kentucky
19	Liquid	1/2 mile south of I-70 on Indiana Hwy. 42
20	Liquid	Kentucky Hwy. 813 Exit 37
21	Liquid	Active slurry pond in Kentucky
22	Liquid	Runoff of Peabody strip mine slurry
23	Liquid	1/2 mile south of I-70 on Indiana Hwy. 42
24	Soil	Hot Sulfur Springs Colorado
25	Soil	Hot Sulfur Springs Colorado
26	Soil	Hot Sulfur Springs Colorado
27	Soil	Hot Sulfur Springs Colorado
28	Soil	Hot Sulfur Springs Colorado
29	Soil	Hot Sulfur Springs Colorado
30	Soil	Pressboard Plant (Dr. Boyden)
31	Soil	Chevron Oil Refinery, Port Arthur, Texas -- West Lagoon Conduit Bank
32	Soil	Chevron Oil Refinery, Port Arthur, Texas -- #6 Tank hold

Sample Number	Type	Site Taken From
33	Soil	Chevron Oil Refinery, Port Arthur, Texas -- 47PH Manifold Conduit
34		
35		
36		
37		
38		
39		
40		
41		
42		
43		
44		
45		
46		
47		
48		

ATTACHMENT A
APPENDIX II
Project Activity from March 15, 1989 to March 15, 1990

1 PROJECT PLANNING

Task Completed.

2 COAL PROCUREMENT AND PREPARATION

2.1 Grinding, Sieving, and Storage

Additional Illinois coal (from Illinois Geological Surv[illegible] [illegible]C-101) has been procured, ground to 200 mesh, homogenized, and stored under nitrogen atmosphere. This coal is in addition to what was previously on hand from a previous coal shipment. Analyses on the new coal will be in subsequent reports.

2.2 Microbial Pyrite and Sulfate Removal

Microbial reactors have been set up for the removal of pyritic sulfur. Cultures employed for this purpose include ATCC Thiobacillus thiooxidans and Thiobacillus ferrooxidans, and a mixed environmental culture extracted from a South American mining site, denoted Sero de Pasco. The first batch of "depyritized" coal was sent to U. of Mississippi, with approximately 90% of the resident pyrite removed. Additional shipments will be made as the coal becomes available.

3 ANALYTICAL PROCEDURES FOR TOTAL ORGANIC SULFUR

3.1 Characterization

Additional characterization will be forthcoming on the coal recently ground and put under nitrogen. These analyses will include a sulfur breakdown, ash, volatiles, moisture, BTU content, and a C:H:N:O breakdown. The effects of storage on this coal will also be monitored by same above analyses.

3.2 Quality Assurance and Control

All of the analytical procedures have been reviewed and approved (by Dr. Rowley) and incorporated into the central file.

3.3

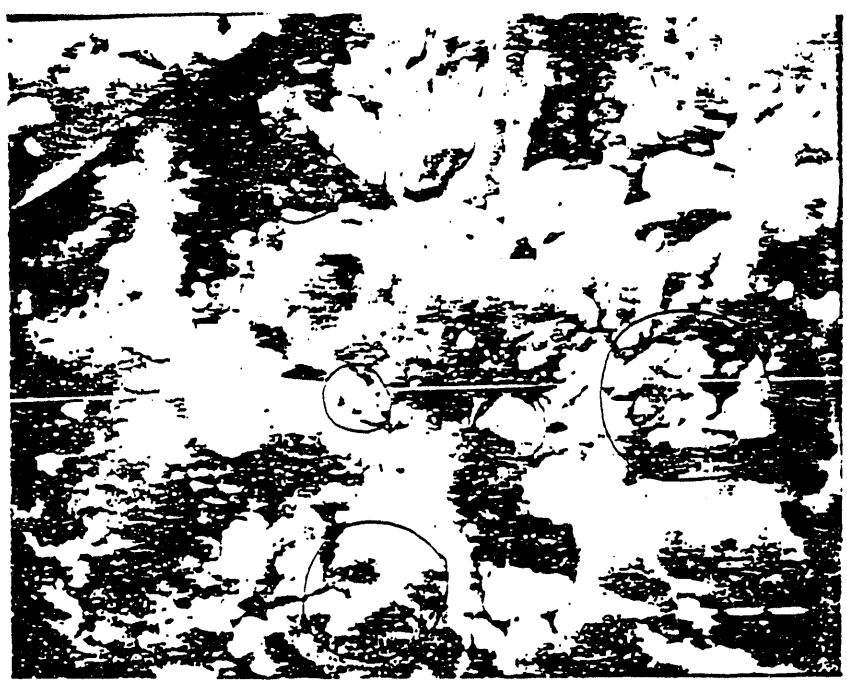
3.4 Microscopic Analysis

3.4.1 EDS-SEM

Work involving the use of this technique for the direct analysis of organic sulfur in coal is continuing. The present areas of emphasis are: (1) sample preparation, (2) optimum magnification, and (3) minimization of the number of required shots. With respect to sample preparation, the traditional 24-hour epoxy mounting has been replaced by a pelletization procedure. A sample of homogenized, 200 mesh coal is compressed under several thousand psi into an one-half inch diameter pellet. The surface of the pellet appears "glassy" to the eye and, under magnification, provides a reasonably planer surface in which crystalline pyrite can be identified (and therefore avoided). A view graph of a representative surface has been included with the areas of pyrite isolated. Resolution was lost when the view graph is photocopied, however, the crystalline pyrite can be identified by looking for signs of a crystalline matrix, e.g. straight edges, triangular areas, cubics, et cetera.

EDS - SEM

Parameters: Voltage 15 eV
TOA 30°



501-020
ASTM % S Total = 2.89
EDS-SEM % S Total = 3.24
450X

Circled areas - pyritic sulfur (rhombic)

Shot location - center of pellet

Sample - Lot # 489-217 (501-020)
Standard

C - 68.50%
H - 4.49%
N - 0.86%

TOTAL SULFUR %
ASTM - 2.89
EDS-SEM - 3.24
% ERROR - 12.11

1 PROJECT PLANNING

Task Completed.

2 COAL PROCUREMENT AND PREPARATION

2.1 Grinding, Sieving, and Storage

Of the approximate 15 lbs of Illinois #6 coal originally ground to -200 mesh and stored under nitrogen, approximately 6 lbs are still to be depyritized via Thiobacillus sp. The coals (both Illinois basin coals) being utilized in the project include lots 1002 and 1003 as defined below:

	lot #1002		lot #1003	
Component	wt%	Deviation -/-	wt%	Deviation -/-
Total Sulfur	3.72	0.06	4.22	0.10
Pyritic Sulfur	pending		0.85	0.01
Sulfate Sulfur	0.27	0.02	0.16	0.02
Organic Sulfur (via diff.)	pending		3.21	0.10
Volatiles	41.5	0.7	51.1	0.50
Ash	10.2	0.1	10.51	0.19
Moisture	8.98	0.09	11.38	0.19
Carbon	64.91	0.97	pending	
Hydrogen	4.82	0.20	pending	
Nitrogen	0.71	0.01	pending	
Oxygen (via diff.)	pending		pending	
Heating Value (BTU/lb_m)	11,950	48	11,145	58

2.2 Microbial Pyrite and Sulfate Removal

The microbial leaching of pyrite (via Thiobacillus sp.) from coal lot 1002 is via a series of 5 gallon reactors (2 wt% slurry). Each different batch is denoted as 1002A, 1002B, et cetera. Because of the smaller batches, final ultimate and proximate analyses will not be performed until the coal is treated, recombined, and homogenized. However, the reactor used for depyritizing coal lot 1003 is approximately 50 gallons and can treat up to 10 lbs of coal per batch (a typical batch lasting anywhere from 2 to 3 weeks). The large size of the 1003 batches allows the economic (ultimate/proximate) analyses of each reactor volume or throughput.

Some of 1002 coal was left on an extra week (batch 1002A) to remove essentially all of the pyrite. This coal is for use as one of the standards for EDS/SEM analyses. As a result of coal 1002A being used in this manner, corroborative ultimate/proximate analyses of this coal was sought through a commercial laboratory (copies included). The analyses of 1002A were as follows (moisture-free):

Component	Our Analyses: lot #1002A wt%	Deviation -/-	Commercial Testing Lab: lot #1002A wt%	Deviation -/-
Total Sulfur	2.35	0.02	2.46	0.10
Pyritic Sulfur	0.09	0.01	0.05	0.05
Sulfate Sulfur	0.17	0.01	0.18	0.02
Organic Sulfur (via diff.)	2.59	0.04	2.23	0.17
Volatiles	37.72	0.3	------	------
Ash	8.91	0.09	9.14	0.30
Moisture	2.90	0.09	2.97	------
Carbon	68.78	pending	68.78	0.30
Hydrogen	4.77	pending	4.77	0.07
Nitrogen	1.49	pending	1.49	0.05
Oxygen (via diff.)	13.36	pending	13.36	------
Heating Value (BTU/lb_m)	11,902	13	------	-------

3 ANALYTICAL PROCEDURES FOR TOTAL ORGANIC SULFUR

3.1 Characterization

Much of the coal characterization is included in the above sections. However, another experiment which was of interest with respect to sulfur analyses was that of cleaning (or not cleaning) the coal after treatment with Thiobacillus sp. for pyrite removal. There was some concern by this team that microorganisms adhering to the surface of the coal could contain internal sulfur (or perhaps inclusion bodies) which, depending on the degree the coal was washed, could affect the sulfur analyses. In July's report several coal washing procedures were presented, including ultrasonic cleaning. The ultrasonic cleaning was tested and the results are tabulated below:

Results of Ultrasonic Cleaning

	Sulfur Content (Wet, mass %)	Moisture (mass %)	Sulfur Content (Dry, mass %)
Before Washing	2.60 ± 0.06	4.20 ± 0.23	2.71 ± 0.06
After Washing	2.33 ± 0.03	16.52 ± 0.65	2.79 ± 0.03

Based on this procedure (and the fact that the coal was so throughly washed), we detected no discernable difference between washed and unwashed coal with respect to sulfur analyses.

3.2 Quality Assurance and Control

All of the analytical procedures have been reviewed and approved (by Dr. Rowley) and incorporated into the central file.

3.4 Microscopic Analysis

3.4.1 EDS-SEM

Work with respect to the use of the EDS-SEM for monitoring of organic sulfur content in microbially treated coals is continuing. Currently, Illinois #6 coal standards (with varying degrees of sulfur content) are being incorporated into a standard library. Standards are prepared by removing essentially all of the resident pyrite from the coal via the use of Thiobacillus sp. and thus circumventing any problems associated with the possible presence of micro-pyrite. Coal sample 1002A is one of the coals to be used as a standard.

The standard analysis technique is a known (although not necessarily widely known or accepted) way through which the results from a particular coal EDS-SEM spectrum are compared with spectra of 'known' organic sulfur contents. A statistical comparison between the known and unknown spectra determines the organic sulfur content for the unknown sample. The closer the unknown organic sulfur content to that of the library standard, the more accurate the analysis. Conference with Dr. Warren Straszheim (who did considerable work with this technique in his Ph.D. studies) at the recent Pittsburgh conference has helped considerably, particularly with working out system 'bugs.' The standard analysis will be that used in conjunction with ASTM procedures to ascertain changes in organic sulfur contents as a result of the use of environmental isolates.

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300
Member of the SGS Group (Societe Generale de Surveillance)

September 2, 1989

PLEASE ADDRESS ALL CORRESPONDENCE TO
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460136 CTE U
FAX: (504) 464-7220

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272
BRUCE H. BOYDEN

Sample identification by
LA. TECH

P.O. 12-4111

Kind of sample reported to us: 32-4111 DOE COAL PROJECT SUBMITTED SAMPLE

Sample taken at: RUSTON, LA.

Sample taken by: UNKNOWN

Date sampled: ------

Date received: August 28, 1989

Analysis Report No. 89-54726

SHORT PROXIMATE - ULTIMATE ANALYSIS

	As Received	Dry Basis	
% Moisture	2.97	xxxxx	
% Carbon	66.74	68.78	
% Hydrogen	4.63	4.77	
% Nitrogen	1.45	1.49	
% Sulfur	2.39	2.46	
% Ash	8.87	9.14	
% Oxygen(diff)	12.95	13.36	
	100.00	100.00	
Btu/lb	xxxxx	xxxxx	MAF xxxxx

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO.

Robert D. Stephens
Manager, St. Rose Laboratory

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 963-9300

SINCE 1908

Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO:
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

September 16, 1989

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

RE: SUBMITTED SAMPLE IDENTIFIED AS 32-4111 DOE COAL PROJECT
P. O. NO. 12-9111
SGS/CTE FILE: 89-54726

SULFUR FORMS - ASTM D 2492 PRECISION

	FORMS OF SULFUR		PERMISSIBLE DIFFERENCES, %	
	As Received	Dry Basis	Same Laboratory	Different Laboratory
% PYRITIC	0.05	0.05	....	....
Coal, under 2%			0.05	0.30
Coal, over 2%			0.10	0.40
% SULFATE	0.17	0.18	0.02	0.04
% ORGANIC (DIFF) ..	2.17	2.23		
% SULFUR	2.39	2.46		

SGS/COMMERCIAL TESTING & ENGINEERING CO.

Robert G. Stephens

Robert G. Stephens, St. Rose Branch Mgr.

PMcD

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

SINCE 1908 — Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO:
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460133 CTE UI
FAX: (504) 464-7220

September 16, 1989

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

RE: SUBMITTED SAMPLE IDENTIFIED AS 32-4111 DOE COAL PROJECT
P. O. NO. 12-9111
SGS/CTE FILE: 89-54726

ULTIMATE ANALYSIS - ASTM D 3176, TABLE 3 PRECISION

	ULTIMATE ANALYSIS As Received	ULTIMATE ANALYSIS Dry Basis	PERMISSIBLE DIFFERENCES, % Same Laboratory	PERMISSIBLE DIFFERENCES, % Different Laboratory
% MOISTURE	2.97	XXXXX	....	....
% CARBON	66.74	68.78	0.30	....
% HYDROGEN	4.63	4.77	0.07	....
% NITROGEN	1.45	1.49	0.05	....
% SULFUR	2.39	2.46	....	....
Coal, under 2%			0.05	0.10
Coal, over 2%			0.10	0.20
% ASH	8.87	9.14		
No carbonates present			0.20	0.30
Carbonates present			0.30	0.50
Coals with more than 12% ash containing carbonates and pyrites			0.50	1.00
% OXYGEN (DIFF)...	12.95	13.36		
	100.00	100.00		

SGS/COMMERCIAL TESTING & ENGINEERING CO.

Robert G. Stephens

Robert G. Stephens, St. Rose Branch Mgr.

PMcD

1 PROJECT PLANNING

Task completed.

2 COAL PROCUREMENT AND PREPARATION

2.1 Grinding, Sieving, and Storage

Task completed.

2.2 Microbial Pyrite and Sulfate Removal

The removal of pyritic sulfur from approximately 16 to 18 lbs of Illinois #6 coal (200 mesh) via the Thiobacillus sp. is completed. Two coal lots of Illinois #6 were used, lot 1002 and lot 1003. As the coal was treated by batches, each batch was designated by lot number followed by a letter; e.g. the first batch of 1002 treated with Thiobacillus sp. was designated 1002A, subsequent batches B, C, et cetera. The original plan was to use several batches and at the end homogenize all the batch runs. However, if a particular coal batch was needed for immediate use, complete analyses were also performed on that batch.

3 ANALYTICAL PROCEDURES FOR TOTAL ORGANIC SULFUR

3.1 Characterization

To date, a synopsis of the treatment of the coals is presented below. Data on the most recently treated and homogenized coals (1002[B,C&D] and 1003[A&B]) will be included in next month's report. All treated coals are stored under nitrogen and will be periodically monitored for property changes.

	Lot/Batch Numbers							
	1002		1002A		1002(B&C)		1002(B,C&D)	
	wt%[1]	SD[2]	wt%	SD	wt%	SD	wt%	SD
Total Sulfur	3.72	0.06	2.85	0.02	2.84	0.08		
Pyritic Sulfur	0.94	0.01	0.09	0.01	0.08	0.01		
Sulfate Sulfur	0.30	0.02	0.17	0.01	0.18	0.01		
Organic Sulfur (via difference)	2.48	0.09	2.59	0.04	2.58	0.10		
Volatiles	41.5	0.7	37.72	0.3	NA[3]	NA		
Ash	10.2	0.1	8.91	0.09	NA	NA		
Moisture	8.98	0.09	2.90	0.09	28.26	0.1		
Carbon	69.18	0.97	68.78	0.3	NA	NA		
Hydrogen	5.03	0.2	4.77	0.07	NA	NA		
Nitrogen	1.51	0.01	1.49	0.05	NA	NA		
Oxygen (via difference)	10.36	1.34	13.20	0.53	NA	NA		
Heating Value, BTU/lb.	13,129	53	11,902	13	NA	NA		

	Lot/Batch Numbers 1003		1003A		1003(A&B)	
	wt%[1]	SD[2]	wt%	SD	wt%	SD
Total Sulfur	4.22	0.10	3.23	0.03		
Pyritic Sulfur	0.96	0.01	0.13	0.01		
Sulfate Sulfur	0.18	0.02	0.09	0.01		
Organic Sulfur (via difference)	3.08	0.10	3.01	0.05		
Volatiles	51.1	0.5	35.95	0.13		
Ash	10.51	0.19	8.86	0.31		
Moisture	11.88	0.19	12.58	0.34		
Carbon	69.00	0.3	68.45	0.3		
Hydrogen	5.12	0.07	4.59	0.07		
Nitrogen	1.49	0.05	1.33	0.05		
Oxygen (via difference)	9.66	0.71	13.54	0.76		
Heating Value, BTU/lb$_m$	12,647	66				

[1]All wt% are on a moisture free basis.
[2]Standard deviation.
[3]Not analyzed.

3.2 Quality Assurance and Control

All of the analytical procedures have been previously approved by the Quality Assurance Officer, Dr. John Rowley. Moreover, to assure continued accuracy of analytical results, periodic analysis have been routinely farmed out to other labs for verification. Two such laboratories included Commercial Testing & Engineering Co. of St. Rose, LA (used routinely for ultimates) and Guelph Chemical Laboratories Ltd. of Ontario, Canada. To date our in-house analyses have been well within acceptable inter-laboratory data reporting.

3.3

3.4 Microscopic Analysis

Studies during this quarter have concentrated on developing and using a standardless analysis (as opposed to a standard analysis) to monitor organic sulfur removal. The coals in use, two Illinois #6 coals 1002 & 1003, have been cleaned of pyrite to where that remaining constitutes approximately 0.10 percent by weight; resident sulfate ranges from 0.10 to 0.20 percent. This translates (via the difference formula) into an organic sulfur content of ca. 2.5 to 3.0 wt%.

Because of the special nature of our coals, the EDS-SEM standard analysis (as delineated in the literature) was considered too laborious (considering the number of samples involved) and perhaps not the optimum route to pursue or duplicate. A standardless analysis on the other hand offered more flexibility. This procedure however for coal sulfur analysis has yet to be developed; to this end, we have made significant progress.

Extensive EDS-SEM analyses have been made on coal 1002, before (Figure 1) and after pyrite removal (Figure 2). In Figure 1, EDS analyses are presented from left to right, down the page, by decreasing magnifications. ASTM total sulfur analyses are reflected in each plot by the straight line. The average percent sulfur and standard deviation for each magnification is presented in the bottom plot. As expected, the average deviation decreased proportionally with decreased magnification. One would expect the average sulfur concentration to be more of an "average" as the area of excitation is increased. In the spot mode, where the area of excitation is small (or the raster of the electron beam is restricted), larger deviations in S concentration were expected and this was reflected experimentally. As more of the coal surface (i.e. volume) is included in the analysis, the S average shows less tendency to vary drastically.

Each of the plots in Figure 1 is composed of 15 shots. This number was random in choice, however, a statistical analysis (students t-test) is currently underway to minimize the number of required shots at each magnification to fall within accepted ASTM levels at a 95% confidence level. The apparent, almost linear, trend (in the bottom most plot) of increasing sulfur concentration with decreasing magnification is also being further elucidated.

Figure 2 consists of the EDS-SEM shots of the same coal after pyrite removal. Additional plots were included to authenticate apparent fundamental behavior or anomalies. For instance in the right most bottom plot (average % sulfur and standard deviation as a function of magnification), there appeared to be a trend from 220X to 70X in the form of decreasing sulfur concentration with decreasing magnification. However, further investigation (i.e. additional shots) revealed no such behavior. Plots containing just 5 points are other examples of reruns at particular magnifications.

Although the causes and effects are by no means firmly established (or for that matter, the deductions and hypotheses composed), what can be said of the data in Figures 1 and 2 to date is this:

i. As expected, there is less standard deviation ("drift" if you will) in the data as the magnification is decreased or the volume [of coal] of excitation is increased.

ii. The optimum magnification (at the NVs chosen) seems to be around 320X.

iii. The most apropos NVs, although originally composed from 100 - %C - %H - %O - %N - %moisture (sometimes) or those elements which EDS cannot detect without a special window, seem to correspond closely with the percent ash (see coal analyses). However, this is an early supposition.

iv. Early indications confirm that the standardless analysis is a viable alternative to the standard analysis for this type of sulfur analysis in coal.

Future directions of experimentation with the EDS include the following concerns:

i. Complete shooting of coals 1003 and 1003(A&B) and/or 1003A in an analogous fashion as was done with coals 1002 and 1002A.

ii. Analyze results of both coals to determine 'optimum' magnification(s) or correction factors at any magnification.

iii. Complete statistical analyses to minimize number of shots to achieve a defendable (and reproducible) level of accuracy.

iv. Obtain more data for the selection of apropos NVs.

v. Analyze iron content data; perhaps, to back out resident pyrite concentration?

vi. Begin routinely using the technique/procedure in tandem with ASTM procedures on inventoried coals being used to test microorganic sulfur removal.

COMMERCIAL TESTING & ENGINEERING CO.

GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO:
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

January 16, 1990

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

Sample identification by
LA. TECH.

SAMPLE IDENTIFIED AS: 1002BCD

Kind of sample reported to us: COAL - SUBMITTED SAMPLE

Sample taken at: UNKNOWN

Sample taken by: UNKNOWN

Date sampled: ------

Date received: January 2, 1990

Analysis Report No. 89-67390

% CARBON (DRY BASIS)	= 69.19
% HYDROGEN (DRY BASIS)	= 4.43
% NITROGEN (DRY BASIS)	= 1.42

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO

Manager, St. Rose Laboratory

COMMERCIAL TESTING & ENGINEERING CO.

GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO:
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

January 16, 1990

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

Sample identification by
LA. TECH.

SAMPLE IDENTIFIED AS: 10038B

Kind of sample reported to us: COAL - SUBMITTED SAMPLE

Sample taken at: UNKNOWN

Sample taken by: UNKNOWN

Date sampled: ------

Date received: January 2, 1990

Analysis Report No. 89-67391

% CARBON (DRY BASIS) = 68.94

% HYDROGEN (DRY BASIS) = 4.60

% NITROGEN (DRY BASIS) = 1.38

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO

Robert A. Stephens

Manager, St. Rose Laboratory

TASK 1 Project Planning

Task complete.

TASK 2 Coal Procurement and Preparation

2.1 Grinding, Sieving, and Storage

Task complete.

Future: We have ordered 20 pounds of Illinois #6 (IBC-101). Half of this coal will be homogenized, ground to pass through a #200 sieve, and rehomogenized. Five pounds each will be ground to 30/0 and 100/0.

2.2 Microbial Pyrite and Sulfate Removal

Task complete.

The removal of pyritic sulfur from Illinois #6 and the Freeport coal is completed.

Depyritized Coal Available:

Illinois #6 (IBC 101)

1002E (Freeport) -------- 2270g
1003AB (Illinois #6) -------- 2360g

minus 300 g to David Boron DOE
minus 300 g to John Kilbane IGT

Future: Depyritized coal will be available as batch removal continues.

1. The large reactor will be started with 200/0 coal. This batch should be available by April 30, 1990.

   We will run a mass balance with a degeneration curve while monitoring pH, temperature, and bacteria count.

2. Efforts to optimize the batch reaction should generate additional depyritized coal. The resultant coal by-product may vary in grain size. Two small (5 gal.) reactors have been constructed to test reactor conditions. The first two runs of these small reactors (sr) will be used to compare the efficiency of reactor sizes.

   sr1 Duplicate large reactor run discussed above.

   sr2 Control run without bacteria to evaluate oxidation of coal matrix. The next runs will be used to evaluate the effects of coal size on the efficiency of depyritization.

TASK 3 Analytical Procedures for Total Organic Sulfur

3.1 Characterization

Analyses of the stock and depyritize coal are included in Table 1. Further analyses of the most recently treated coal (1002E) await the return of carbon analysis from the commercial laboratory. These analyses will be available for the next report. All treated coals are stored under nitrogen.

3.2 Quality Assurance and Control

We continue to run multiple samples to check in house accuracy as prescribed by Quality Assurance Officer, Dr. John Rowley. Moreover, to assure continued accuracy of analytical results, periodic analysis have been routinely farmed out to other labs for verification. To date, our in house analyses have been well within acceptable inter-laboratory data reporting. Commercial analyses of carbon, hydrogen, and nitrogen are included in Appendix A.

3.4 Microscope Analysis

We finished basic data collection with the SEM/EDS system this last quarter. Most of our efforts have been dedicated to statistical analysis of these data. Our first concern was to find a statistical measure that fit the distribution of our data. We analyzed the data for normal, lognormal, truncated lognormal, maximum-minimum uniform, and standard uniform distribution. The result of these analyses for the two end points and a middle value are included in Table 2. We used a Kolomogorov-Smirnov (K-S) test to evaluate the fit of each of our data sets to these distributions (Table 2). Our data is best described as having a normal or lognormal distribution. Normal and lognormal plots of spot mode and 100X are included in Figure 1.

Once we had a reasonable representation of our data distribution, we were able to ask how many samples were necessary to predict the mean with a 95 percent certainty to fall within ASTM tolerances. We ran student "t" test on each distribution. Again the results for spot mode, 100X and 30X are reported (Table 3). These analyses suggest that four to five spectra should predict the mean within ASTM tolerances as long as we keep the magnification at or below 100X (Figure 2).

Table 1. Batch Analyses of Microbial Depyritized Coal

	Lot/Batch Numbers									
	1002		1002A		1002(3&C)		1002(B,C3D)		1002E	
	wt%[1]	SD[2]	wt%	SD	wt%	SD	wt%	SD	wt%	SD
Total Sulfur	3.72	0.06	2.35	0.02	2.34	0.08	3.00	.02		
Pyritic Sulfur	0.94	0.01	0.09	0.01	0.08	0.01	0.264	.02		
Sulfate Sulfur	0.30	0.02	0.17	0.01	0.18	0.01	0.232	.01		
Organic Sulfur (via difference)	2.48	0.09	2.59	0.04	2.58	0.10	2.50	0.05		
Volatiles	41.5	0.7	37.72	0.3	NA[3]	NA	40.40	±.14		
Asn	10.2	0.1	8.91	0.09	NA	NA	7.02	.13		
Moisture	8.98	0.09	2.90	0.09	28.26	0.1	12.55	.36		
Carbon	69.18	0.97	68.78	0.3	NA	NA	69.19	0.3		
Hydrogen	5.03	0.2	4.77	0.07	NA	NA	4.43	0.07		
Nitrogen	1.51	0.01	1.49	0.05	NA	NA	1.42	0.05		
Oxygen (via difference)	10.36	1.34	13.20	0.53	NA	NA	14.94	0.57		
Heating Value, BTU/lb_m	13,129	53	11,902	13	NA	NA	11,995	60		

[1] All wt% are on a moisture free basis.
[2] Standard deviation.
[3] Not analyzed.

Table 1 (cont'd) Batch Analyses of Microbial Depyritized Coal

	Lot/Batch Numbers									
	1003		1003A		1003(A&B)		1004		1004A	
	wt%[1]	SD[2]	wt%	SD	wt%	SD	wt%	SD	wt%	SD
Total Sulfur	4.22	0.10	3.23	0.03	3.34	±0.07	2.79	±.05	1.07	±.02
Pyritic Sulfur	0.96	0.01	0.13	0.01	0.130	0.008	1.26	±.03	.25	±.03
Sulfate Sulfur	0.18	0.02	0.09	0.01	0.117	0.002	0.654	±.03	.417	±.009
Organic Sulfur (via difference)	3.08	0.10	3.01	0.05	3.09	0.09	0.88	±.11	.40	±.06
Volatiles	*51.1	0.5	35.95	0.13	45.83	±.20	30.09	±.15	35.07	±.24
Ash	10.51	0.19	8.36	0.31	6.95	.09	*15.47	*.98	12.49	±.14
Moisture	11.38	0.19	12.58	0.34	12.22	.22	2.24	±.03	8.00	±.18
Carbon	69.00	0.3	68.45	0.3	68.94	0.3	73.57	.30	74.97	.30
Hydrogen	5.12	0.07	4.59	0.07	4.50	0.07	4.43	.07	4.54	.07
Nitrogen	1.49	0.05	1.33	0.05	1.25	0.05	1.30	.05	1.39	.05
Oxygen (via difference)	9.66	0.71	13.54	0.76	15.02	0.58	2.44	1.45	5.54	.58
Heating Value, BTU/lb_m	12,647	66	11,732	537	11,599	149	12,962	±52	*13,460	89

[1] All wt% are on a moisture free basis.
[2] Standard deviation.
[3] Not analyzed.
*Confirmed.

Table 2. Statistical Test of Best Fit of SEM/EDS Data

A. Spot Mode

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.4687	3.4230	0.0355	3.6400	3.4687
Variance	0.3470	0.0282	0.0303		
Stand. Dev.	0.5890	0.1680	0.1741		
Range				2.3314	2.0405
K-S test	0.125202	0.094013	0.095132	0.213848	0.136609

B. Magnification = 100X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.4573	4.4511	0.0466	4.4300	4.4573
Variance	0.0591	0.0030	0.0033		
Stand. Dev.	0.2431	0.0547	0.0573		
Range				0.8914	0.8421
K-S test	0.168889	0.168461	0.163465	0.175320	0.127733

C. Magnification = 30X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.8613	4.8564	0.0510	4.9200	4.8613
Variance	0.0527	0.0022	0.0024		
Stand. Dev.	0.2296	0.0467	0.0491		
Range				0.8457	0.7954
K-S test	0.183656	0.174524	0.174998	0.268807	0.197294

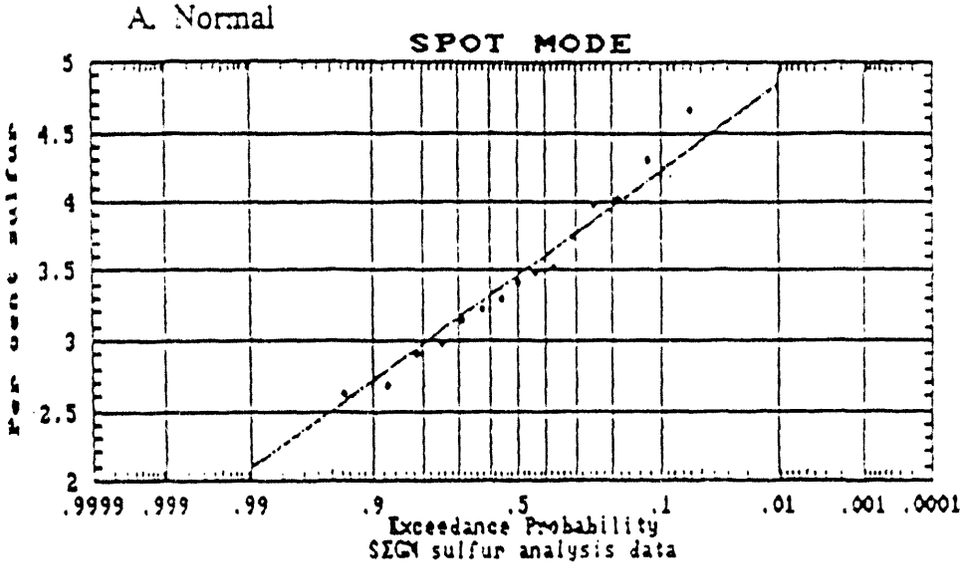
Table 3. Student "T" Tolerance Test of Normal Distribution of SEM/EDS Data

		Sample #
A.	Spot Mode	
	Tolerance = 0.3469	
	Normal distribution	= 15
	Lognormal distribution	= 15
	Doubly truncated lognormal distribution	= 16
	Uniform distribution using maximum likelihood	= 13
	Uniform distribution using sample data	= 12
B.	Magnification = 100X	
	Tolerance = 0.4457	
	Normal distribution	= 5
	Lognormal distribution	= 5
	Doubly truncated lognormal distribution	= 5
	Uniform distribution using maximum likelihood	= 6
	Uniform distribution using sample data	= 6
C.	Magnification = 30X	
	Tolerance = 0.4861	
	Normal distribution	= 4
	Lognormal distribution	= 4
	Doubly truncated lognormal distribution	= 4
	Uniform distribution using maximum likelihood	= 5
	Uniform distribution using sample data	= 5

----NOTE: 9999 indicates >31----

Figure 1. Statistical Analyses of SEM/EDS Sulfur Data

A. Normal



B. Lognormal

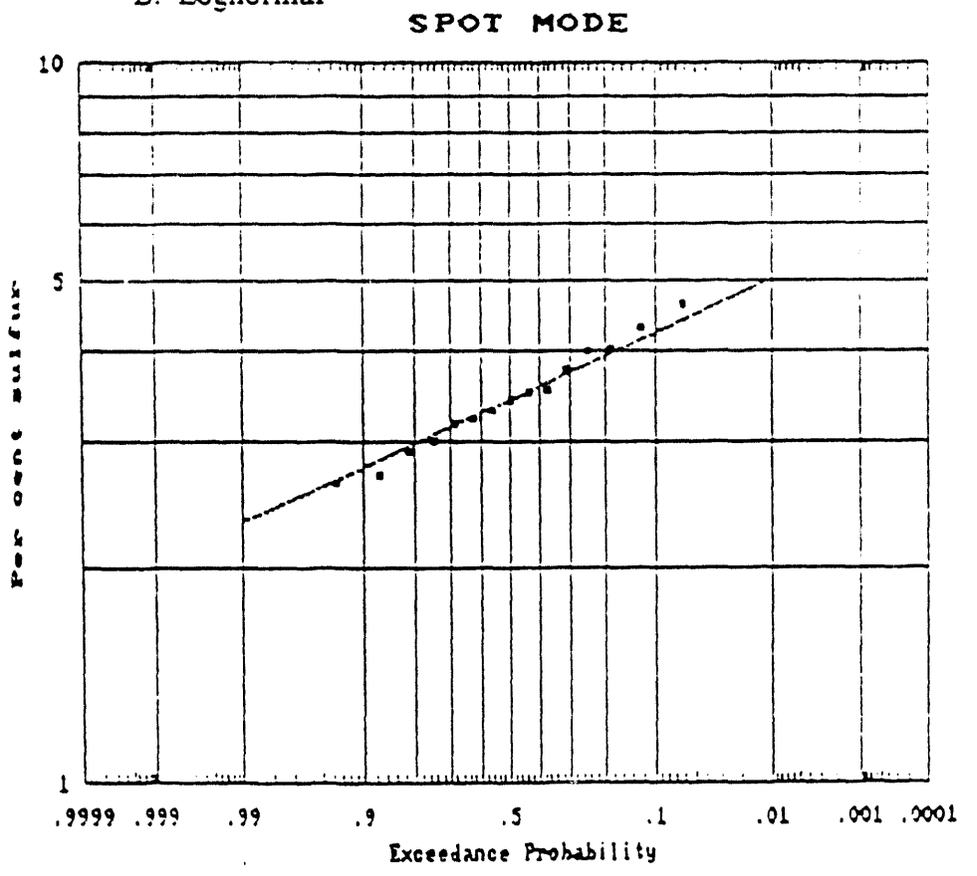
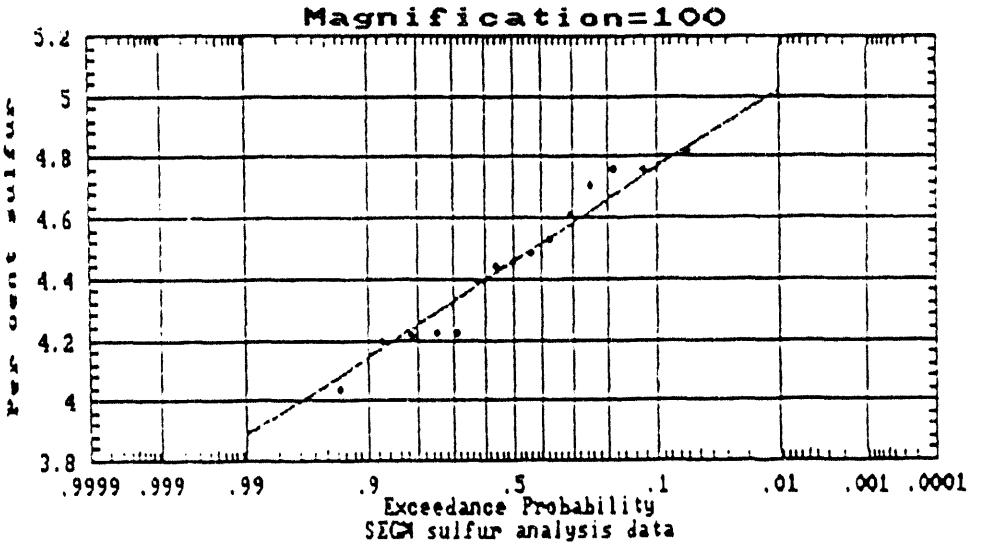


Figure 1 (cont'd) Statistical Analyses of SEM/EDS Sulfur Data

C. Normal



D. Lognormal

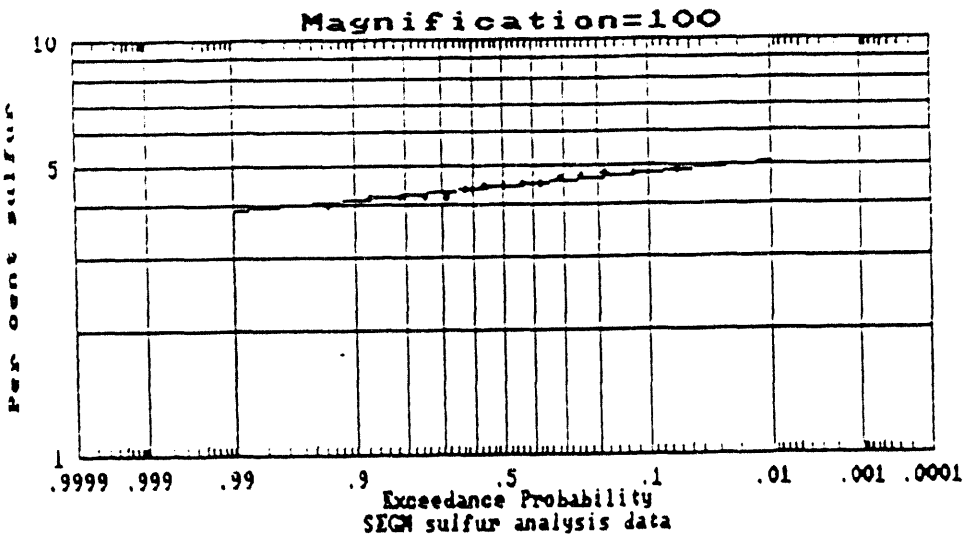
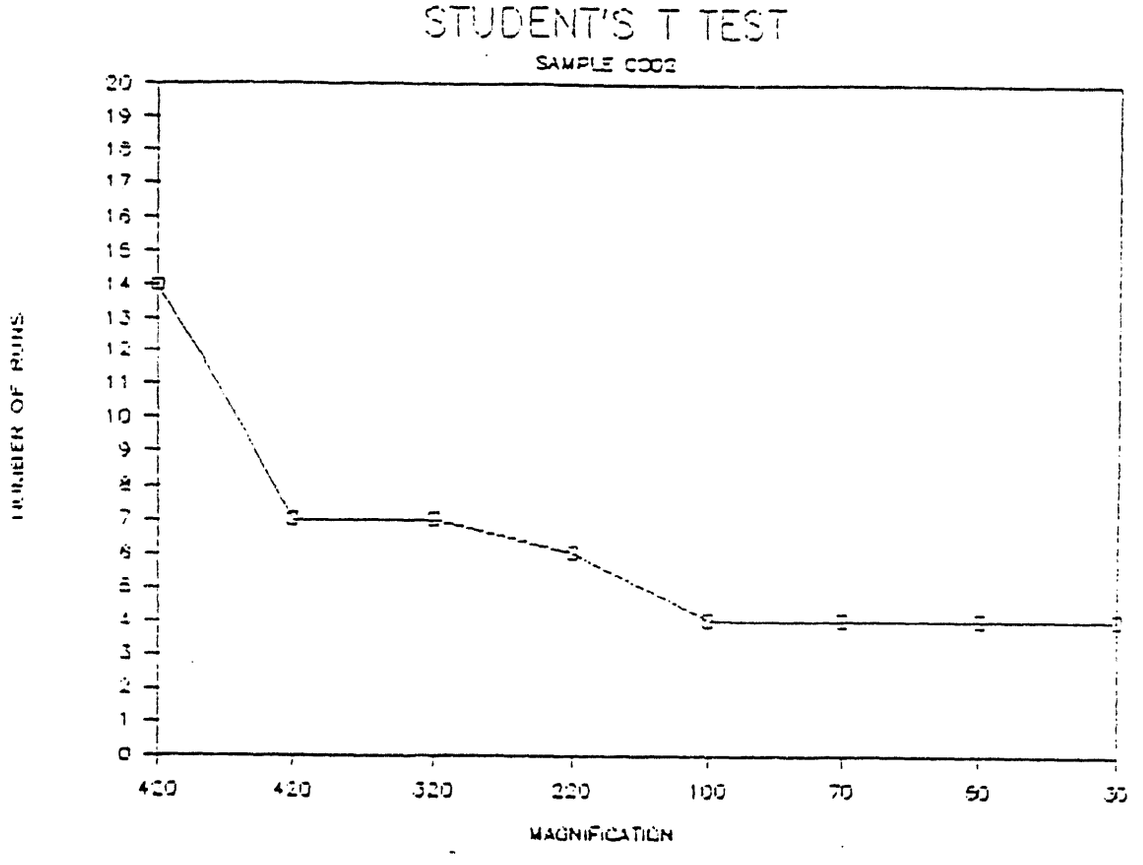


Figure 2. Tolerance Test of Normal Distribution



APPENDIX A

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

January 16, 1990

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

Sample identification by
LA. TECH.

SAMPLE IDENTIFIED AS: 1002BCD

Kind of sample reported to us: COAL - SUBMITTED SAMPLE

Sample taken at: UNKNOWN

Sample taken by: UNKNOWN

Date sampled: ------

Date received: January 2, 1990

Analysis Report No. 89-57390

% CARBON (DRY BASIS) = 69.19

% HYDROGEN (DRY BASIS) = 4.43

% NITROGEN (DRY BASIS) = 1.42

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO

Robert D. Stephens

Manager, St. Rose Laboratory

COMMERCIAL TESTING & ENGINEERING CO.
GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

Member of the SGS Group (Societe Generale de Surveillance)

PLEASE ADDRESS ALL CORRESPONDENCE TO
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

January 16, 1990

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

Sample identification by
LA. TECH.

SAMPLE IDENTIFIED AS: 1003AB

Kind of sample reported to us	COAL - SUBMITTED SAMPLE
Sample taken at	UNKNOWN
Sample taken by	UNKNOWN
Date sampled	------
Date received	January 2, 1990

Analysis Report No. 89-67391

% CARBON (DRY BASIS) = 68.94

% HYDROGEN (DRY BASIS) = 4.50

% NITROGEN (DRY BASIS) = 1.25

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO

Robert D. Stephens

Manager, St. Rose Laboratory

COMMERCIAL TESTING & ENGINEERING CO.

GENERAL OFFICES: 1919 SOUTH HIGHLAND AVE., SUITE 210-B, LOMBARD, ILLINOIS 60148 • (312) 953-9300

Member of the SGS Group (Societe Generale de Surveillance)

March 19, 1990

PLEASE ADDRESS ALL CORRESPONDENCE TO
151 JAMES DRIVE WEST, ST. ROSE, LA 70087
TELEPHONE: (504) 467-5522
TELEX: 460135 CTE UI
FAX: (504) 464-7220

LOUISIANA TECH UNIVERSITY
P. O. BOX 10348
RUSTON LA 71272

Sample identification by
LA.TECH

Kind of sample reported to us: COAL - SUBMITTED SAMPLES

Sample taken at: UNKNOWN

Sample taken by: UNKNOWN

Date sampled: ------

Date received: March 16, 1990

Analysis Report No. 89-59273

	NO. 1004	NO. 1004A
% CARBON (DRY BASIS) =	73.57	74.97
% HYDROGEN (DRY BASIS) =	4.43	4.54
% NITROGEN (DRY BASIS) =	1.30	1.39
% SULFUR (DRY BASIS) =	2.65	1.26

Need Standard Deviations for all of these numbers

Emmett

Respectfully submitted,
COMMERCIAL TESTING & ENGINEERING CO.

Manager, St. Rose Laboratory

ATTACHMENT A
APPENDIX III
Activity from March 15, 1990 to Project Completion (April 20, 1992)

INTRODUCTION.

On May 21, 1990, Dr. Michael Hsieh and I formally assumed responsibility for the microbial coal desulfurization project. I will be serving as Project Director throughout the remainder of the contract period, while Dr. Hsieh is providing expertise in the area of biological coal treatment.

The following discussion summarizes the work done over the last quarter and outlines planned activities in the upcoming months in relation to the Tasks as outlined in the contractual agreement.

TASK OVERVIEW.

Task 1 - Project Planning.

Task Complete.

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete.

Current coal supplies (raw and depyritized) are being inventoried to determine amounts available for further treatment. All of the raw coal on hand (Illinois #6 IBC-101) is being ground to 200 mesh particle for subsequent depyritization. As described in the April 20 report (Task 2.1) the remaining unground coal comprises approximately 10 pounds of the initial 20 pounds ordered. Portions of this lot of coal have previously been ground to 200 mesh and treated for pyrite removal (Batch Runs 1005A & B) and are currently being tested for sulfur content.

Additionally, 20 pounds of Illinois #6 IBC-101 have been ordered (arrival of this shipment is expected in July). This coal will be riffled and portions of the lot will be ground to various particle sizes (including 200 mesh) for future evaluations of the kinetic rates and extent of depyritization as a function of particle size. Literature sources have reported successful microbial attack upon pyritic inclusions on particles sized up to 60 mesh (Andrews et al., 1987).

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

Depyritization of Illinois #6 (IBC-101) coal remains an ongoing procedure in order to provide a supply of depyritized coal for various organic sulfur removal studies. All analytic and coal handling procedures are currently under review to ensure reliability and continuity of the project with the recent change of personnel (including most of the staff of student workers conducting analytic procedures).

Previous depyritization runs for each of the three batch reactors (April and May monthly reports, Task 2.2) are being analyzed for the extent of pyrite removal. The apparent cessation of microbial depyritization for Reactors 1005A & B is being investigated. For future batch runs, the operating conditions and parameters monitored will include agitation rate (rpm), pH levels, initial cell concentrations of inocula, inoculum sizes, etc. Close monitoring of batch runs offers the potential for conducting studies for developing a kinetic model describing the depyritization rate for the Thiobacillus consortium used in this project.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.2 Quality Assurance and Control.

As with previous raw and treated coals, in-house analytical results will be checked periodically with outside lab testing.

3.4 Microscope Analysis.

Task complete.

Task 4 - Organic Sulfur Removal.

The following discussion outlines the activities and procedures which have been initiated as an effort to isolate organic sulfur utilizers.

Forty-one samples of natural inocula, obtained from various sources including soils, slurry ponds, coals, and oil sludges (collected samples were refrigerated to minimize microbial activity) were inoculated onto 82 petri plates of screening medium, Trypticase Soy Agar with dibenzothiophene (DBT) added in solution. DBT was dissolved in glycerol and added to the medium to give a final concentration of 1 g/L DBT (0.1% w/w). Inoculum dilutions were prepared by adding 1 g of sample to 4 mL of sterile, distilled water. For each sample prepared, streaks were made onto two plates, each with a different pH value (2.4 and 6.7, respectively). The plates at the lower pH were prepared to screen for strong acidophiles (considering the attractiveness of having an organism operating in the same pH range as Thiobacillus sp.). A control plate was prepared for the medium at pH 6.7. Of course, this procedure only screens for organisms that are capable of growing in the presence of DBT, though not necessarily utilizing it.

The plates were incubated a minimum of 72 hours at room temperature. Specific colonies showing strong growth were then selected from each plate. These colonies were again streaked onto fresh plates containing the same medium in order to purify the working culture and eliminate the presence of contaminants. Again, plates were incubated for a minimum of 72 hours at room temperature.

Organisms from the purified colony-separation plates were placed in a broth screening medium identical to the Tryptic Soy/DBT medium (but without agar)to produce a workable quantity of the purified strains. This procedure was carried out for each of the cultures isolated. These broth cultures were also incubated for at least 72 hours.

Planned work.

For each culture isolated, a preparation of 150 mL of basal salts medium containing depyritized coal will be inoculated with 15 mL of seed culture from the Tryptic Soy broth culture. The basal medium with 3 g of coal will be added to a 250 mL Erlenmeyer flask, autoclaved at 121 °C for 15 minutes to sterilize the culture environment. The 15 mL of seed inoculum will be added following cooling of the autoclaved medium. Once inoculated, the flask will be placed on a shaker bed at an agitation rate of 180 rpm and at room temperature (approximately 27 °C). The culture will be incubated in this environment for at least two weeks after which the coal will be filtered from the culture broth, treated for biomass removal from the coal particles, dried and analyzed for organic sulfur content. It is possible that the incubation period may have to be increased in order to observe significant changes in the sulfur content, provided that strong microbial activity is observed, and that supplemental medium additions may be necessary to prevent nutrient limitations over such a long incubation period.

For cultures which show positive results, the shaker bed inoculation procedure will be repeated. However, it is proposed that two samples be run for each positive culture -- one with glucose and one without to examine the potential of increasing the extent of microbial attack on organic sulfur in the presence of high energy substrates. Positive cultures can then be identified and characterized.

Throughout this process, measures are being taken to maintain stock cultures in a viable state until they have been proven positive or negative in their ability to attack the organic sulfur fraction in coal. Regular transfers of cultures into similar, fresh medium will be carried out. Cultures which show positive performance will carefully be preserved with standard microbiological techniques (e.g. lyophilization, refrigeration, ultra-freezing, etc.) in addition to periodic transfer and incubation of stock cultures.

Finally, plans are underway for the construction of equipment which would enable testing of these cultures in fed batch or continuous flow reactors. This will allow further optimization of process operating conditions.

Andrews, G., M. Darroch, and T. Hannson, "Bacterial Removal of Pyrite from Concentrated Coal Slurries", Biotech. and Bioeng., 32:813-320, 1988.

Table 1. Batch Analyses of Microbial Depyritized Coal

	Lot/Batch Numbers									
	1002		1002A		1002(B&C)		1002(B,C&D)		1002E	
	wt%[1]	SD[2]	wt%	SD	wt%	SD	wt%	SD	wt%	SD
Total Sulfur	3.72	0.06	2.85	0.02	2.84	0.08	3.00	.02	2.75	.020
Pyritic Sulfur	0.94	0.01	0.09	0.01	0.08	0.01	0.264	.02	.119	.006
Sulfate Sulfur	0.30	0.02	0.17	0.01	0.18	0.01	0.232	.01	.133	.001
Organic Sulfur (via difference)	2.48	0.09	2.59	0.04	2.58	0.10	2.50	0.05	2.50	.027
Volatiles	41.5	0.7	37.72	0.3	NA[3]	NA	40.40	±.14	40.47	.46
Ash	10.2	0.1	8.91	0.09	NA	NA	7.02	.13	9.80	.62
Moisture	8.98	0.09	2.90	0.09	28.26	0.1	12.55	.36	18.16	.56
Carbon	69.18	0.97	68.78	0.3	NA	NA	69.19	0.3	67.79	.243
Hydrogen	5.03	0.2	4.77	0.07	NA	NA	4.43	0.07	4.52	0.0
Nitrogen	1.51	0.01	1.49	0.05	NA	NA	1.42	0.05	1.32	.028
Oxygen (via difference)	10.36	1.34	13.20	0.53	NA	NA	14.94	0.57	13.82	.916
Heating Value, BTU/lb_m	13,129	53	11,902	13	NA	NA	11,995	60	10,771	130

[1] All wt% are on a moisture free basis.
[2] Standard deviation.
[3] Not analyzed.

Table 1 (cont'd) Batch Analyses of Microbial Depyritized Coal

	Lot/Batch Numbers									
	1003		1003A		1003(A&B)		1004		1004A	
	wt%[1]	SD[2]	wt%	SD	wt%	SD	wt%	SD	wt%	SD
Total Sulfur	4.22	0.10	3.23	0.03	3.34	±0.07	2.79	±.05	1.07	±.02
Pyritic Sulfur	0.96	0.01	0.13	0.01	0.130	0.008	1.26	±.03	.25	±.03
Sulfate Sulfur	0.18	0.02	0.09	0.01	0.117	0.002	0.654	±.03	.417	±.009
Organic Sulfur (via difference)	3.08	0.10	3.01	0.05	3.09	0.09	0.88	±.11	.40	±.06
Volatiles	*51.1	0.5	35.95	0.13	45.83	±.20	30.09	±.15	35.07	±.24
Ash	10.51	0.19	8.36	0.31	6.95	.09	*15.47	*.98	12.49	±.14
Moisture	11.88	0.19	12.58	0.34	12.20	.22	2.24	±.03	8.00	±.18
Carbon	69.00	0.3	68.45	0.3	68.94	0.3	73.57	.30	74.97	.30
Hydrogen	5.12	0.07	4.59	0.07	4.50	0.07	4.43	.07	4.54	.07
Nitrogen	1.49	0.05	1.33	0.05	1.25	0.05	1.30	.05	1.39	.05
Oxygen (via difference)	9.66	0.71	13.54	0.76	15.02	0.58	2.44	1.45	5.54	.58
Heating Value, BTU/lb_m	12,647	66	11,732	537	11,599	149	12,962	±52	*13,460	89

[1] All wt% are on a moisture free basis.
[2] Standard deviation.
[3] Not analyzed.
*Confirmed.

TASK 3 Analytical Procedures for Total Organic Sulfur

3.1 Characterization

Task complete.

Final analyses of the stock and depyritized coal are included in Table 1. All treated coals are stored under nitrogen.

3.2 Quality Assurance and Control

To assure continued accuracy of analytical results, each of the depyritized coal samples were sent out to "Commercial Testing and Engineering Company". These final analyses are included in the Appendix A. To date, our in-house analyses have been well within ASTM acceptable error for inter-laboratory data reporting.

3.4 Microscope Analysis

Task complete.

Statistical analyses of the data sets for 1002 and 1002a were completed. Our first concern was to find a statistical measure that fit the distribution of our data. We analyzed the data for normal, lognormal, truncated lognormal, maximum-minimum uniform, and standard uniform distribution. The results of these tests are included in Tables 2 and 3. We used a Kolomogorov-Smirnov (K-S) test to evaluate the fit of each of our data sets to these distributions (also Table 2). While there is some variation, our data is best described as having a normal or lognormal distribution. Normal and lognormal plots for each data set are included in Figures 1 and 2.

Once we had a reasonable representation of our data distribution, we were able to ask how many samples were necessary to predict the mean with a 95 percent certainty to fall within ASTM tolerances. We ran student "t" tests on each distribution to discern the minimum sample size. The results of these tests are summarized in Tables 4 and 5. These analyses suggest that four to five spectra should predict the mean within ASTM tolerances as long as we keep the magnification at or below 100X (Figure 3).

Table 2. Statistical Test of Best Fit of SEM/EDS Data for C002

A. Spot Mode

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.4687	3.4230	0.0355	3.6400	3.4687
Variance	0.3470	0.0282	0.0303		
Stand. Dev.	0.5890	0.1680	0.1741		
Range				2.3314	2.0405
K-S test	0.125202	0.094013	0.095132	0.213848	0.136609

B. Magnification = 420X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.7400	3.7228	0.0387	3.6650	3.7400
Variance	0.1342	0.0100	0.0108		
Stand. Dev.	0.3664	0.1000	0.1038		
Range				1.4743	1.2691
K-S test	0.132631	0.150090	0.149428	0.181298	0.134533

C. Magnification = 320X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.8987	3.8805	0.0404	3.7600	3.8987
Variance	0.1461	0.0102	0.0110		
Stand. Dev.	0.3822	0.1011	0.1050		
Range				1.5086	1.3239
K-S test	0.143063	0.159826	0.159167	0.221212	0.131221

D. Magnification = 220X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.0667	4.0524	0.0422	4.0000	4.0667
Variance	0.1215	0.0076	0.0083		
Stand. Dev.	0.3485	0.0873	0.0909		
Range				1.2571	1.2074
K-S test	0.094872	0.107409	0.106708	0.196591	0.137110

Table 2. Statistical Test of Best Fit of SEM/EDS Data for C002 (Cont.)

E. Magnification = 100X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.4573	4.4511	0.0466	4.4300	4.4573
Variance	0.0591	0.0030	0.0033		
Stand. Dev.	0.2431	0.0547	0.0573		
Range				0.8914	0.8421
K-S test	0.168889	0.168461	0.168465	0.175320	0.127733

F. Magnification = 70X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.5413	4.5361	0.0475	4.4750	4.5413
Variance	0.0504	0.0025	0.0027		
Stand. Dev.	0.2245	0.0498	0.0521		
Range				0.9029	0.7775
K-S test	0.137861	0.128500	0.128954	0.261656	0.148673

G. Magnification = 50X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.6800	4.6764	0.0491	4.7300	4.6800
Variance	0.0364	0.0016	0.0018		
Stand. Dev.	0.1908	0.0403	0.0423		
Range				0.6629	0.6608
K-S test	0.195870	0.192029	0.192166	0.257184	0.181800

H. Magnification = 30X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	4.8613	4.8564	0.0510	4.9200	4.8613
Variance	0.0527	0.0022	0.0024		
Stand. Dev.	0.2296	0.0467	0.0491		
Range				0.8457	0.7954
K-S test	0.183656	0.174524	0.164998	0.268807	0.197294

Table 3. Statistical Test of Best Fit of SEM/EDS Data for C002A

A. Spot Mode

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.0767	3.0214	0.0312	3.1650	3.0767
Variance	0.3727	0.0387	0.0412		
Stand. Dev.	0.6105	0.1966	0.2029		
Range				2.3657	2.1149
K-S test	0.159655	0.129666	0.130650	0.167814	0.136554

B. Magnification = 420X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	2.9127	2.9047	0.0299	2.8700	2.9127
Variance	0.0490	0.0060	0.0063		
Stand. Dev.	0.2214	0.0773	0.0796		
Range				0.8229	0.7669
K-S test	0.147148	0.149854	0.149798	0.208681	0.141506

C. Magnification = 320X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	2.8433	2.8369	0.0292	2.8500	2.8433
Variance	0.0392	0.0048	0.0051		
Stand. Dev.	0.1979	0.0696	0.0717		
Range				0.7314	0.6854
K-S test	0.113065	0.108833	0.108635	0.149610	0.149244

D. Magnification = 220X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	2.7993	2.7892	0.0287	2.8350	2.7993
Variance	0.0626	0.0077	0.0082		
Stand. Dev.	0.2503	0.0878	0.0904		
Range				0.9486	0.8670
K-S test	0.178132	0.163942	0.164368	0.220934	0.172353

Table 3. Statistical Test of Best Fit of SEM/EDS Data for C002A (Cont.)

E. Magnification = 100X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	2.6300	2.5965	0.0267	2.8550	2.6300
Variance	0.2086	0.0262	0.0278		
Stand. Dev.	0.4567	0.1620	0.1667		
Range				1.5200	1.5820
K-S test	0.210258	0.182356	0.183168	0.369079	0.224146

F. Magnification = 70X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	2.4287	2.3949	0.0245	2.7050	2.4287
Variance	0.2033	0.0281	0.0297		
Stand. Dev.	0.4509	0.1677	0.1723		
Range				1.7257	1.5620
K-S test	0.277763	0.238348	0.239409	0.468074	0.301794

G. Magnification = 50X

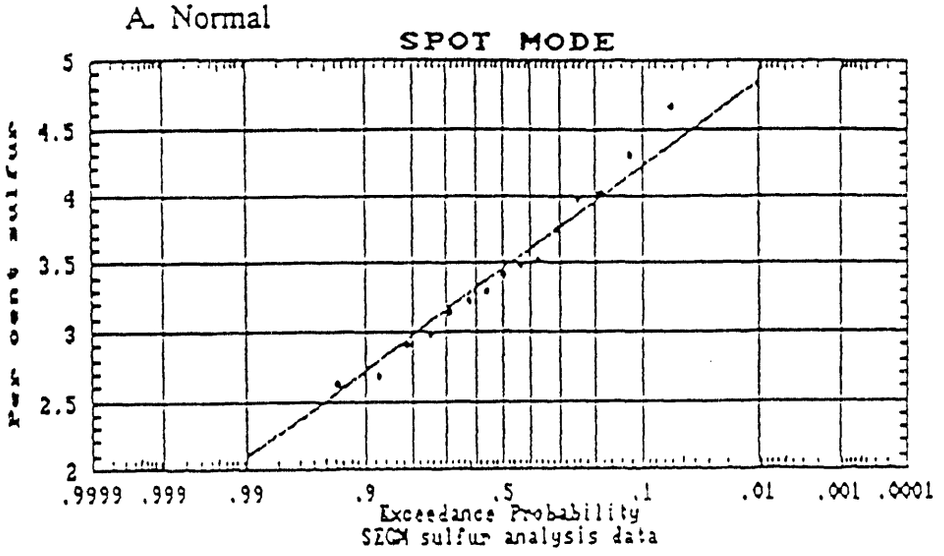
	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.3053	3.2980	0.0341	3.2250	3.3053
Variance	0.0505	0.0049	0.0052		
Stand. Dev.	0.2247	0.0698	0.0721		
Range				0.9714	0.7784
K-S test	0.136072	0.150225	0.149767	0.259069	0.162243

H. Magnification = 30X

	Normal	Lognormal	Truncated Lognormal	Maxlike Uniform	Sampdata Uniform
Mean	3.3327	3.3321	0.0345	3.3200	3.3327
Variance	0.0043	0.0004	0.0004		
Stand. Dev.	0.0656	0.0197	0.0204		
Range				0.2514	0.2274
K-S test	0.142161	0.142538	0.142559	0.194696	0.113710

Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002

A. Normal



B. Lognormal

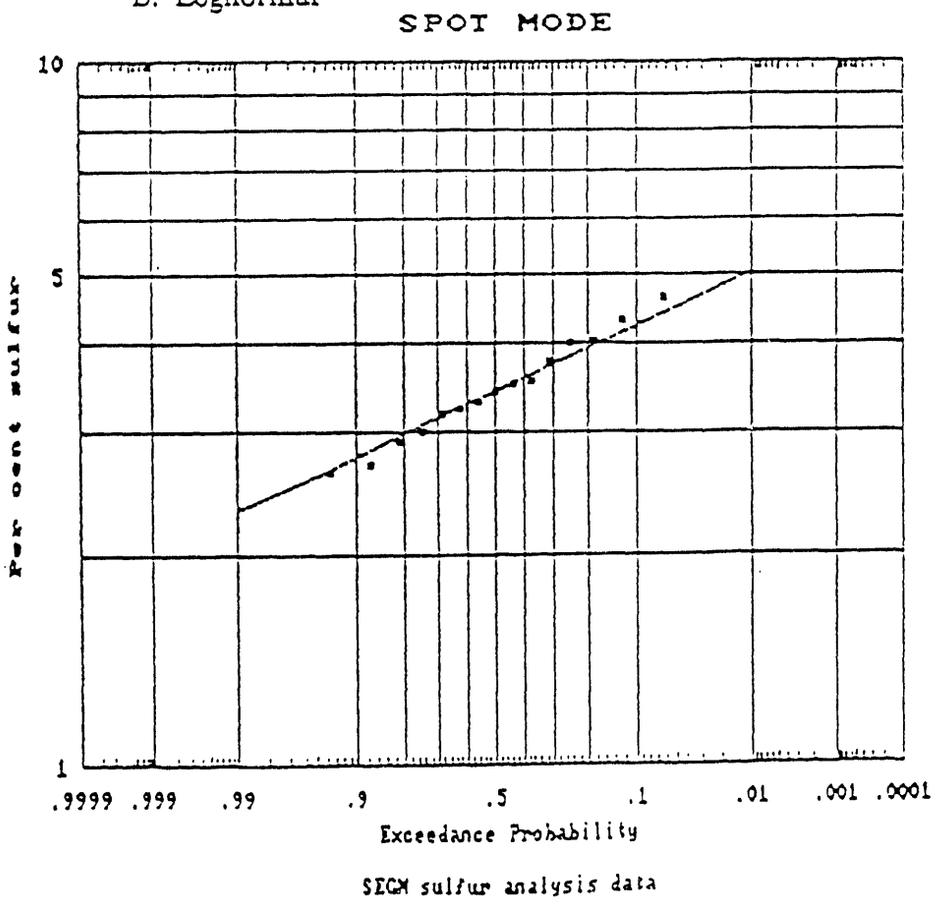
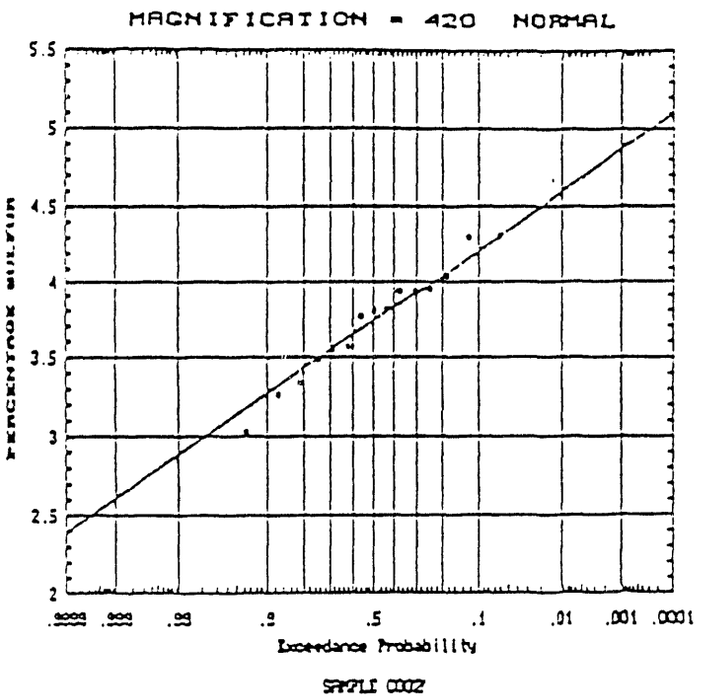


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



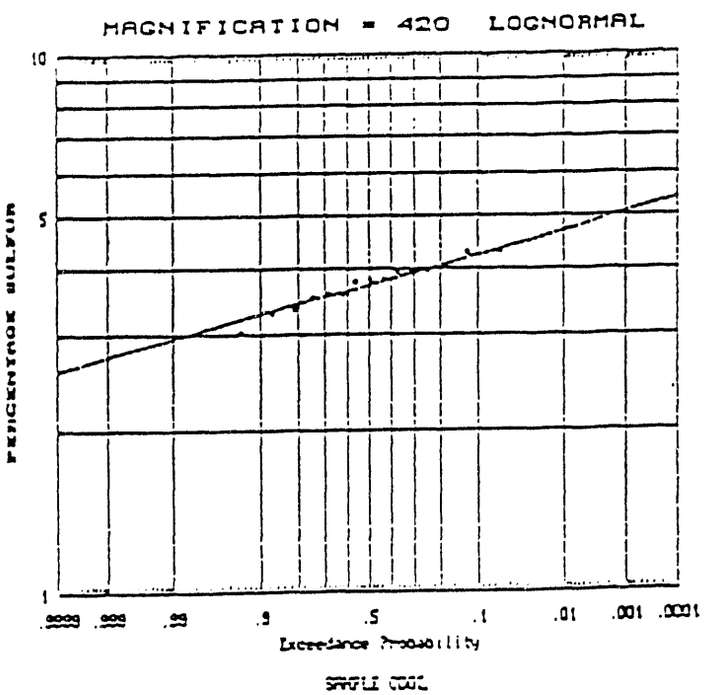
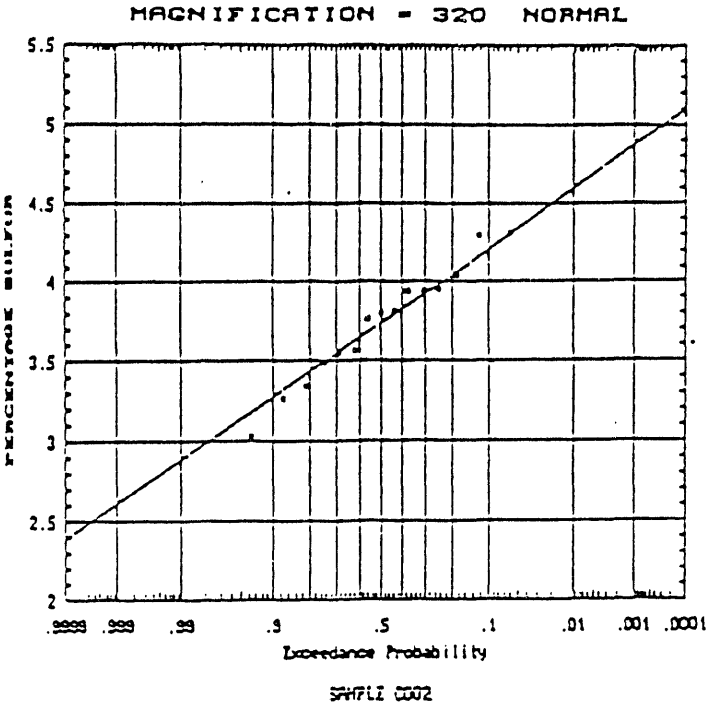


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



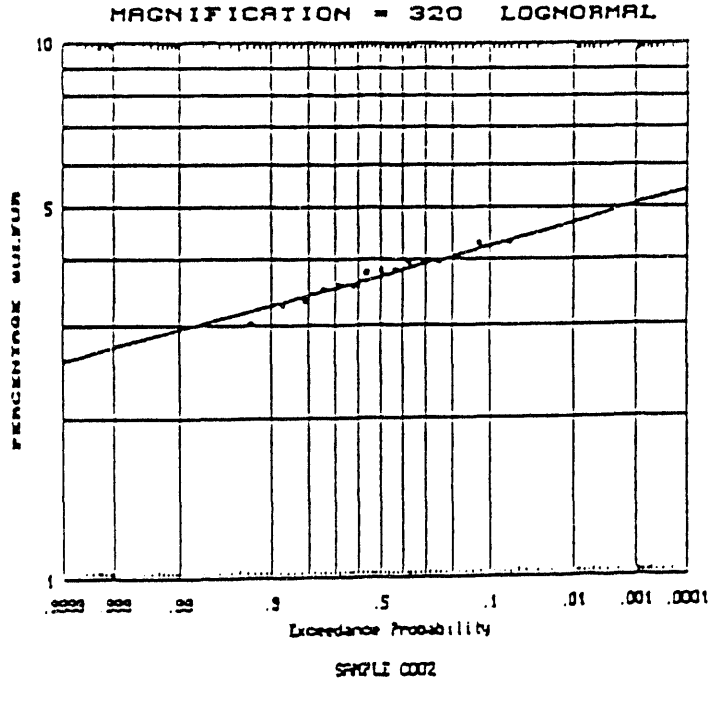
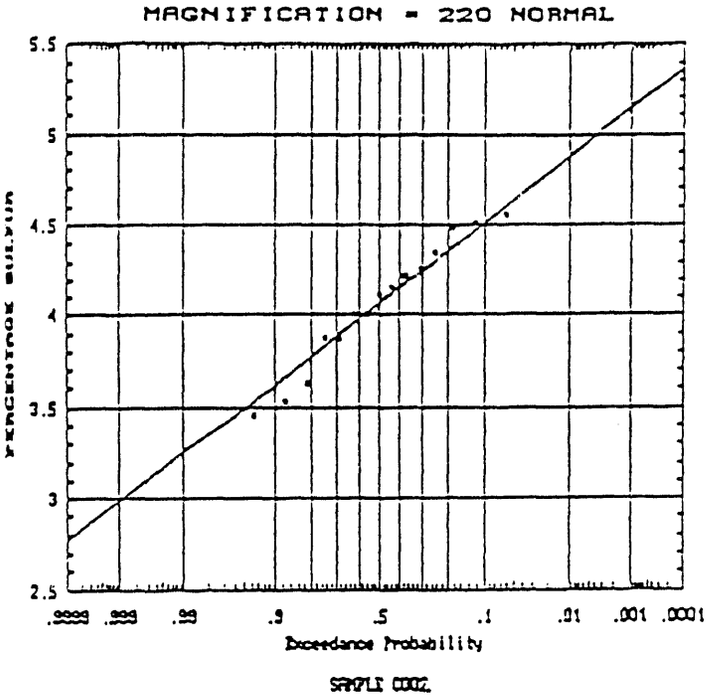


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



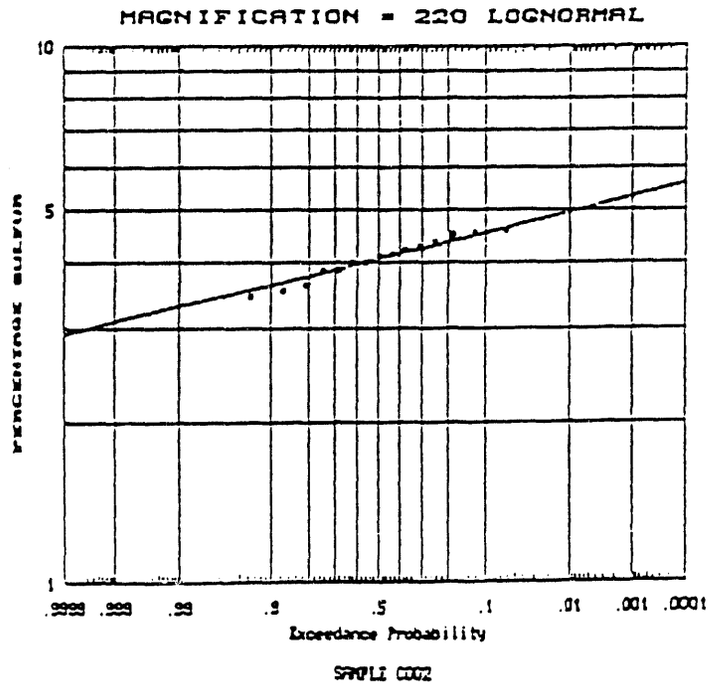
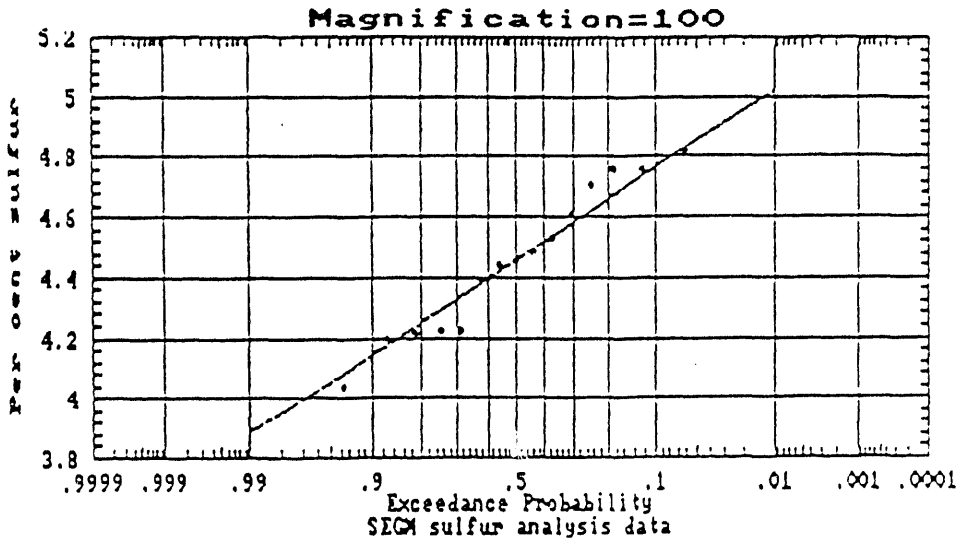


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)

C. Normal



D. Lognormal

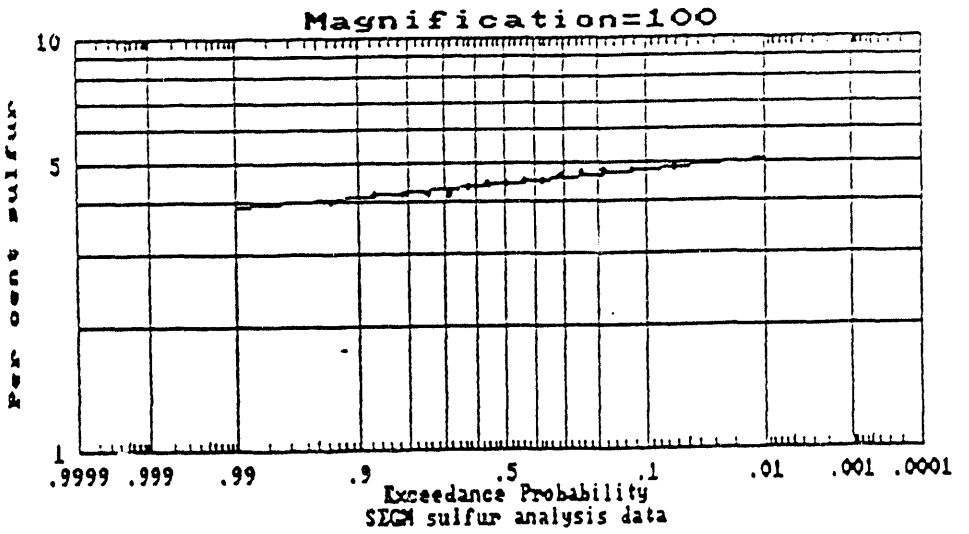
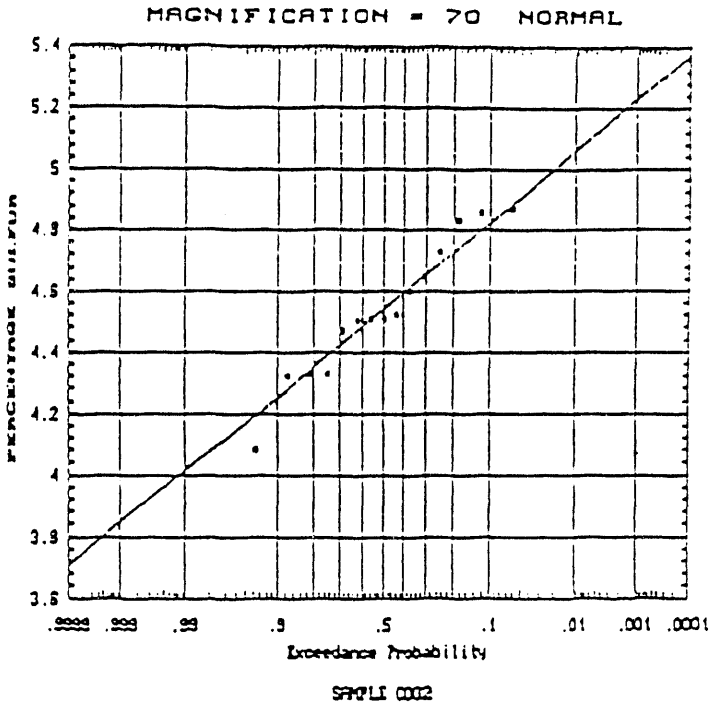


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



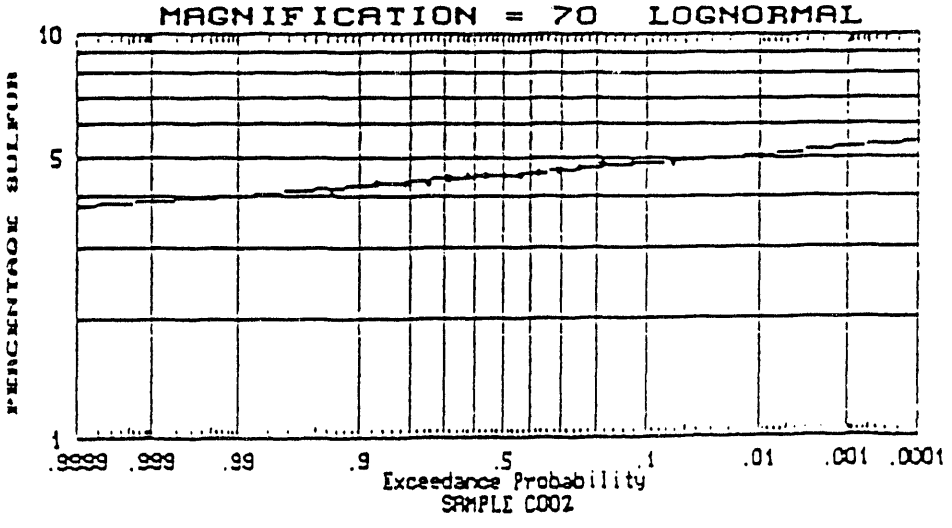
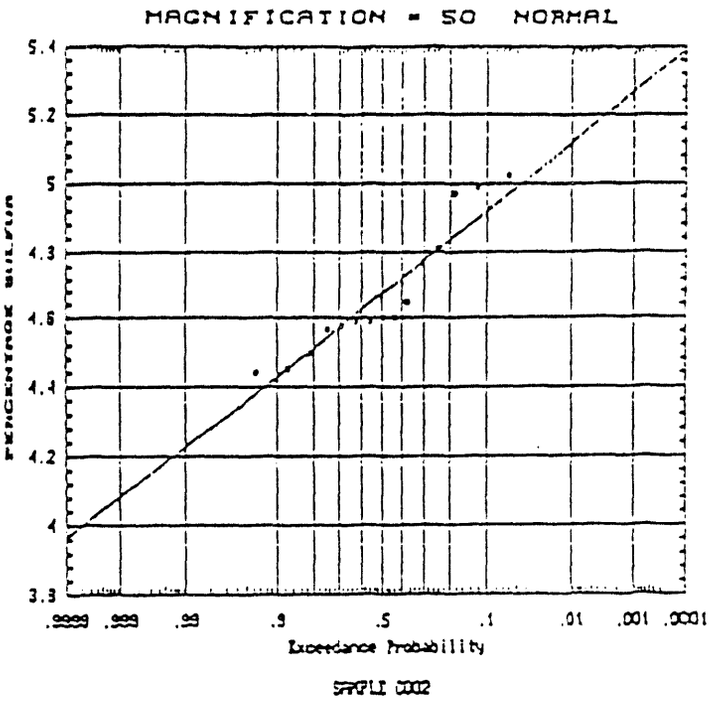


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



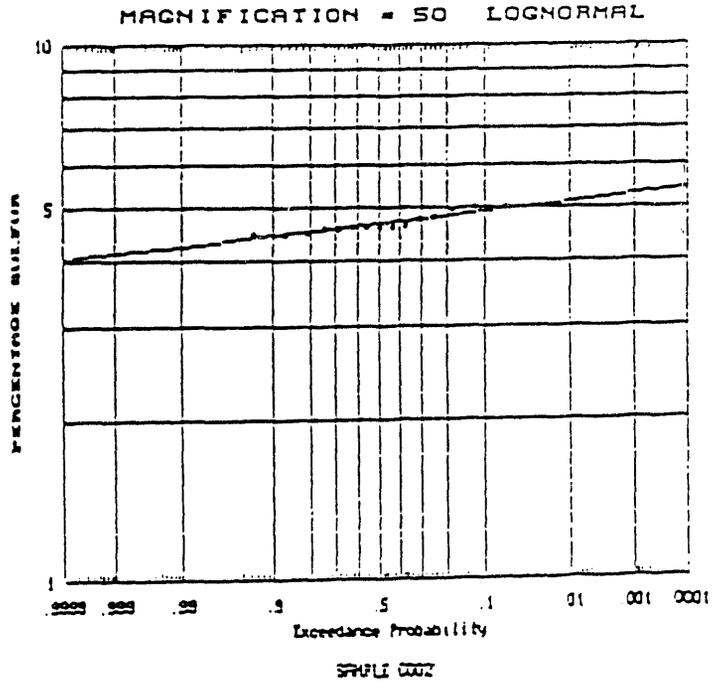
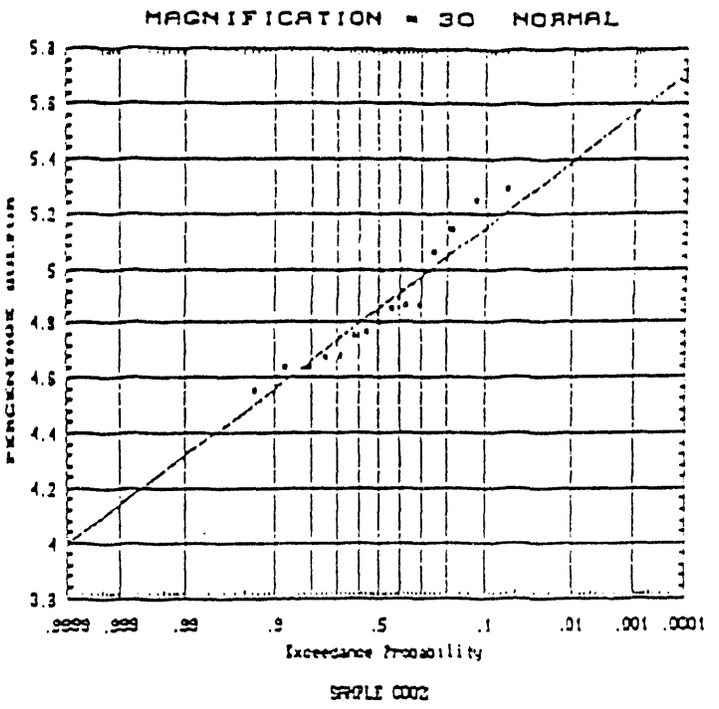


Figure 1. Statistical Analyses of SEM/EDS Sulfur Data for C002 (Cont.)



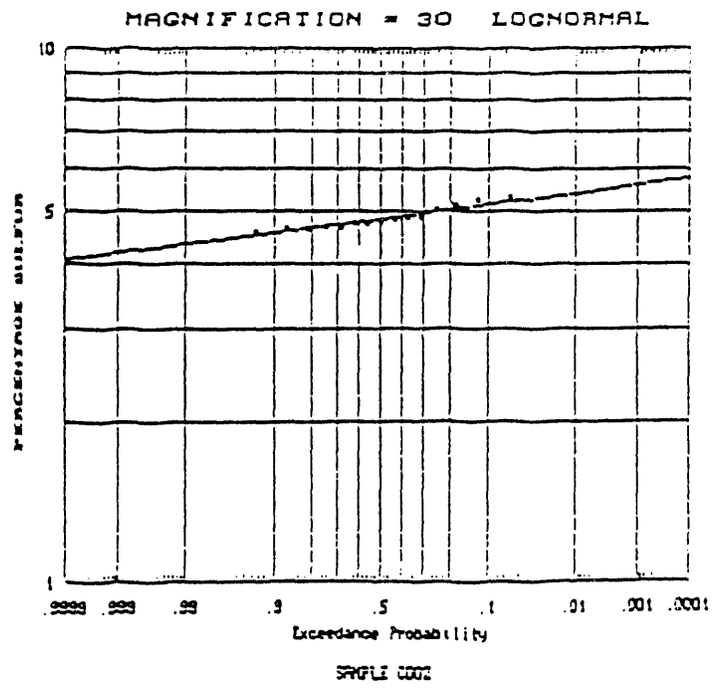
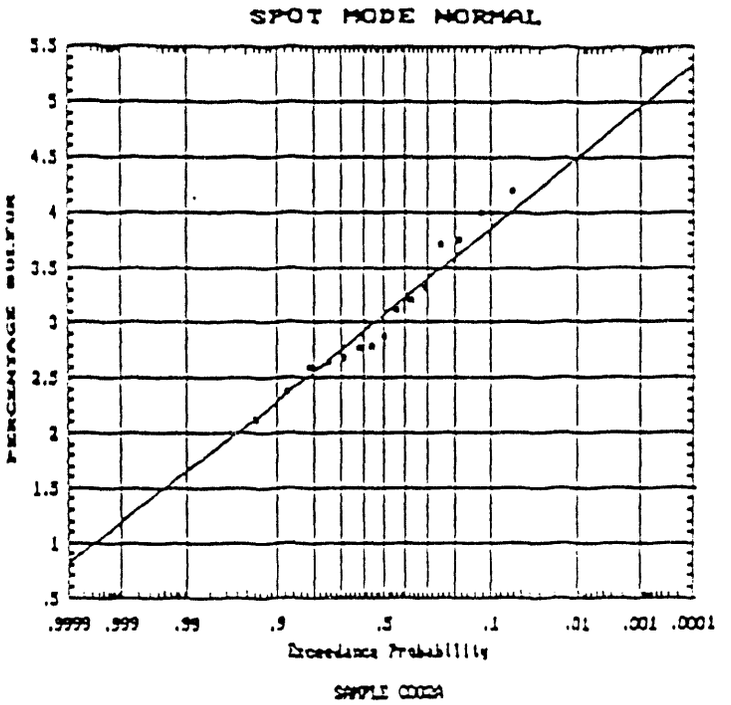


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A



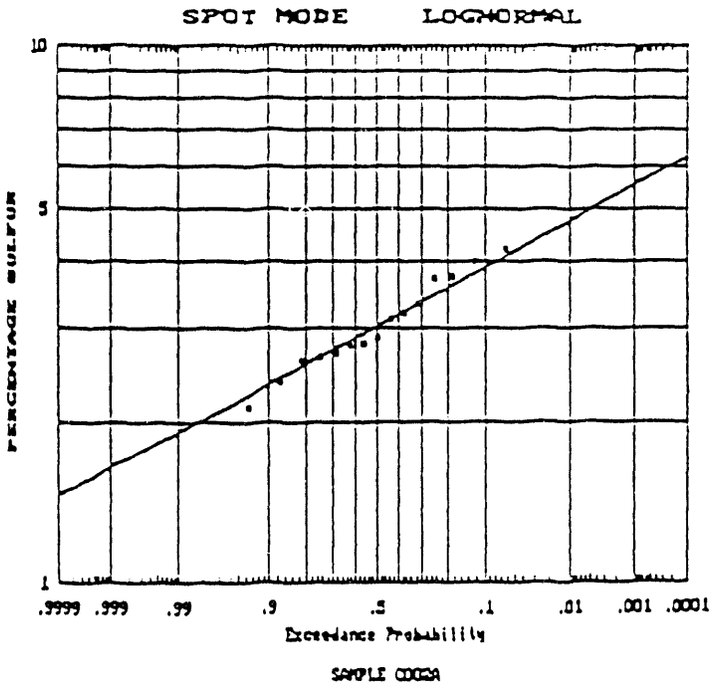
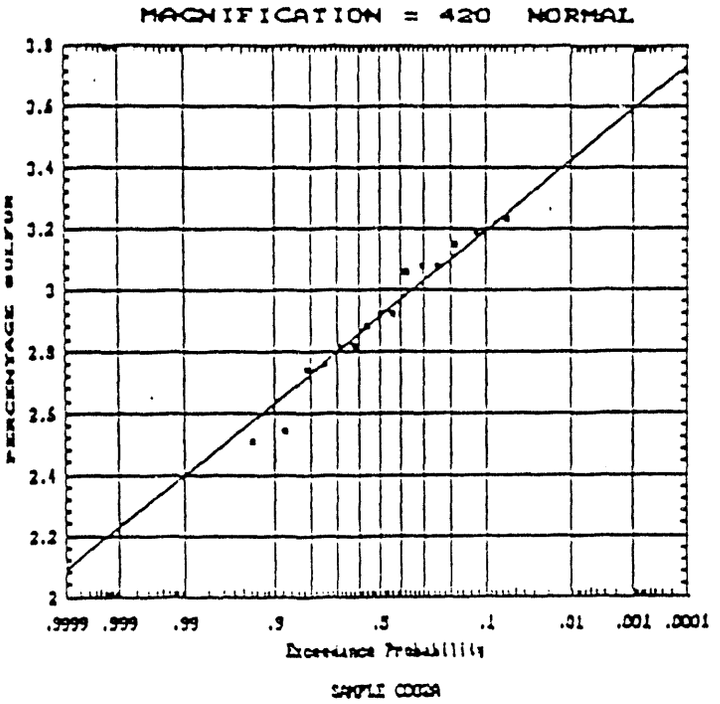


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)



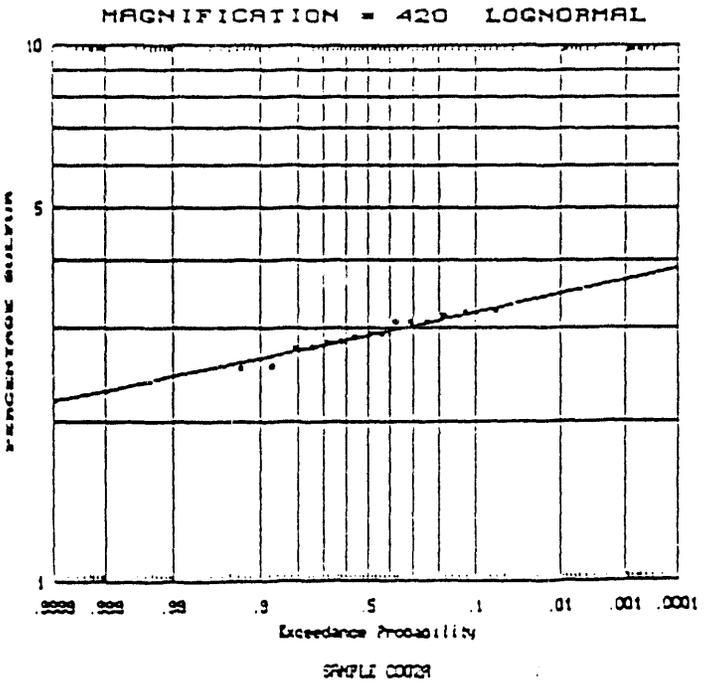
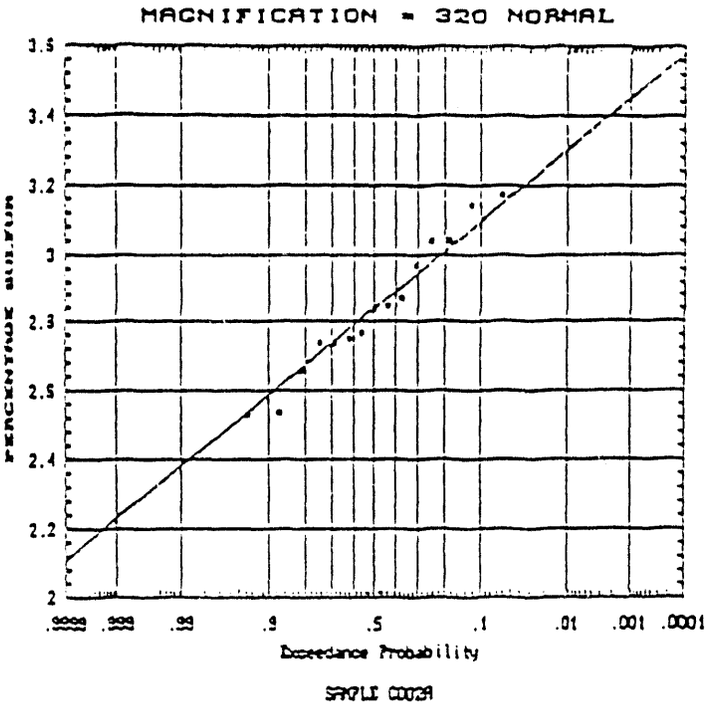
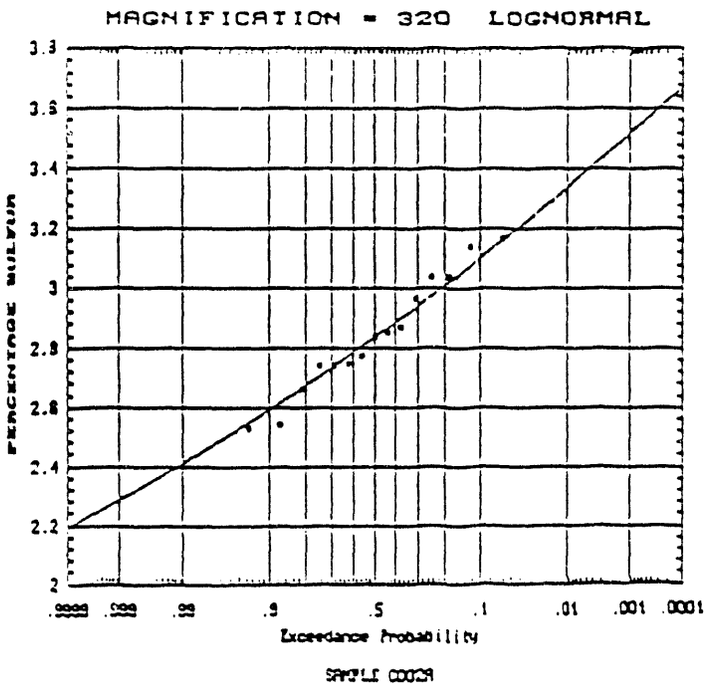


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)





AIIM

Association for Information and Image Management

1100 Wayne Avenue, Suite 1100
Silver Spring, Maryland 20910

301/587-8202

Centimeter

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 mm

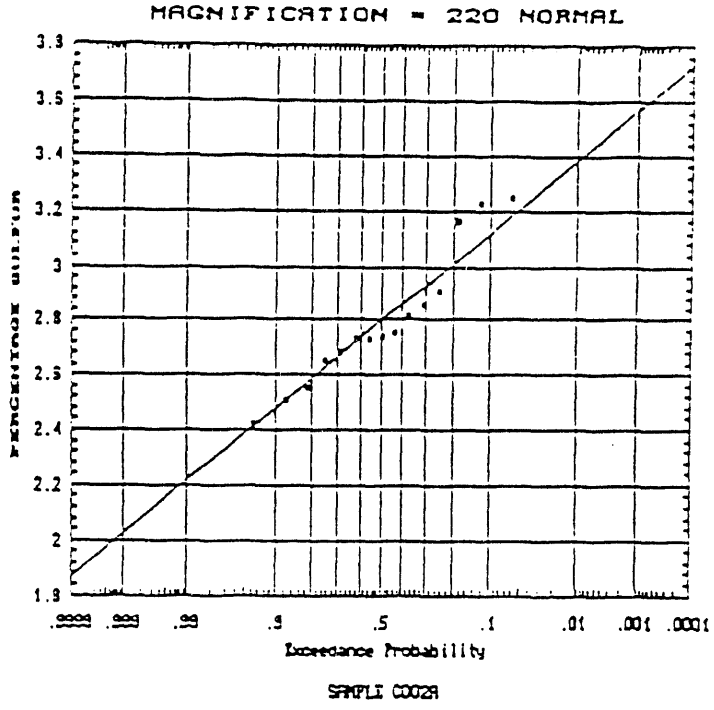
1 2 3 4 5

Inches

MANUFACTURED TO AIIM STANDARDS
BY APPLIED IMAGE, INC.

2 of 3

Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002-A (Cont.)



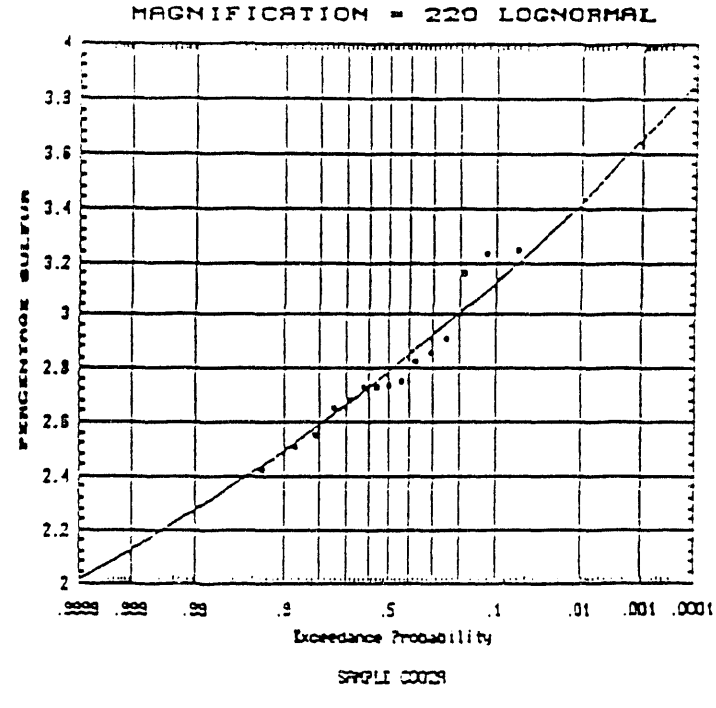
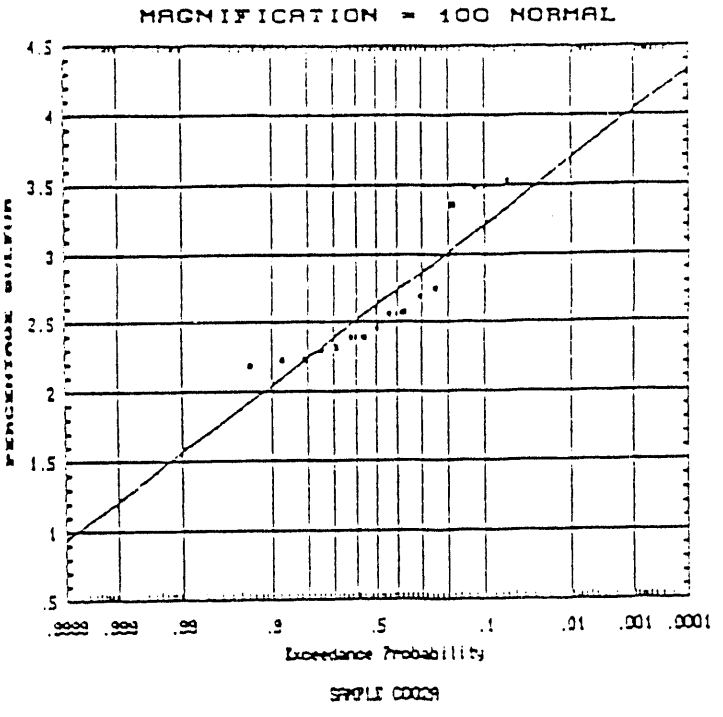


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)



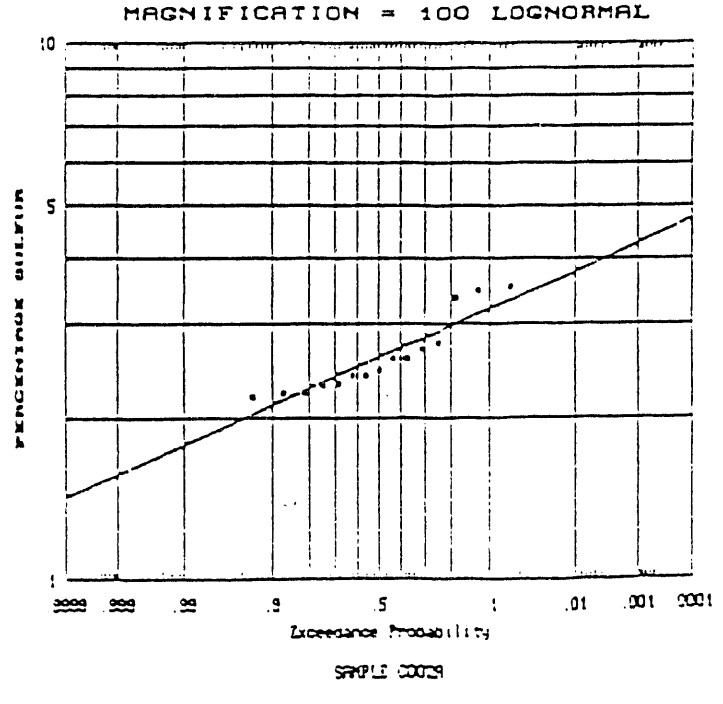
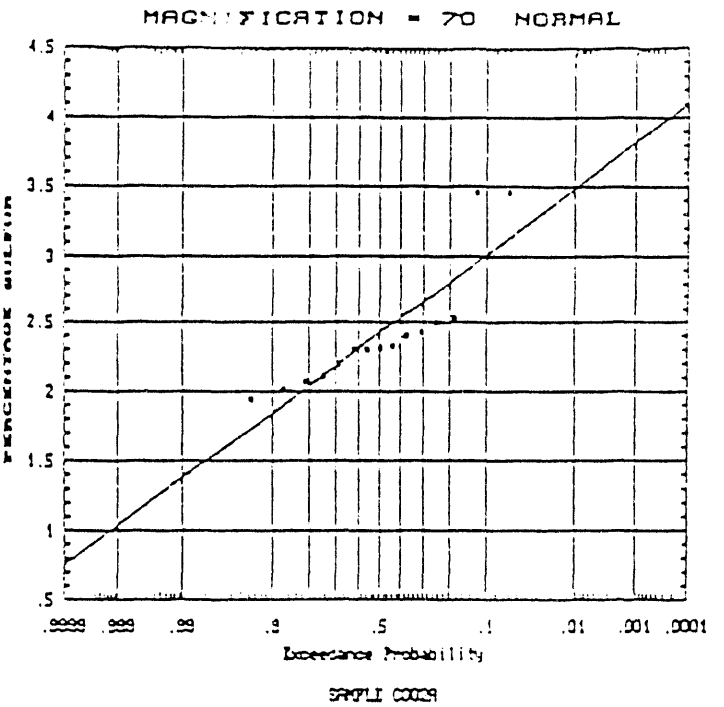


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)



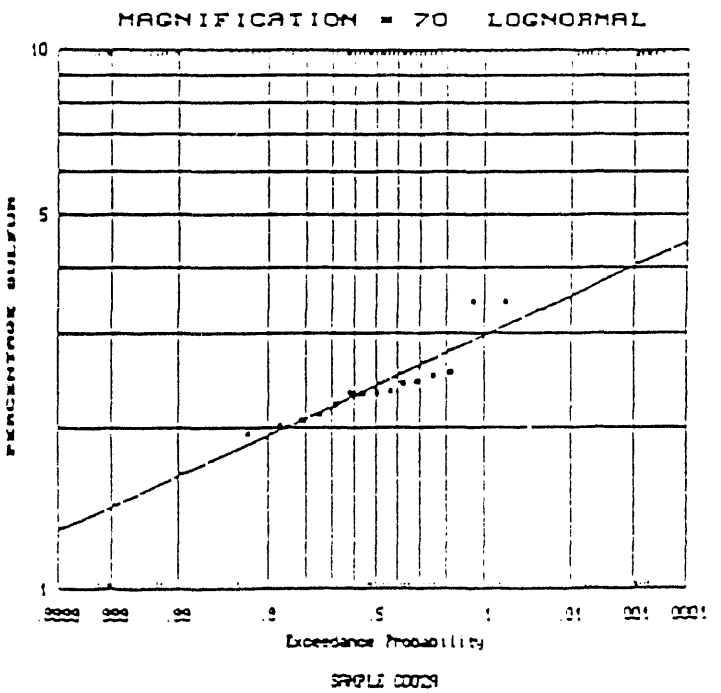
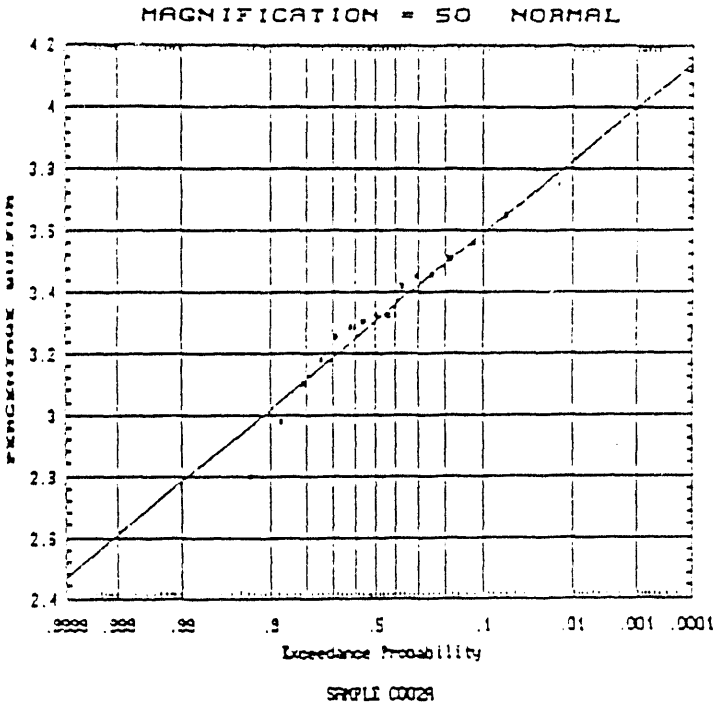


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)



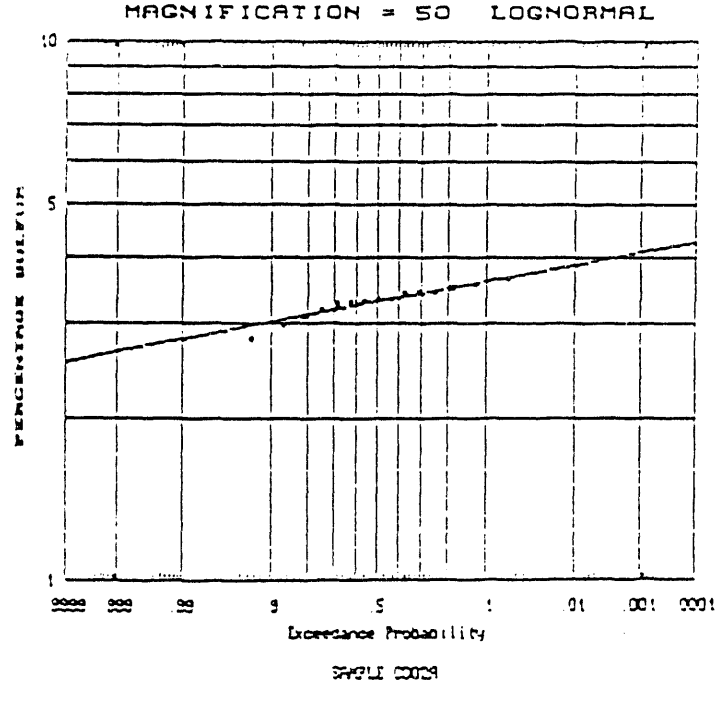
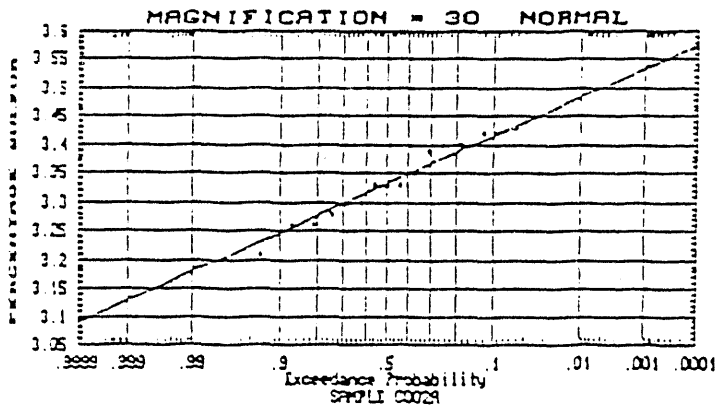


Figure 2. Statistical Analyses of SEM/EDS Sulfur Data for C002A (Cont.)



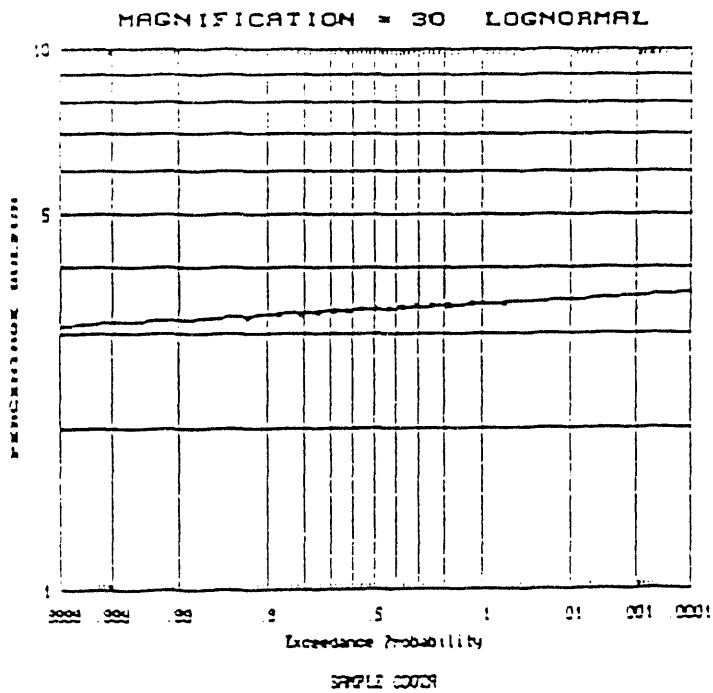


Table 4. Student "T" Tolerance Test of Normal Distribution of SEM/EDS Data for C002

		Sample #
A.	Spot Mode	
	Tolerance = 0.3469	
	Normal distribution	= 15
	Lognormal distribution	= 15
	Doubly truncated lognormal distribution	= 16
	Uniform distribution using maximum likelihood	= 13
	Uniform distribution using sample data	= 12
B.	Magnification = 420X	
	Tolerance = 0.3740	
	Normal distribution	= 7
	Lognormal distribution	= 8
	Doubly truncated lognormal distribution	= 8
	Uniform distribution using maximum likelihood	= 9
	Uniform distribution using sample data	= 8
C.	Magnification = 320X	
	Tolerance = 0.3890	
	Normal distribution	= 7
	Lognormal distribution	= 8
	Doubly truncated lognormal distribution	= 8
	Uniform distribution using maximum likelihood	= 9
	Uniform distribution using sample data	= 8
D.	Magnification = 220X	
	Tolerance = 0.4067	
	Normal distribution	= 6
	Lognormal distribution	= 7
	Doubly truncated lognormal distribution	= 7
	Uniform distribution using maximum likelihood	= 8
	Uniform distribution using sample data	= 7

Table 4. Student "T" Tolerance Test of Normal Distribution of SEM/EDS Data for C002 (Cont.)

		Sample #
E.	Magnification = 100X	
	Tolerance = 0.4457	
	Normal distribution	= 5
	Lognormal distribution	= 5
	Doubly truncated lognormal distribution	= 5
	Uniform distribution using maximum likelihood	= 6
	Uniform distribution using sample data	= 6
F.	Magnification = 70X	
	Tolerance = 0.4540	
	Normal distribution	= 4
	Lognormal distribution	= 4
	Doubly truncated lognormal distribution	= 5
	Uniform distribution using maximum likelihood	= 6
	Uniform distribution using sample data	= 5
G.	Magnification = 50X	
	Tolerance = 0.4680	
	Normal distribution	= 4
	Lognormal distribution	= 4
	Doubly truncated lognormal distribution	= 4
	Uniform distribution using maximum likelihood	= 5
	Uniform distribution using sample data	= 5
H.	Magnification = 30X	
	Tolerance = 0.4861	
	Normal distribution	= 4
	Lognormal distribution	= 4
	Doubly truncated lognormal distribution	= 4
	Uniform distribution using maximum likelihood	= 5
	Uniform distribution using sample data	= 5

Table 5. Student 'T' Tolerance Test of Normal Distribution

of SEM/EDS Data for C002A

		Sample #
A.	Spot Mode	
	Tolerance = 0.3076	
	Normal distribution	= 19
	Lognormal distribution	= 20
	Doubly truncated lognormal distribution	= 21
	Uniform distribution using maximum likelihood	= 14
	Uniform distribution using sample data	= 13
B.	Magnification = 420X	
	Tolerance = 0.2912	
	Normal distribution	= 6
	Lognormal distribution	= 6
	Doubly truncated lognormal distribution	= 6
	Uniform distribution using maximum likelihood	= 7
	Uniform distribution using sample data	= 7
C.	Magnification = 320X	
	Tolerance = 0.2843	
	Normal distribution	= 5
	Lognormal distribution	= 6
	Doubly truncated lognormal distribution	= 6
	Uniform distribution using maximum likelihood	= 7
	Uniform distribution using sample data	= 6
D.	Magnification = 220X	
	Tolerance = 0.2799	
	Normal distribution	= 7
	Lognormal distribution	= 7
	Doubly truncated lognormal distribution	= 7
	Uniform distribution using maximum likelihood	= 8
	Uniform distribution using sample data	= 8

Table 5. Student "T" Tolerance Test of Normal Distribution

of SEM/EDS Data for C002A (Cont.)

		Sample #
E.	Magnification = 100X	
	Tolerance = 0.2630	
	Normal distribution	= 15
	Lognormal distribution	= 15
	Doubly truncated lognormal distribution	= 15
	Uniform distribution using maximum likelihood	= 12
	Uniform distribution using sample data	= 12
F.	Magnification = 70X	
	Tolerance = 0.2428	
	Normal distribution	= 17
	Lognormal distribution	= 15
	Doubly truncated lognormal distribution	= 16
	Uniform distribution using maximum likelihood	= 14
	Uniform distribution using sample data	= 13
G.	Magnification = 50X	
	Tolerance = 0.3305	
	Normal distribution	= 5
	Lognormal distribution	= 6
	Doubly truncated lognormal distribution	= 6
	Uniform distribution using maximum likelihood	= 7
	Uniform distribution using sample data	= 6
H.	Magnification = 30X	
	Tolerance = 0.3330	
	Normal distribution	= 3
	Lognormal distribution	= 3
	Doubly truncated lognormal distribution	= 3
	Uniform distribution using maximum likelihood	= 4
	Uniform distribution using sample data	= 4

Project Report for 6/21/90-9/20/90

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-9-90
Report Period: June 21, 1990
Sept.20, 1990

INTRODUCTION

Depyritization and general screening procedures for potential microbial organic sulfur utilizers have continued throughout the past quarter. Sample analyses and associated reporting have been delayed somewhat as a result of new student technicians replacing the former work group and the loss of the departmental secretary.

The ongoing work which has been conducted during the past quarter is summarized in the following discussion.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

The twenty pound shipment of IBC-101 (to be designated Lot 1007) has been received, riffled and placed storage. The inert gas used as a blanket for storage has been changed from nitrogen to argon as per the discussion at the 6th Annual Coal Contractors Conference. This coal is being ground to 200 X 0 mesh for use in the future as needed.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

In order to compare the effects of LOPOSO medium (the supporting medium for *Thiobacillus sp.* for depyritization) on the degree of depyritization in batch culture, a run was conducted in three flasks, each with varying initial conditions.

Table 1 shows the initial conditions set for each flask. The operating conditions for each batch culture were identical with 50 grams of 200 X 0 coal carefully measured into each flask.

Table 1. Batch Reactor Initial Conditions

Description	1	2	3
Medium	water only	LOPOSO only	LOPOSO + organisms
Temp. °C	25	25	25
Rx. pH	2.5	2.5	2.5
% coal slurry	5	5	5

As presented, 1000 ml of water and 50 g of 200 X 0 mesh coal (raw coal #1005) were added to Flask 1. Flask 2 received LOPOSO medium (composition presented in Table 2) and coal. The third flask, in addition to LOPOSO medium and 50 grams of coal, received a 10% v/v inoculum of *Thiobacillus sp.* The culture inoculum consisted of equal volumes (50 mL each) of *Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans*. Each of these cultures have been maintained in their respective ATCC media as specified in earlier reports. This 100 mL inoculum was added to 900 mL LOPOSO medium.

Table 2. LOPOSO Medium Constituents

Component	Composition (g/L)
NH_4Cl	0.63
KH_2PO_4	0.10
$MgCl_2 \cdot 6H_2O$	0.85
KCl	0.10
$CaCl_2 \cdot 2H_2O$	0.008
NaCl	0.123

Upon inoculation of Flask 3, the three flasks were placed in a constant temperature environment (T=25 °C) on stirring plates with agitation rates between 200-400 rpm.

Each reactor pH was monitored periodically and maintained at 2.5 with 1.5 N NaOH. Table 3 presents the periodic "preadjustment" pH values for the three flasks.

It should be noted that after pH measurement, the pH of each flask was returned to pH=2.5.

Table 3. pH Values as a Function of Time.

Time (hrs)	Flask 1	Flask 2	Flask 3
0.0	2.60	2.52	2.51
24.5	2.45	2.41	2.31
95.5	2.70	2.33	2.12
121.5	2.68	2.17	2.21
168.5	2.54	2.15	2.19
336.5	2.37	2.13	2.10
480.5	experiment discontinued		

As indicated in the representative data of Table 3, the pH dropped significantly in both Flask 2 with LOPOSO and Flask 3 with LOPOSO plus organisms. On day 14 (hrs=336.5), a slurry sample estimated to yield approximately 5 grams of coal was removed from each of the three flasks for subsequent sulfur analyses. These analyses have yet to be conducted. Upon discontinuation of the experiment, the coal slurry was filtered, wash with distilled water to remove organisms, and dried for sulfur analyses.

Attempts to close the mass balance have met with several difficulties. The initial coal samples weighed into each of the three flasks were carefully measured to 50 grams per flask. However, in trying to effectively wet and mix the coal into the medium, considerable "dusting" occurred with the likely loss of some coal to the atmosphere. Additionally, coal sampling during the experiment incurred some coal loss through adherence to the sides of filtration equipment, filter paper, etc. In summary, the post-experimental coal weights are as follows (with 50 grams as the initial weight for each flask):

Flask 1 (water only) - 44.7 g
Flask 2 (LOPOSO) - 45.4 g
Flask 3 (LOPOSO+BUGS) - 46.1 g

The post-experimental dry coal weights presented above were obtained by drying the washed coal slurries in an air flow oven

until no measurable mass change occurred (+/- 0.01 g).

This experiment will be repeated with planned improvement in coal handling procedures. Sulfur analyses will be forwarded as obtained.

An additional batch reactor has been started for depyritizing a sizeable amount of coal. For this batch run (designated Experiment 10051A), a 5% slurry of 200 X 0 mesh coal was prepared in LOPOSO medium--1275 grams of coal in 25.5 liters of medium. The pH was adjusted to 2.5 with a reaction temperature of 30 °C and an agitation rate of approximately 200 rpm. The coal slurry was inoculated with 1 liter of 2% coal slurry, previously inoculated with equal volumes of Thiobacillus ferrooxidans and Thiobacillus thiooxidans. Additionally, approximately 150 mL of each Thiobacillus pure culture were added to ensure the viability of the inoculum.

Samples have been obtained on a regular basis and reactor pH monitored closely. Sulfur analyses are forthcoming as students complete training in the analytical area.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.2 Quality Assurance and Control.

Cross-checks of in-house analyses periodically conducted.

3.4 Microscopic Analyses.

Task complete.

Task 4 - Organic Sulfur Removal.

As described in the Quarterly Progress Report for March-June, 1990, cultures from raw inocula sources have been placed in basal salts medium with depyritized coal and allowed to incubate at 27 ° C with an agitation rate of 180 rpm for approximately 14 days. Upon completion of the incubation period, the coal samples were filtered and washed to remove biomass, air-dried, and stored in a dessicator for sulfur analysis. These samples will be analyzed for total sulfur and compared to the total sulfur values for the depyritized coal used as a source for the experiment.

Cultures for which the sulfur analyses show significant changes will be further subjected to testing.

Sulfur analyses for the cultures tested will be completed as students gain proficiency in the necessary analytical methods.

Work to construct a system of reactors and a temperature controlled cabinet which would allow better control of process parameters in microbial coal desulfurization studies has been delayed in order to concentrate on new staff orientation and training and to set up and monitor the batch depyritization reactor.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-12-90
Report Period: September 21, 1990
December 20, 1990

INTRODUCTION

The ongoing work which has been conducted during the past quarter is summarized in the following discussion.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

The twenty pound shipment of IBC-101 (Lot 1007) has been ground to 200X0 mesh. riffled and placed storage. An additional twenty pound shipment (Lot 1008) has been received. It will be ground to various mesh sizes for future studies.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

During this past quarter, experimentation was conducted in order to compare the effects of LOPOSO medium (the supporting medium for Thiobacillus sp. for depyritization) on the degree of depyritization in batch culture. Analysis of the samples for the completed experiment is underway.

Table 1 shows the initial conditions set for each flask. The operating conditions for each batch culture were identical with 50 grams of 200 X 0 coal carefully measured into each flask.

Table 1. Batch Reactor Initial Conditions

Description	1	2	3
Medium	water only	LOPOSO only	LOPOSO + organisms
Temp. °C	25	25	25
Rx. pH	2.5	2.5	2.5
% coal slurry	5	5	5

As presented, 1000 ml of water and 50 g of 200 X 0 mesh coal (raw coal #1005) were added to Flask 1. Flask 2 received LOPOSO medium (composition presented in Table 2) and coal. The third flask, in addition to LOPOSO medium and 50 grams of coal, received a 10% v/v inoculum of Thiobacillus sp. The culture inoculum consisted of equal volumes (50 mL each) of Thiobacillus thiooxidans and Thiobacillus ferrooxidans. Each of these cultures have been maintained in their respective ATCC media as specified in earlier reports. This 100 mL inoculum was added to 900 mL LOPOSO medium.

Table 2. LOPOSO Medium Constituents

Component	Composition (g/L)
NH_4Cl	0.63
KH_2PO_4	0.10
$MgCl_2 \cdot 6H_2O$	0.85
KCl	0.10
$CaCl_2 \cdot 2H_2O$	0.008
NaCl	0.123

Upon inoculation of Flask 3, the three flasks were placed in a constant temperature environment (T=25 °C) on stirring plates with agitation rates between 200-400 rpm.

Each reactor pH was monitored periodically and maintained at 2.5 with 1.5 N NaOH. Table 3 presents the periodic "preadjustment" pH values for the three flasks.

It should be noted that after pH measurement, the pH of each flask was returned to pH=2.5.

Table 3. pH Values as a Function of Time.

Time (hrs)	Flask 1	Flask 2	Flask 3
0.0	2.60	2.52	2.51
24.5	2.45	2.41	2.31
95.5	2.70	2.33	2.12
121.5	2.68	2.17	2.21
168.5	2.54	2.15	2.19
336.5	2.37	2.13	2.10
480.5	experiment discontinued		

As indicated in the representative data of Table 3, the pH dropped significantly in both Flask 2 with LOPOSO and Flask 3 with LOPOSO plus organisms. On day 14 (hrs=336.5), a slurry sample estimated to yield approximately 5 grams of coal was removed from each of the three flasks for subsequent sulfur analyses. These analyses have yet to be conducted. Upon discontinuation of the experiment, the coal slurry was filtered, wash with distilled water to remove organisms, and dried for sulfur analyses.

Attempts to close the mass balance have met with several difficulties. The initial coal samples weighed into each of the three flasks were carefully measured to 50 grams per flask. However, in trying to effectively wet and mix the coal into the medium, considerable "dusting" occurred with the likely loss of some coal to the atmosphere. Additionally, coal sampling during the experiment incurred some coal loss through adherence to the sides of filtration equipment, filter paper, etc.

In summary, the post-experimental coal weights are as follows (with 50 grams as the initial weight for each flask):

Flask 1 (water only) - 44.7 g
Flask 2 (LOPOSO) - 45.4 g
Flask 3 (LOPOSO+BUGS) - 46.1 g

The post-experimental dry coal weights presented above were obtained by drying the washed coal slurries in an air flow oven until no measurable mass change occurred (+/- 0.01 g).

An additional batch reactor was started for depyritizing a sizeable amount of coal. For this batch run (designated Experiment 10051A), a 5% slurry of 200 X 0 mesh coal was prepared in LOPOSO medium--1275 grams of coal in 25.5 liters of medium. The pH was adjusted to 2.5 with a reaction temperature of 30 °C and an agitation rate of approximately 200 rpm. The coal slurry was inoculated with 1 liter of 2% coal slurry which had been grown for approximately two weeks, previously inoculated with equal volumes of Thiobacillus ferrooxidans and Thiobacillus thiooxidans. Additionally, approximately 150 mL of each Thiobacillus pure culture were added to ensure the viability of the inoculum. The microbial activity of the 2% coal slurry as detected by a decrease in the pyrite percentage had not been verified by analysis at the time the 25.5 liter batch was started. Therefore, the additional pure cultures of Thiobacillus sp. were added to ensure an ample bacterial cell concentration to initiate depyritization.

Samples were obtained on a regular basis and reactor pH monitored closely. The initial and final pyritic sulfur analyses are presented below in Table 4.

Table 4. Pyritic Sulfur as a Function of Time for the 5% Coal Slurry.

Time (days)	% Pyrite
0	0.638 +/- 0.028
76	0.393 +/- 0.005

As seen in Table 4, only approximately 38% of the pyritic sulfur was removed over a rather extended reactor operating period. The extent of this period (76 days) results from the absence of sulfur analyses for the samples obtained throughout the batch operation. This is due to an ongoing training of new student workers. This coal will be retained for subsequent "retreatment" at a later date.

It should be noted that the solids concentration for this batch was higher (5%) than for batches of coal treated previously (2%). During the treatment of this batch of coal, samples of the slurry were periodically examined for the presence of cells. Though cells were detected, the determination of microbial depyritizing activity could not be made in the absence of sulfur analyses.

Another batch of raw coal is currently being treated in a manner identical to the methods

presented above. However, the solids concentration for this batch has been set at 2%. Progress is being made in obtaining reliable sulfur analyses from the lab thus allowing for closer monitoring of reaction progress for this batch.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.2 Quality Assurance and Control.

Cross-checks of in-house analyses periodically conducted.

3.4 Microscopic Analyses.

Task complete.

Task 4 - Organic Sulfur Removal.

Procedures for the selection of definitive sulfur utilizers from the list of isolates presented in monthly progress Report No. PETC-10-90 are being developed with a focus on promoting organic sulfur utilization through the application of mutagenesis techniques to these cultures.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-3-91
Report Period: December 21, 1990
March 20, 1991

INTRODUCTION

The research activities which have been conducted during this time period are addressed below.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

An additional twenty pound supply of Illinois #6 coal (IBC-101) has been received and is available for grinding as needed.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

A batch treatment for depyritizing a sizeable portion of coal is planned for the latter stages of the month. The reactor vessel which has been used previously is being altered in an attempt to increase the rate and extent of depyritization. The reactor has a 136 liter working liquid volume in which a 2% coal slurry (mass %) will be mixed with LOPOSO medium and operated at a reaction temperature of 30 °C. The reactor impeller has been changed from a three-blade axial flow impeller to a four-blade axial flow impeller having a greater blade surface area and longer blade length (in an attempt to promote greater homogeneity in mixing). Additionally, the inlet air source has been affixed to the bottom of the conical reactor vessel to inhibit settling of the coal particles. The successful operation of this reactor scheme will be followed with a detailed figure and description in subsequent monthly progress reports.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.2 Quality Assurance and Control.

Cross-checks of in-house analyses periodically conducted.

3.4 Microscopic Analyses.

Task complete.

Task 4 - Organic Sulfur Removal.

Task complete.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-6-91
Report Period: March 21, 1991
June 20, 1991

INTRODUCTION

The ongoing work which has been conducted during the past quarter is summarized in the following discussion.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete. Ground coal (200 X 0 mesh, Illinois #6 IBC-101) has been placed under Argon storage awaiting depyritization treatment.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

A coal batch of approximately 1.5 kg is being treated for removal of pyrite a by T. ferrooxidans and T. thiooxidans mixed culture. Samples obtained during the treatment are awaiting analysis.

As the work at LTU has been comprised primarily of depyritization efforts, an update in reviewing the literature is being conducted to search for areas in which new work may be pressed forward here. One area that has captured our attention is the employment of water-in-oil emulsions and reverse micelle processes for the enhancement of mass transfer between the coal substrate and microbial biocatalysts (Lee and Yen, 1990). This study looks promising for improving the total sulfur content of particulate coal, and our focus this next quarter will be toward possible contributions to this area of research.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.4 Microscope Analysis.

During the spring quarter, one of our graduate students completed his master's thesis entitled, "Advanced Scanning Electron Microscopy and Energy Dispersive Spectroscopy Techniques for Sulfur Analysis in Coal". This document will be mailed under separate cover.

Task 4 - Organic Sulfur Removal

The completion of the contractual agreement with Dr. Bailey Ward at the University of Mississippi has brought this portion of the work to a close. This portion of the project's final report should be forthcoming this summer and will be forwarded upon receipt.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-9-91
Report Period: June 21, 1991
September 20, 1991

INTRODUCTION

The ongoing work which has been conducted during the past quarter is summarized in the following discussion.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete. All ground coal (200 X 0 mesh, Illinois #6 IBC-101) which has been placed under Argon storage has now been treated for pyrite removal. Additional coal has been ground for the reverse micelle studies currently underway.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

During the past quarter, a coal batch of 1.8 kg (200 X 0 mesh, Illinois #6 IBC-101) was treated for removal of pyrite by a *T. ferrooxidans* and *T. Thiooxidans* mixed culture. Samples obtained during the treatment were analyzed.

The batch reactor run for this experiment was comprised of a 5% coal slurry in LOPOSO medium (see Table 1 below for medium composition) and an inoculum of *Thiobacillus* sp. (*T. ferrooxidans* and *T. thiooxidans*). The inoculum consisted of a 1 liter 2% coal slurry, incubated for 48 hours prior to inoculation of the large batch reactor. In turn, the inoculum was prepared by inoculating the 2% coal slurry from 50 mls each of the two *Thiobacillus* sp. stock cultures. Prior to inoculation, air flow and agitation was established. The agitation rate was set at approximately 200 rpm.

Table 1. Composition of LOPOSO medium.

Component	g/L
NH_4Cl	0.63
KH_2PO_4	0.10
$MgCl_2 \cdot 6H_2O$	0.85
KCl	0.10
$CaCl_2 \cdot 2H_2O$	0.008
NaCl	0.123

Prior to addition of the culture inoculum, the contents of the batch reactor were allowed to equilibrate to the reaction temperature of 30 °C and were adjusted to a pH of 2.5. Samples were obtained regularly and pH was readjusted to 2.5 daily after inoculation.

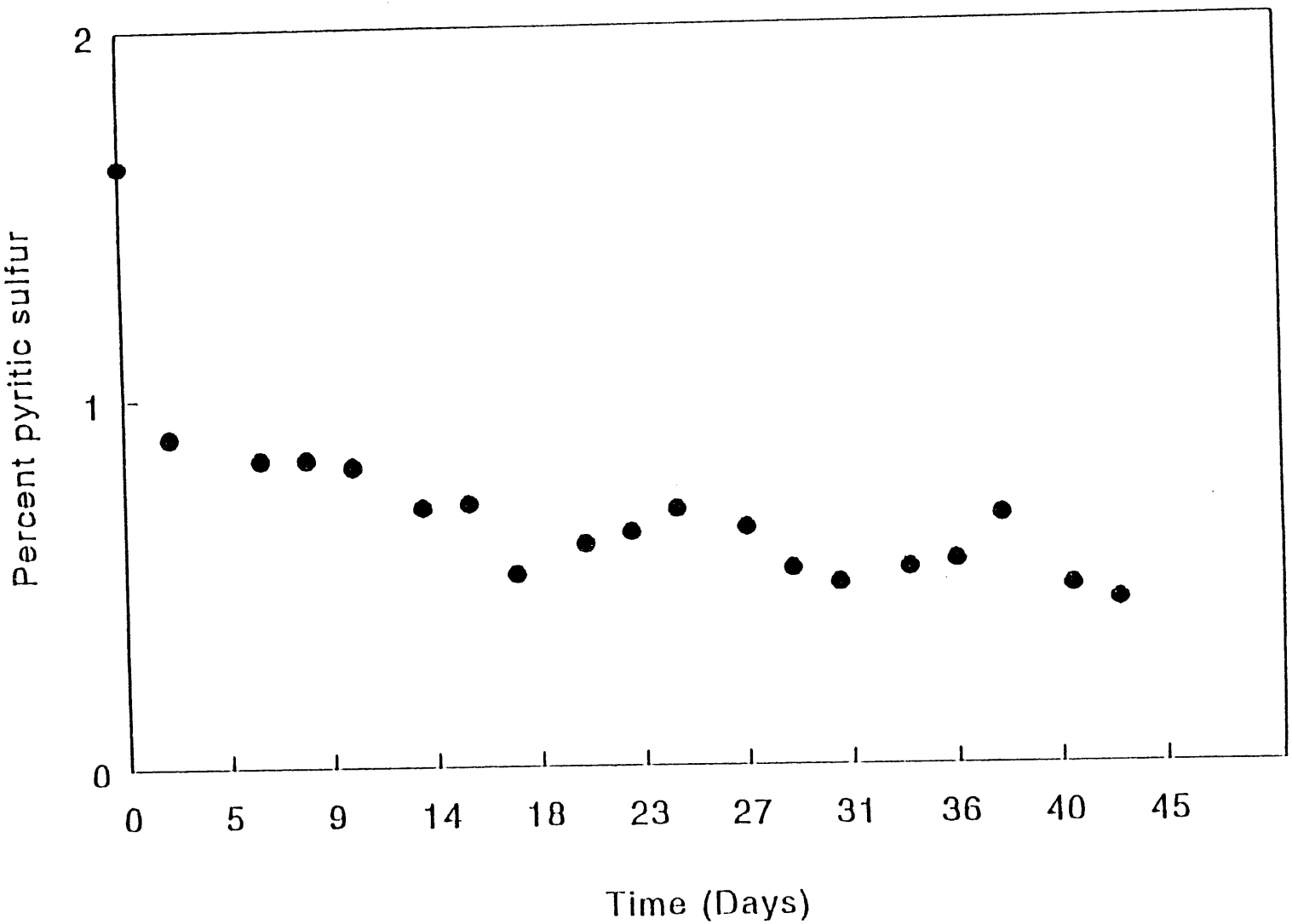
As described in PETC Report No. PETC-8-91, samples which were obtained were allowed to sit until the coal particles settled. Afterwards, the supernatant was decanted. The remaining coal was washed (with vigorous agitation) repeatedly with distilled water to remove microorganisms potentially adhering to the coal surface. This process was repeated three times after which the coal was filtered through a Buchner funnel under vacuum filtration. The coal filter cake was then dried in an air-flow oven at 105-110 °C to complete dryness. With the subsequent encounter of some difficulties in starting up the Atomic Absorption Analyzer, the coal was allowed to equilibrate with laboratory air. Each of the samples were then treated together for pyritic sulfur determination and corrected for the presence of moisture in the laboratory air (6% moisture) at the time of analysis. The percent moisture was determined by ASTM procedures for moisture in coal samples. The results of the pyrite analysis as obtained through atomic absorption techniques (described in ASTM Method D2492-84) are presented in Table 2 and Figure 1.

As seen in Figure 1, the percent (by mass) of pyritic sulfur dropped approximately 45% in the first 48 hours after inoculation. This was accompanied by a drop in reactor pH from 2.5 to approximately 2.1. The reactor pH was corrected to 2.5 with NaOH and monitored daily thereafter. However, as seen from the data presented, the rate of depyritization slowed dramatically after this initial period. Reactor operation was continued to observe the apparent ultimate extent of depyritization. The lengthy reaction time largely negates the utility of this experiment. After approximately 43 days, the coal had experienced a mass percentage loss in pyritic sulfur of approximately 71% which is well below reported literature values for pyrite removal in excess of 90% (Andrews and Maczuga, 1982; Hoffman *et al*, 1981).

Table 2. Percent Pyritic Sulfur As a Function of Time.

Time (days)	% Pyrite (by mass)
0	1.63
2	0.90
6	0.84
8	0.84
10	0.82
13	0.71
15	0.72
17	0.53
20	0.61
22	0.64
24	0.70
27	0.65
29	0.54
31	0.50
34	0.54
36	0.68
38	0.49
41	0.45

Fig 1. Percent Pyritic Sulfur With Time
Raw Coal Batch #1007.



A number of factors have been cited as contributing to a decrease in microbial pyrite oxidation including a decrease in *T. ferrooxidans* activity resulting from the release of organics into the aqueous phase (Eligwe, 1988); the precipitation of jarosite and iron oxides which inhibit bacterial activity through limiting oxygen mass transfer (Torma and Banhegyi, 1984); and the presence of competitive acidophilic bacteria contaminating the system. The latter bacterial contamination might have occurred in the batch run presented above through the use of nonsterile coal in the batch depyritization reactor. Such organisms may be resident upon the coal itself. In an effort to eliminate the possibility of such contamination, subsequent batches of coal will be autoclaved prior to addition to the medium--the sterilization of which is not practical with the current reactor design. To ensure the purity of our stock cultures, a plan of rapid serial dilutions has been undertaken with gradual lowering of the pH from 2.5 to near 1.8 with each subsequent dilution in order to eliminate possible contamination of the stock cultures by acidophilic organisms.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.4 Microscope Analysis.

Task complete.

Task 4 - Organic Sulfur Removal

The contractual agreement with Dr. Bailey Ward at the University of Mississippi was completed during the second quarter of this year. The compilation of his results is underway and will be incorporated into the project final report following completion of our no-cost contract extension.

LITERATURE CITED

G. Andrews and J. Maczuga, *Biotechnol. Bioeng. Symp. Ser.*, **12**, 337 (1982).

C. Eligwe, *Fuel*, **67**, 451, (1988).

M. R. Hoffman, B. C. Faust, F. A. Panda, H. H. Koo, and H. M. Tsuchiya, *Appl. Environ. Microbiol.*, **42**, 259, (1981).

A. E. Torma and I. G. Banhegyi, *Trends in Biotechnol.*, 2, 13, (1984).

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-12-91
Report Period: September 21, 1991
December 20, 1991

INTRODUCTION

As described in last month's report, preparatory work for the reverse micelle study has continued with the rapid serial dilution of our *Thiobacillus* sp. stock cultures at a low Ph (approximately 1.8-2.0) to ensure a strong inoculum in a logarithmic growth phase.

Total sulfur analyses will be run on samples obtained from experimental work for the reverse micelle study. After relocating this equipment in a more convenient laboratory, several difficulties have been encountered in setting up and operating this equipment. These problems continue to be addressed this month.

The research activity conducted during the current reporting period is summarized below.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

In addition to the serial dilution procedures for preparation of *Thiobacillus* sp. stock cultures (which are continuing), a preliminary experiment for the comparison of organic-phase, reverse-micelle depyritization to the typical aqueous phase culture has been initiated. The experimental set-up and procedures are described below.

In this initial experiment, only *Thiobacillus ferrooxidans* was used in the various batch cultures. Stock cultures are maintained in Ferrous Iron Medium (composition shown in Table 1 below).

Ferrous Iron Medium

Part I (autoclavable)	Part II (filter sterilizable)
1000 mL dist. H_2O	500 mL dist. H_2O
0.50 g K_2HPO_4	84 g $FeSO_4 \cdot 7H_2O$
0.50 g $(NH_4)_2SO_4$	25 mL 1N H_2SO_4
0.50 g $MgSO_4 \cdot 7H_2O$	
3.14 mL conc. H_2SO_4	

Stock cultures are maintained in 250 Erlenmeyer flasks containing 200 mL of Ferrous Iron medium with parts I and II mixed in the proportion of 144 mL and 36 mL, respectively. The medium pH is adjusted to 2.5 through base (NaOH) or acid (H_2SO_4) as necessary prior to transfer of the inoculum to fresh medium. The inoculum size is typically 10% v/v for stock culture maintenance.

For initiation of the reverse micelle experiment, one liter of Ferrous Iron medium was prepared and adjusted to pH 2.1. To it was added 100 mL of *Thiobacillus ferrooxidans* inoculum from a 7-day old culture. After incubation at 28 °C and 100 rpm (agitation rate) for five days, the cells were harvested by centrifugation. The culture medium was spun down at 10 °C for 30 minutes at 5,000 rpm (approximately 3,500 g). The cell pellet which was obtained was resuspended in 3 mL of distilled water (previously adjusted to 2.5). From this 3 mL cell concentration, 1 mL was added to the reverse micelle formulation (described below), 1 mL was added to a control and the remaining milliliter was retained and resuspended in Ferrous Iron medium for stock culture maintenance.

The reverse micelle was prepared in a 2 L flask in the following manner:

- 500 mL light mineral oil
- 500 mL n-heptane
- 1 mL Tween 80 (surfactant)
- 1 mL bacterial suspension (described above)
- mixture agitated with a stirring bar for 1 hour

After preparation of the reverse micelle system and the batch reactor controls, 20 grams of 100X0 mesh coal was added to each to form a 2% slurry. Table 2 presents each batch reactor condition.

Table 2. Batch Reactor Description

Flask No. 1 - Experimental reverse micelle system

Flask No. 2 - Control #1 (reverse micelle without biocatalyst)

Flask No. 3 - Control #2 (water @ pH=2.5 + 1 mL cells)

After each 2 liter flask had been prepared (as in Table 2), a stirring bar was placed in each for agitation and the media were autoclaved (prior to addition of the biocatalyst). Agitation was then accomplished by stirring plates in a temperature controlled cabinet (temp.= 30 °C).

Samples were obtained from each batch reactor every 12 hours with 50 mls removed per sample. Each sample was filtered through a Whatman No. 1 filter under vacuum filtration. The aqueous phase sample, only, was washed with distilled water. The organic phase samples were not washed. All samples were then dried 12 hours in an air-flow oven at 105 °C and stored in a dessiccator awaiting total sulfur analysis. This analysis will be provided as soon as current analytical equipment problems are resolved.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.4 Microscope Analysis.

Task complete.

Task 4 - Organic Sulfur Removal

Task complete.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Quarterly Report

Report No. PETC-3-92
Report Period: January 21, 1992
March 20, 1992

INTRODUCTION

The research activities conducted this month include the reverse micelle study (previously reported in Report No. PETC-2-92) and the initiation of related work concerning the growth characteristics of *Thiobacillus* sp. This experimentation is outlined below.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

As described in last month's report, a second experiment for investigating the depyritization activity of *T. ferrooxidans* in a reverse micelle system was initiated this month. However, similiar difficulties experienced with the first experiment (as described in PETC Report No. PETC-1-92) were encountered and the experiment was discontinued. The investigation of the mixed-culture (*T. ferrooxidans/T. thiooxidans*) depyritization activity in aqueous phase culture is continuing to ensure culture viability.

Additionally, an experiment has been started to investigate the effects of the organic phase upon desulfurization. A search of the literature has shown that the reverse micelle alone (without organisms) removes sulfur to some degree. Studies are being initiated to investigate the degree of desulfurization with and without organic solvent pretreatment.

The capability of the heptane to remove sulfur will be reported upon completion of this experimental work.

In order to examine the potential of reducing the lag phase in microbial growth and depyritization, a study is underway to investigate cellular activity in early exponential phase growth (as compared to later, stationary-phase growth).

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.4 Microscope Analysis.

Task complete.

Task 4 - Organic Sulfur Removal

Task complete.

Biodesulfurization Techniques: Application of Selected Microorganisms for Organic Sulfur Removal from Coals

Louisiana Tech University
Contract No. DE-AC22-88PC88854

Monthly Report

Report No. PETC-4-92
Report Period: March 21, 1992
April 20, 1992

INTRODUCTION

The past month's research activities are reported below.

TASK OVERVIEW

Task 2 - Coal Procurement and Preparation.

2.1 Grinding, Sieving, and Storage.

Task Complete.

2.2 Microbial Removal of Pyrite and Sulfate.

Task complete.

Comparative experiments have been conducted to examine the effects of "seed culture" age, the presence of a surfactant (Tween 80) and pretreatment of the coal with n-heptane on the rate and extent of desulfurization. These studies have been initiated as a result of attempts to formulate a reverse micelle system (as described in the literature) for improving the bacterial depyritization of coal. Problems with dispersion of the aqueous, microbial phase in the organic phase have resulted (discussed in previous reports) thereby prompting an investigation of several conditions associated with the reverse micelle system. These experiments are described below. Resulting experimental analysis will be provided as it becomes available.

EXPERIMENT #1

Following the initial reverse micelle experiments, a standard test has been conducted to reexamine the ability of our *T. ferrooxidans* strain to remove pyrite from the Illinois #6 coal (200 X 0 mesh). The experiment was conducted in 2 liter flasks containing one

liter of LOPOSO medium plus a nitrogen source (0.6 g/L NH_4Cl) and 20 g of coal for a 2% (w/w) slurry. Flasks containing media and coal were autoclaved for 15 minutes at 121 °C.

The bacterial inoculum consisted of a pure culture of *T. ferrooxidans* that had been growing for seven days at 30 °C and 120 rpm in modified 9K medium with 30 g/L $FeSO_4 \cdot 7H_2O$ as the energy source. Typical cell concentrations of 1O^7 - 10^8 cells/ml were achieved.

Cells were filtered from the 9K medium using 0.2 micron filters. Cells were then resuspended by agitating the filter in 50 mL of distilled water at pH 2.5 (adjusted by the addition of 1N HCl). The resuspension of cells contained 10^9 - 10^10 cells/mL. Cell concentrations were obtained by the measurement of the optical density in a spectrophotometer and through correlation to actual cell concentrations for *T. ferrooxidans* cited in published sources.

The two-liter flasks were then inoculated with 25 mL of the bacterial resuspensions to produce approximately 5X10^7 cells/mL in the 2% (w/w) coal slurry. Incubation was carried out at 30 °C and 120 rpm for 9 days.

The decrease in total sulfur was monitored with time by analysis on a LECO induction furnace. The experimental flasks removed approximately 75% of the pyritic sulfur in 10 days, while the control removed only approximately 10% of the pyritic sulfur.

EXPERIMENT #2

The next experiment was identical to the one described above except that a 5% (w/w) coal slurry was used with a surfactant (Tween 80), and cells were harvested after only two days of incubation to obtain cells in logarithmic growth. The purpose of this experiment was to examine both the effect, if any, of the added surfactant and the seed culture age upon the rate and extent of depyritization. The number of cells per mL was not recorded because of the presence of fine iron particulates in the resuspension.

The experimental flasks (both with and without surfactant) showed a similar reduction of 40% in pyritic sulfur in 12 days as compared to the uninoculated control in which a sulfur removal of only 10% was observed. It should be noted that the total sulfur removed in the experimental flasks was significantly lower for a comparable length of time than the experiment described above. It is likely that this is due, in part, to a lower cell count than in the previous, older culture.

EXPERIMENT #3

In this experiment, 50g of coal was washed for 8 hours in 100 mL of n-heptane, ethanol, and distilled water at 30 °C and 120 rpm in 1 liter flasks. The washed coal was then filtered and dried overnight in an air-flow oven at 110 °C.

The washed coal was then inoculated with cells harvested as before (Experiment #1) from a 7-day old culture. The experiment was done in 1 liter flasks containing 500 mL of LOPOSO medium and 10 g of washed coal for a 2% (w/w) slurry.

The initial results indicate that, prior to bacterial inoculation, the n-heptane washed coal experienced a 15% reduction in pyritic sulfur while washing with ethanol and distilled water removed only about 1%. After three days of incubation with the bacterial inoculation, the n-heptane flask showed about 30% pyritic sulfur removal as compared to only about 10% in the other two flasks (one with ethanol-washed and water-washed coal, respectively).

In each of the experiments described above, it is assumed that 1) the sulfatic sulfur is solubilized upon contact of the coal with the medium and 2) the organic sulfur remains unaltered by the aforementioned treatments thereby leaving only the pyritic sulfur to be removed by bacterial action. This allows the use of total sulfur analysis as a means of following the depyritization of the coal. However, further investigation into the action of heptane (upon all forms of sulfur) is warranted and more detailed sulfur analyses may be required.

Future experimentation is planned as follows:

1) n-heptane removal of sulfur as a function of time
2) effects of other organic solvents (e.g. acetone, benzene, etc. for removal of sulfur)
3) determination of the effect of each of the above treatments on *T. ferrooxidans* and *T. thiooxidans* activity in shake flasks
4) the acclimation of *Thiobacillus* cultures to FeS_2 use by the addition of small amounts of elemental sulfur to growing cultures.

Reporting on these results will be made in the May monthly report and the Project Final Report.

Task 3 - Analytical Procedures for Total Organic Sulfur.

3.1 Characterization.

Task complete.

3.4 Microscope Analysis.

Task complete.

Task 4 - Organic Sulfur Removal

Task complete.

ATTACHMENT B

Attachment B contains a complete, chronological description of Task 4--Organic Sulfur Removal, as conducted by Dr. Bailey Ward at the University of Mississippi.

MICROBIAL EXTRACTION OF NONPYRITIC SULFUR FROM COAL

BAILEY WARD, Ph.D.

Principal Investigator

Environmental Microbiology Laboratories
Department of Biology
The University of Mississippi
University, Mississippi 38677

Principal Research Team

Joe E. Lepo, Ph.D.: Research Associate
Carol J. Stevens, Ph.D.: Research Associate
Ralph F. Purdy, M.S.: Research Assistant

FINAL REPORT

Subcontract research in collaboration with Louisiana Tech University
Department of Chemical Engineering
U.S. Department of Energy Contract No. DE-AC22-88PC88854

November 5, 1991

SUMMARY

Several hundred bacterial cultures isolated from a variety of natural sites were tested for their ability to desulfurize selected model coal organosulfur compounds. Initial focus was on detection of biodesulfurization of dibenzothiophene (DBT) via a proposed pathway involving the intermediates, DBT-5-oxide, DBT-5-dioxide (-sulfone), and 2-(2-hydroxyphenyl)benzenesulfonic acid, from which removal of sulfate would give the product, 2,2'-dihydroxybiphenyl. Several screening methods initially were used to monitor desulfurization activity (on different organosulfur compounds): the Lehigh spray-plate assay (DBT), growing cell suspensions (DBT or DBT-sulfone), resting cell slurries (DBT or DBT-sulfone), or a sulfur-stress assay in which the only source of sulfur was organic (DBT or DBT-sulfone).

Numerous cultures produced colored "Kodama-type" products from DBT, but not from DBT-sulfone, via a carbon-destructive, non-desulfurizing pathway. Because the Kodama products interfered with detection of desulfurization activity, DBT-sulfone became the substrate of choice for subsequent screening assays. Although several cultures from a variety of sources exhibited activity (removal or disappearance from solution) on DBT-sulfone, no products were detected by the assays for which an inorganic-sulfur-sufficient medium was used.

Only two of our isolates, UMX9 and UMX3, and the strain IGT S-8, provided by the Institute for Gas Technology as a reference culture, would grow on DBT or DBT-sulfone as a sole source of sulfur under the sulfur-stress assay. Under sulfur-stress conditions, a desulfurized product was detected only for UMX9 and IGTS8. The desulfurized product was identified as 2-hydroxybiphenyl (2-phenylphenol). Biodesulfurization activity for all three organisms occurred only for growing cultures (vs. washed, resting cell suspensions), and was depressed by free sulfate, although more so for UMX3 and IGTS8 than for UMX9. All three organisms exhibited similar biodesulfurization activity on a variety of organic sulfur substrates. Comparative tests on carbohydrate utilization (48 substrates using API CH kit) revealed that only UMX9 would grow on glucose, and that only IGTS8 would grow on L-arabinose. None of the three cultures exhibited good growth on DBT, DBT-sulfone, or 2-phenylphenol as sole sources of carbon, compared to growth on glycerol, and none produced Kodama-type colored products from DBT. Taxonomic studies (including MIDI analysis) revealed UMX3 to be similar to IGTS8, whereas UMX9 exhibited characteristically different features. All of the organisms exhibited Rhodococcus-like features. Assays of biodesulfurization activity as a function of temperature or pH revealed further differences between UMX9 and UMX3/IGTS8. Under optimized assay conditions for each organism, UMX9 exhibited ca. 30% greater biodesulfurization activity than did IGTS8 and UMX3, which were about identical in activity.

Cultures UMX3 and UMX9 were tested for ability to grow on biodepyritized coal as sole source of sulfur, under the conditions of the sulfur-stress assay, with glycerol and glucose as carbon sources. Relative to appropriate controls consisting of growth medium plus coal only or medium plus cells only, both UMX9 and UMX3 exhibited better growth on coal as sole source of sulfur. Evidence of physical deterioration of the coal was noted for some biotreated samples. No data have yet been obtained on coal chemistry, thus we can only suggest that organic sulfur was removed from the coal.

INTRODUCTION AND BACKGROUND

Combustion of sulfur-containing fossil fuels results in the liberation of sulfoxides into the atmosphere where they react with water to produce sulfuric acids. Precipitation laden with the acids forms "acid rain", which poses a multitude of threats to the environment. The continued use of coals in general, and high-sulfur coals in particular, will require technologies designed to eliminate or reduce sulfur emissions. Two approaches can be taken: elimination of sulfur from post-combustion gases or pre-combustion coal

desulfurization, the latter being the subject of the present report. The predominant forms of sulfur in coals are 1) inorganic pyrite crystals existing as aggregates cloistered among, but not bound to, the organic coal matrix and 2) "organic" sulfur bound to carbon atoms. On the average, about one-half of the total sulfur in a "typical" coal is organic. The bulk of pyritic sulfur can be removed by a variety of chemical, physical, and biological technologies, but efficient methods for removing organic sulfur are lacking.

Because it would be desirable to remove coal organic sulfur without loss of fuel value, we proposed to search for bacteria capable of cleaving C-S bonds in model coal compounds or of coal itself. Previous reports (1,2) already had established the potential for using bacteria to remove organic sulfur from coals, a process that will be called **biodesulfurization** in the present report. A pathway was proposed (2) for selective enzymatic cleavage of C-S bonds in thiophenic model compounds, with emphasis on dibenzothiophene (DBT). The proposed pathway was called the "4S" pathway in reference to the sequence of sulfur oxidation via DBT-5-oxide, DBT-5-dioxide (-sulfone), 2-(2-hydroxyphenyl) benzenesulfonic acid, and liberation of free sulfate ion, resulting in the desulfurized product, 2,2'-dihydroxybiphenyl. A variant of the 4S pathway leading to 2-hydroxybiphenyl has been reported by Kilbane (3,4). Another possible pathway of biodesulfurization has been suggested (5) in which ring cleavage accompanies or follows desulfurization, with benzene and derivatives occurring as transient intermediates. (The 4S pathway is illustrated in Fig. 1, App. 8) For effective biodesulfurization of fuel coals, the desired activity would be specific cleavage of the bonds of any sulfur bound to coal carbon. The fact of attendant ring cleavage of model compounds might be inconsequential inasmuch as such compounds likely do not exist in coal. That is, a microorganism that removes sulfur from DBT along with degradation of the parent substrate might only remove sulfur from coal.

Our objectives were to obtain one or more novel microbial systems that specifically desulfurized DBT or DBT derivatives (with or without ring cleavage, but with focus on 4S-type activity), to characterize the biodesulfurization activity and the organisms themselves, and to test the superior cultures on biodepyritized coal.

The project developed and matured over a period of about thirty months, with progress and noteworthy events chronicled in the forms of monthly and quarterly reports, and in three manuscripts published as proceedings (6,7,8). Detailed and timely descriptions of methods, data collected, and interpretations appeared in the aforementioned reports, some or portions of which are appended to the present final report (in an approximately chronological sequence) to serve as references. For convenience (and to avoid having to rewrite that which already has been written) we will refer the reader to the Appendices for details of methodology, experimental rationale and setups, data, and results obtained as the project developed. This final report is presented as a chronology of events leading to a focus on the three (out of hundreds tested) organisms--cultures UMX3, UMX9, and IGTS8--that exhibited definite biodesulfurization, defined herein as **ability to grow on an organosulfur substrate as a sole source of sulfur**. For convenience of reporting, details of new methodologies applied for the last few months of the project are presented in Appendix 7. This approach allows for a narrative description of general approaches with focus on important findings and interpretations. The project culminated with a presentation and manuscript (App. 8) for the Third International Symposium on Biotechnology of Coal and Coal-derived substances, Essen, Germany, September 23-24, 1991.

PROJECT CHRONOLOGY

We initially focused on isolating from natural sources bacteria that exhibited one or more of the reactions associated with the 4S pathway. Because DBT is a common component of petroleum, we reasoned that sites of petroleum contamination might harbor bacteria capable of desulfurizing thiophenic compounds. We collected samples of weathered or fresh, refined or crude petroleum that had been mixed with soils or water in streams, ditches, or sumps near oil wells, pipelines, or storage or transfer facilities. At the time of the

initiation of the project, we were aware that Professor Steven Krawiec of Lehigh University had reported (INEL Program Review, January, 1989 and personal communications;later published, ref. #9) on the isolation from fuel-oil-contaminated soil of several (actually numerous) bacteria that gave evidence of production of 2,2'-dihydroxybiphenyl from DBT. Dr. Kraweic's apparent success reinforced our initial focus on collecting samples from oil-contaminated sites. We also collected samples from several sites where coals had been mixed with moist soils, and from an activated sludge wastewater facility.

Over the course of the project, several methods were tested as screening assays to detect and monitor biodesulfurization activity. Our desire was to use a rapid and simple regime that would allow for screening many isolates for 4S activity. Initially, we tried the Lehigh spray-plate assay (9;App.1,2) on a few hundred cultures, but found no convincing indications of (presumptive) 4S activity (DBT-dependent production of fluorescent products). Unless stated otherwise, an inorganic sulfur-sufficient medium, designated 21C (App. 1, p.3; ref.10) was used for all assays. This was the medium formulation exactly as used by Professor Krawiec (9) for detecting 4S activity for the Lehigh isolates via the spray-plate assay, thus we presumed that our use of the same formulation would yield good results in our hands.

Our initial lack of success using the spray-plate assay prompted us to secure cultures of several of the Lehigh University (Professor Steven Krawiec et al.) collection; these cultures had been reported to exhibit presumptive 4S activity via spray-plate assay and in some cases to produce 2,2'-dihydroxybiphenyl (9). After consultation with Dr. Krawiec and his associate, Diane Dutt, to ensure similarity of techniques, we tested the Lehigh isolates C18, F23, B24, A4, C1, C2, D20, E1, and F14. It was our aim to use the Lehigh cultures as references to test our methodologies and analytical techniques; we wanted to be sure that we were not missing 4S activity due to faulty techniques. A few of the Lehigh isolates did produce blue fluorescent products from DBT, but these same cultures also produced colored products, a phenomenon consistent with "Kodama" metabolism of DBT resulting in sulfur-containing yellow/orange/red products (11) which also fluoresce blue under short wavelength irradiation (12). After many trials and manipulations, we concluded that the spray-plate method did not allow us to differentiate between possible production of 4S products and conspicuous production of Kodama products. In fact, all presumptive positive 4S activity was due either to confirmed Kodama products or to unidentified but undesulfurized products. In no case for any isolate, including the nine Lehigh isolates, did we detect a desulfurized product from DBT, although several isolates would remove DBT from solution. For these and subsequent experiments, we used thin-layer (TLC) and gas chromatography (GC), and GC/mass spectrometry (GC/MS) to monitor or identify products. Refer to App.1-5 for details of analytical protocols and to App. 3 and 5 for a description of our experiences with the Lehigh Plate Assay.

As we were testing the spray-plate assay, we also began to use shake-flask cultures to screen for biodesulfurization activity, both of crude or "raw" mixed cultures obtained directly from collected samples or of purified cultures. For these trials, we added DBT crystals to standard 21C medium inoculated with cells, then incubated cultures plus DBT-only and cell-only controls at 30°C (the standard temperature of all assays, unless otherwise noted). Developed cultures were extracted with organic solvent (App.2 which was analyzed by GC for amount of DBT remaining and appearance of 4S products. We also compared growing cells (shake-flask) with resting cell suspensions. Appendix 3 contains descriptions of the analytical methods. We were not able to detect desulfurized products for any culture tested (although some DBT disappeared from the medium for some cultures). We encountered problems with interference by DBT crystals of cell-density (O.D.) measurements so we began using DBT-sulfone as a substrate because of its greater water solubility than DBT. We used a water-saturated DBT-sulfone (0.166mM) solution (no crystals) for subsequent tests. We reasoned that any organism capable of desulfurizing DBT via the 4S pathway would also desulfurize DBT-sulfone. Although several cultures would readily remove DBT-sulfone from solution, in what appeared to be cometabolic reactions, we did not detect any products of possible biodesulfurization reactions. In short, we were not getting any evidence of desulfurization, only removal of the substrate. We should note here that we never were able to confirm biodesulfurization activity for any of the Lehigh isolates. Refer to App. 3 and 5 and ref. No. 6 and 7 for details.

We also tested numerous cultures (that removed DBT or DBT-sulfone) for release of sulfate into the medium. We used at first the ASTM turbidimetric ($BaCl_2$ precipitation, App. 1) method then turned to ion chromatography (App. 5). We were not able to demonstrate significant increases in sulfate due to microbial activity on the thiophenic substrates; yet we had no real evidence that desulfurization was occurring. We wonder if cometabolic release of sulfate from organosulfur compounds in a sulfate-sufficient medium would indeed result in measurable increase in total sulfur. We became concerned that the presence of free sulfate in the culture medium might repress biodesulfurization activity. During this stage of the project we learned of Dr. John Kilbane's work at the Institute of Gas Technology (IGT). Dr. Kilbane described (3,4) a "sulfur bioavailability assay" in which an isolate designated IGT S-8 would grow on DBT as a sole source of sulfur; the organism, identified by Dr. Kilbane as a Rhodococcus rhodocrous, produced the product 2-hydroxybiphenyl, designated in the present report as 2-phenylphenol. Thus IGT S-8 exhibited a variant of the proposed 4S pathway. Dr. Kilbane sent a filtered (cell-free) supernatant prepared at IGT for us to analyze to provide independent confirmation of the biodesulfurized product, 2-phenylphenol. The supernatant was from a spent culture medium in which DBT had served as the sole sulfur source for IGT S-8 prepared at IGT. We confirmed (by UV spectral analysis, GC/MS, GC, and TLC) presence of 2-phenylphenol in the filtered supernatant. We realized that we had been devoting our efforts to the wrong approach for obtaining biodesulfurizing isolates. We modified our standard 21C medium by replacing all sulfate salts with chloride salts to yield a sulfur-free medium, which then was used for our **Sulfur-Stress Assay**, in which the only source of sulfur was DBT-sulfone (water-saturated solution) or other organosulfur substrates. Glucose and glycerol were provided as organic carbon sources to favor growth of a variety of organisms. Each trial consisted of three sets of culture-tube preparations containing 10 ml of medium:

SET 1. DBT-sulfone + inoculum;
SET 2. inoculum only in sulfur-free medium;
SET 3. DBT-sulfone + inoculum + added sulfate [0.166mM].

Cultures were considered to exhibit biodesulfurization when good growth occurred in **SET 1** tubes and continued after several (five or more) successive transfers; with no to minimal growth in **SET 2** tubes; and with good growth in **SET 3** tubes which also allowed us to check for possible inhibition by the organosulfur substrate. All glassware was rinsed in HCl to remove residual sulfur. Measurements of optical density (O.D.) provided indications of cell growth. Culture extracts were analyzed by GC and GC/MS to monitor desulfurization activity (removal of organosulfur substrate and appearance of desulfurized product).

The reader here is referred to App. 7 and 8 for details of methodology and procedures for the latter phases of the project.

For time-course analyses of DBT-sulfone removal and appearance of 2-phenylphenol via sulfur-stress assays, we also used spectrometric UV scans to monitor and quantify biodesulfurization. Because DBT-sulfone and 2-phenylphenol gave distinguishable spectral peaks in the range 200-400 nm, this method allowed us to monitor directly the aqueous, cell-free supernatants of test cultures (DBT-sulfone supplied as water-saturated solution of 0.166 mM). We used a Milton Roy Spectronic 1201 coupled to a computerized SpecScan program that allowed for curve area analysis and differentiation. Cultures were grown under sulfur-stress conditions, shaken in flasks at 30° C, with 1.5 ml samples withdrawn at time intervals, centrifuged at 10,000 x g for 10 min, then read directly via spectrometry.

Using the sulfur-stress assay, we retested most of our original isolates, including the Lehigh cultures, and several hundred cultures from the University of Alabama, Huntsville/ collection (isolated from superfund sites-Professor Alfred Mikell et al.). We also obtained IGT S-8 from IGT (Dr. John J. Kilbane) to serve as a reference culture. Of the first several hundred cultures tested, only IGTS8 grew on DBT or DBT-sulfone as a sole source of carbon. However, during subsequent trials, a mixed culture, designated UMX, exhibited positive activity on the sulfur-stress assay. The aforementioned filtered supernatant from IGT

arrived in an apparently non-sterile container (the sample was faintly turbid), and we took no special aseptic precautions in handling the sample. We at once fractionated the sample, then extracted one fraction for analysis; the other fraction was dispensed to a non-sterile culture tube. Subsequent examination of the reserve fraction indicated the presence of cells, so we decided to attempt to culture them. Our interest was piqued by the presumption that the filtrate should have contained no free sulfur; no DBT was detectable by our GC or GC/MS analysis. Even if the solution contained a variety of bacterial cells, the lack of available sulfur should have prevented or at least severely restricted growth. However, organisms having a low sulfur requirement might at least survive the sulfur-stressed environment. A mixed culture derived (initially in 1/10 strength tryptic soy broth) from the filtrate grew in the sulfur-stress assay on DBT-sulfone as sole source of sulfur and produced 2-phenylphenol (App. 5, Fig. 1). Our first thought was that IGT S-8 had slipped past the filter at IGT, and was present in the consortium. Examination of the consortium after growth on the sulfur-stress assay revealed a predominance of two pseudomonads along with several other organisms (App. 5). Upon separation and repeated tests of members of the consortium, only two isolates in pure culture, UMX3 and UMX9, grew well on the sulfur-stress assay. Analysis of extracts revealed that only UMX9 produced 2-phenylphenol, and that UMX3 did not produce a detectable product (App. 5,8). As evidenced by staining reactions, cell and colony morphology, and growth habit in liquid media, UMX3 appeared to be identical to IGT S-8, with the exception of lack of 2-phenylphenol in extracts of UMX3. The UMX9 appeared to be unlike either UMX3 or IGTS8 (App. 6). Thus, we concluded that IGT S-8 was not present in the consortium. The UMX cultures represented a fortuitous circumstance in which the original filtrate served as an enrichment and selection medium favoring the survival of organisms capable of desulfurizing the minuscule amount of DBT that must have remained in solution; or perhaps sulfur was being recycled from biomass. Our subsequent tests revealed that the nondesulfurizing members of the original consortium would grow in the sulfur-stress medium only when the desulfurizers were present. Moreover, the nondesulfurizers usually were the dominant members of the consortia; indeed, desulfurizers were not apparent in the mix when it was viewed via microscopy, yet they could be isolated by serial dilutions and plate culture on sulfur-stress media. We concluded that the desulfurizer(s) liberated excess sulfur from the organosulfur substrate, and that the other organisms scavenged the free sulfur, leaving just enough for survival of the desulfurizers.

Inasmuch as by this time the project was nearing final stages, and considering the fact that UMX3 and UMX9 were the only isolates that we had obtained that exhibited biodesulfurization activity, we elected to concentrate our remaining efforts on characterizing these cultures in comparison to IGT S-8; the remainder of this report focuses on these activities, and the manuscript in App. 8 describes the results of our investigations.

Our objectives were to reveal additional taxonomic or physiological differences among the three organisms, and to compare the three with respect to biodesulfurization activity. For these assays, we used the spectrometric method described earlier. Details of methodology are given in App. 7. Five replicate cultures were used for each experimental variable vs. each tested organism; data are reported as the mean of the five replicates, unless stated otherwise.

Taxonomic/physiological Studies. Our approach to taxonomic differentiation was to have the cultures analyzed by MIDI analysis (App. 7) and to apply the API carbohydrate utilization test to determine differences, if any, in carbohydrate metabolism. We also compared physiological responses of each culture with respect to ability to degrade and perhaps grow on DBT-sulfone or 2-phenylphenol, and checked each for production of Kodama-type products from DBT. Not only would differences in ability to degrade or utilize DBT-sulfone or 2-phenylphenol indicate genetic differences, but also would provide important information in assessing degree to which biodesufurization occurred. For example, if UMX3 rapidly degraded 2-phenylphenol, then its mechanism of biodesulfurization of DBT-sulfone might be the same as that of IGTS8, with the additional capacity for ring cleavage. Moreover, biodegradation of 2-phenylphenol would obscure the actual rate of production. For these tests, we used spectrometric analysis and compared experimentals vs. controls vs. time of incubation.

Kilbane (13) reported that the fatty-acid profile of IGT S-8 revealed Rhodococcus rhodocrous, thus we applied a similar technique to support our own taxonomic work. Our rationale for use of the API test was that because carbohydrate utilization likely represents metabolic pathways genetically distinct from biodesulfurization of thiophenes, such differences would reflect true taxonomic differences among the organisms. Moreover, Kilbane (personal communication) stated that IGT S-8 would not grow on glucose as a carbon and energy source.

We should note that none of the three cultures (UMX3, UMX9, or IGT S-8) produced "Kodama"-type colored products from DBT or DBT--sulfone, and no such products were detected by GC/MS.

The results of the MIDI analysis reported in App. 6 are not conclusive. Both UMX3 and IGT S-8 exhibited similar profiles, but none of the three cultures gave a good match with any organism in the library, including Rhodococcus rhodocrous and a variety of other species of Rhodococcus. We can now only state that UMX3 and IGT S-8 are obviously different from UMX9 and that all three exhibit Rhodococcus-like features.

Figures 2-4, App.8 contain representative time-course assays of growth, disappearance of DBT-sulfone, and appearance of 2-phenylphenol for each of the three organisms assayed under sulfur-stress conditions, with glycerol and glucose as carbon sources, and with DBT-sulfone as sulfur source.

When DBT-sulfone was supplied as sole carbon and sulfur source or 2-phenylphenol sole carbon only (with 0.16 mM sulfate added), increase in cell density after 120 h of incubation was about the same as occurred when no carbon source was added. Some reduction in both 2-phenylphenol and DBT-sulfone occurred for all three organisms. We attribute the slight increase in cell density to carryover carbon/energy source from the starter culture. Small decreases in DBT-sulfone of 0.04-0.05 mM occurred for all three organisms, indicating some removal by the cells. Data and discussion are presented in App. 8.

The results of the API CH tests are presented in Table 5, App. 6. Culture UMX9 would grow on glucose, whereas neither UMX3 nor IGTS8 would do so, and only IGTS8 would grow on L-arabinose. Our working conclusion from the data available is that there are distinct taxonomic differences among the three organisms. UMX3 and IGTS8 appear to be closely related, with physiological differences exhibited in L-arabinose utilization and production of 2-hydroxybiphenyl. The UMX9 appears to be a quite different organism from the other two, yet it possesses the ability to desulfurize DBT or DBT-sulfone to 2-hydroxybiphenyl, and to utilize glucose as a carbon substrate.

Comparative Biodesulfurization Activities. We wanted to determine the differences, if any, that existed among the three cultures with respect to rate and degree of biodesulfurization under a variety of different conditions. We made time-course studies of cell density (O.D.) and disappearance of DBT-sulfone as functions of different carbon substrates (glucose or glycerol) or added sulfate to test for repression of activity. The results of first tests using the different carbon sources are presented in App. 6, Fig. 1 & 2; the effects of added sulfate are described in Fig. 5-7, App. 8, which also presents a discussion.
We also tested each culture for effects of Ph 6, 7, & 8 (30°C) and temperatures of 25, 30, 37, & 41°C (pH 7) on biodesulfurization to identify the best of each variable for each culture, then compared the three organisms under optimized conditions for each. These data along with discussion are presented in App. 8. The reader is referred to the manuscript of App. 8 for additional discussion, conclusions, and relevant references.

Tests on Coal. It was only near the end of the project that we had obtained biodesulfurizing strains, thus only a limited amount of work could be accomplished with coal. Details of the coal tests are presented in App. 6. In summary, UMX3 or UMX9 exhibited greater growth on biodepyritized coal as a sole source of sulfur than on control medium. Physical deterioration of the coal was evident after microbial growth. Glucose and glycerol were supplied, thus it is unlikely that the organisms were utilizing coal carbon.
We were not able to have chemical analyses done, thus we only can state that apparently the coal provided

a source of sulfur; whether organic or not is not known now.

SUBCONTRACT PROPOSAL TASK SUMMARY

The subcontracted efforts were addressed to Task 4 - Organic Sulfur Removal of the Louisiana Tech proposal. The subcontract proposal identified three Subtasks to be performed at the University of Mississippi. Our success at accomplishing each of these efforts is summarized below.

Subtask 4.1 - Desulfurization with OC7-A

Dr. Joseph Fernandes of Louisiana Tech supplied us with a culture identified as OC7-A. The suspension contained several organisms, none of which appeared to be similar to OC7-A, and none of which exhibited any evidence of biodesulfurization. Further work under Subtask 4.1 was not warranted.

Subtask 4.2 - Search for New Organisms

The narrative of the preceding pages 1-6, and the appended materials document our broad efforts and narrow success under this Subtask, which became the primary focus of the research.

We were disappointed that work with coal was limited, due to the fact that the project was almost at end and no additional funds for personnel, supplies, and services were available. As a major component of the overall project, personnel at Louisiana Tech were to provide organic-sulfur analyses using the SEM/EDX system at LTU, in addition to running other coal chemistry analyses on the biologically treated coals. Unfortunately, by the time that we had generated biotreated coals (biodepyritized at LTU), work at LTU had shifted to other focuses. Inasmuch as our funds were exhausted, we were unable to have the biotreated coals tested for amount and kinds of sulfur removed.

Subtask 4.3 - Plasmid-mediated Techniques

Louisiana Tech subcontracted separately with Dr. David Graves, Department of Chemistry, University of Mississippi, for the plasmid work to be done under this Subtask. Our own efforts focused on tests of cell-free extracts of UMX9 and UMX3 (prepared by nitrogen-decompression or French press rupture) revealed no detectable biodesulfurization activity. Some activity was detectable for the cell-particle fractions, but this was greatly reduced when equated with a comparable-biomass, whole-cell preparation. Further work in this direction was not done in favor of other efforts. A tentative conclusion is that the biodesulfurization activity is not mediated by extracellular enzymes.

Dr. Graves analyzed several of our isolates for plasmids during the first year of the project, but none of these ultimately exhibited true biodesufurization, thus the data were not relevant. Toward the end of the project, Dr. Graves tested UMX3, UMX9, and IGT S-8; no plasmids were identified, thus further work under this Subtask was not warranted.

REFERENCES

1. Knecht, A.T. Jr. "Microbial oxidation of dibenzothiophene and its possible application in the desulfurization of coal and petroleum." Ph..D. Dissertation, Louisiana State University, Baton Rouge, 53 pages (1961).

2. Isbister, J.D., and E.A. Kobylinski. "Microbial desulfurization of coal," In: Y. Attia (ed.), Coal and science technology: processing and utilization, p. 627-641. Elsevier/North Holland, Amsterdam (1985).

3. Kilbane, J.J. "Sulfur-specific microbial metabolism of organic compounds." Resources, Conservation, Recycling, 3:69-79 (1990).

4. Kilbane, J.J. "Biodesulfurization: Future prospects in coal cleaning." Proceedings: Seventh International Pittsburgh Coal Conference, pp. 373-382 (September, 1990)

5. van Afferden, M., S. Schacht, J. Klein, and H.G. Truper. "Degradation of dibenzothiophene by Brevibacterium sp. DO." Arch Microbiol. 153:324-328 (1990).

6. Ward, B., J.E. Lepo, F.R. Purdy, and C.J. Stevens. "Studies on biodesulfurization of model coal organosulfur compounds." Proceedings: First International Symposium on the Biological Processing of Coal, Orlando, Florida, P45-51 EPRI GS-6970 (May, 1990).

7. Lepo, J.E., R.F. Purdy, and B. Ward. "Microbial activity on model coal organosulfur compounds." Proceedings: Sixth Annual Coal Preparation, Utilization, and Environmental Control Contractors' Conference (Pittsburgh, Pennsylvania) pp. 226-233 (August, 1990)

8. Purdy, R.F., B. Ward, and J.E. Lepo. "Microbial extraction of sulfur from model coal organosulfur compounds". Proceedings: Third Symposium on Biotechnology of Coal and Coal-derived Substances, 23-24 September, 1991, Essen, Germany.

9. Krawiec, S. "Bacterial desulfurization of thiophenes." Developments in Industrial Microbiology. 31:103-114 (1990).

10. Guirard, B.V., and E.E. Snell. "Biochemical factors in growth." In: G. Gerhardt (ed.) Manual of Methods for General Bacteriology, American Society for Microbiology, Washington , D.C., USA (1981).

11. K. Kodama, K. Umehara, K Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. "Identification of Microbial Products from Dibenzothiophene and its Proposed Oxidation Pathway." Agr. Biol. Chem. Vol 37, pp. 45-50 (1973)

12. Monticello, D.J., D. Bakker, and W.R. Finnerty. "Plasmid-mediated degradation of dibenzothiophene by Pseudomonas species". App. Environ. Microbiol. Vol 49 (4):756-760. 1985

13. Kilbane, J. J. "Biodesulfurization: Future prospects in coal cleaning." Proceedings: Seventh Annual International Pittsburgh Coal Conference, University of Pittsburgh, Pittsburgh, Pennsylvania, USA (September, 1990).

APPENDIX I

BIOLOGICAL SAMPLES

DBT-FLUORESCENCE SPRAY-PLATE ASSAY

CULTURE MEDIA

GAS CHROMATOGRAPHY METHODS

ASTM SULFATE ASSAY

LEHIGH UNIVERSITY CULTURES

SOLVENT EXTRACTION METHODS

CHROMATOGRAM OF ORGANIC SULFUR STANDARDS

TELEFAX TRANSMISSION

Date: 3-3-89

To: Brace Boyden, Interim Project Manager
Department of Chemical Engineering
Louisiana Tech University
Ruston, LA
Telephone: 318-257-2885 (LTU FAX No. 318-257-3356)

From: Bailey Ward, Principal Investigator
Carol J. Stevens, Research Associate

The University of Mississippi
Laboratory of Environmental and Applied Microbiology
Department of Biology
University, MS
Telephone: 601-232-7203

Re: Quarterly Report for the Period December 15, 1988 - March 15, 1989. Biodesulfurization Project.

Personnel

Beginning on January 1, 1989, Dr. Carol J. ("C.J") Stevens joined the project as a Postdoctoral Research Associate. Dr. Stevens received her Ph.D. in Microbiology in December, 1988 from The Ohio State University, where she studied the physiology of Thiobacillus ferrooxidans. Also on January 1, Mr. Mannish Petrekh, and on January 15, Mrs. Kathleen Odum began work on the project as research assistants.

Apparatus

During the last of January and first of February, two water-bath (culture) shakers and a Hewlett Packard Model 5890A gas chromatograph with Model 3396A integrator were installed.

Screening for 4S Bacteria

Methods: Bacteria that were potentially positive for the 4S pathway were screened by two methods. The first method was developed at LeHigh University by Dr. Steven Krawiec and his staff, the other was developed at INEL by Tom Ward and Diane Key. The Krawiec method involved growing bacteria on solid 21C agar, spraying the plates with 3% DBT (w/v) in ethyl ether, allowing the plates to dry, and then observing the plates under a 254nm light source with the intention of observing the production of the fluorescent compound 2,2'-dihydroxybiphenyl (Krawiec's staff claimed the results should be positive within 2 hours). The TTC assay involved growing bacteria on TTC agar that had first been sprayed with 3% (w/v) DBT in ethyl ether. Those bacteria able to

utilize the DBT produced an excess of electrons that were used to reduce the TTC dye to a red color and therefore produced red colonies. Bacteria unable to utilize DBT could not reduce the dye and produced white colonies. The red colonies were then transferred to TTC agar without DBT and were allowed to grow. The lack of excessive carbon source and hence electrons resulted in white colonies; red colonies were regarded as negative because the bacteria were not limited by the yeast extract.

Sample Sources: Bacteria to be screened were isolated from the following sources: (a) the soil from a coal yard in Memphis, TN; (b) the soil contaminated with oil from an oil company in Oxford, MS; (c) a natural crude-oil seepage site at Beaver Dam Creek, MS (these samples were obtained by a state agency in 1985 and had been stored; plans to obtain fresh samples have been hampered by flooding in the area); (d) airborne bacteria that contaminated medium containing DBT. Due to our inability to reproduce the positive results obtained by Krawiec's lab with their method, we also obtained some of their cultures (A4, B24, C1, C2, C18, D20, E1, F14, F23) to use as positive controls.

We made numerous unsuccessful attempts to obtain DBT-dependent fluorescence (of presumed 4S products) from microbial isolates obtained from our sample sources. We double-checked to insure that our techniques conformed to those used by Dr. Steve Krawiec and his colleague, Diane Dutt at LeHigh University. We tested nine of Dr. Krawiec's isolates, but could not detect DBT-dependent fluorescence from any culture using the three-percent diethyl ether solution of DBT described by the LeHigh team. During a series of exploratory tests in our lab, Dr. Stevens discovered that vivid DBT-dependent fluorescence occurred when a 0.1% solution of DBT in ether was sprayed onto developed colonies of the LeHigh isolate strain C-18. This finding confirmed the reports by Dr. Krawiec. We note here that a very light coating of DBT gave the best results, e.g., the amount of DBT crystals deposited on the agar surface produced a barely detectable "sheen" when viewed at an angle under room fluorescent lighting. Table I presents our data on presumptive 4S and "Kodama" activity of the LeHigh cultures.

Details of our procedures are as follows: We used a "Chromist" (Gelman Cat. No. 15901) sprayer to deliver one short burst of DBT solution directly onto the agar surface (in a fume hood with 100 ft/min velocity air flow). The culture dish lid was replaced after about min of drying. Preparations were monitored at room temp. (ca 25°C) from time "zero" at 15 min intervals up to 2 hours, or after incubation at 30C overnight. Fluorescence was monitored under a Spectroline Model ENF-260C, 254 nm UV lamp (about 430 uW/cm^2 at 15 cm), with plates held at an angle at 5-6 cm from the lamp fitted to a Spectroline Model CM-10 viewing cabinet. Subsequent tests using 0.1% DBT on the other LeHigh isolates resulted in detectable fluorescence by five of the nine tested. Some of the LeHigh isolates produced

colored "Kodama"-type products, as did several of our own source sample isolates sprayed with 3% DBT. We cannot now explain why the LeHigh isolates did not exhibit 4S-type fluorescence when sprayed with 3% DBT. It is likely that the LeHigh team applied considerable less of the 3% solution than we applied using the commercial pressurized spray applicator. Yet we still do not see fluorescence for three of the LeHigh strains.

Media: Cultures were maintained and tested on the medium 21C[1] prepared as described below:

Stock solutions: (autoclaved) 50g NH_4Cl/500 ml water; 69.25 g KH_2PO_4/500 ml water; 50.25 g Na_2HPO_4/500 ml water; (filter-sterilized) 30 mg glucose/100 ml water. Vitamin stock (filter-sterilized): 0.5 mg biotin, 50 mg nicotinic acid, and 25 mg thiamine-HCl/ 50 ml water; Hutner's mineral base (filter-sterilized).

To prepare (per liter final volume):

1. 10 ml NH_4Cl, 20 ml KH_2PO_4, and 20 ml Na_2HPO_4 stocks in 920 ml distilled water, autoclaved, then cooled.

2. The following filter-sterilized solutions are added aseptically to the solution from 1. above: 20 ml of trace metals (Hutner's vitamin-free trace metal mix), 1 ml of vitamin mix, and 10 ml glucose. If solid medium is required, 15 g of agar is added prior to autoclaving. In certain circumstances, the glucose and vitamin mixtures are replaced by yeast extract.

TTC agar: This medium contained the same basal salts as the 21C medium (10 ml NH_4CL, 20 ml each KH_2HPO_4 and Na_2HPO_4) as well as 0.25 g yeast extract, 0.025 g triphenyltetrazolium chloride (TTC), 15 g agar, and 930 ml distilled water. The medium was autoclaved for 15 min at 121°C, 15 psi. When cool, 20 ml of Hutner's trace metal mix were added.

The TTC assay indicated that several of the bacteria screened (SOC-3, S2, S4, S8, SOM1-17-1, SOM1-17-3a, SOM1-17-3b, SOM1-17-4, SOM1-17-5, SOM1-17-6a, SOM1-17-6b; Krawiec's cultures) were capable of utilizing DBT, but does not indicate if usage is via the Kodama or 4S pathways. These bacteria can be further screened by the 0.1% DBT fluorescence assay, determining the products of DBT degradation via GC,and possibly by measuring SO_4^{2-} release.

---

[1] Guirard. B.V., and E.E. Snell. Biochemical Factors in Growth. In: Manual of Methods for General Bacteriology (G. Gerhardt, ed.). American Society for Microbiology (publishers), Washington, D.C. 1981.

Analysis of DBT Degradation

Gas Chromatrography

DBT (5% w/v) in acetone was filter sterilized and 1 ml aliquots were dispensed into sterile 125 ml Erlenmeyer flasks. After drying overnight, 50 ml of the 21C medium were added to each flask. The flasks were inoculated with 10 ml of culture that had grown overnight in 21C medium. The flasks were then incubated at 30°C, 125 rpm for 48 h. The DBT and hopefully its by-products were extracted by the following method. After pre-rinsing all glassware with chloroform, 0.5 ml of 0.1% thianthrene was added to a 50 ml centrifuge (1/flask). The culture was added to the centrifuge tube, 9 ml of chloroform were added to the flask and then transferred to the centrifuge tube. The tubes were inverted 50 times, centrifuged, and the organic layer saved. The tubes were extracted twice more with 8 ml chloroform each time for a final total of 25 ml. Then 1 ul of the extract was injected into the gas chromatograph (FID detector, 15 ml/min He as the carrier gas, 10 m HP-5 column). Standard chromatograms have been prepared and preliminary trials have begun on assays for DBT bioconversions by the LeHigh cultures as well as some of the UM isolates.

Sulfate Assay

The release of sulfate from DBT by microorganisms can potentially be measured using the ASTM turbidimetric method of sulfate analysis. So far, we are still trying to determine the reproducibility and detection limits for using this assay. Cultures are grown in 100 ml of 21C media in 250 ml Erlenmeyer flasks, incubated at 30°C, 125 rpm, for 48 h. Samples are centrifuged and filtered to reduce turbidity. Then 50 ml of the sample (or a diluted sample) are mixed with 10 ml of glycerin (diluted 1:1 with water) and 5 ml of NaCl solution (240 g NaCl, 20 ml conc. HCl in 1 liter of water). The spectrophotometer is zeroed with the sample and then 0.3 g of $BaCl_2$-$2H_2O$ crystals are added and the sample stirred for 1 min, then allowed to be static for 4 min, then stirred for 15 seconds and the absorbance read at 390 nm. The amount of sulfate present is determined by comparison to a calibration curve. The amount of sulfate used or released by the bacteria is determined by comparing the inoculated cultures to an uninoculated control. Thus far, the calibration curve is reproducible and it appears that the detection limits for sulfate used by pure cultures in 21C media may be within a useful range, provided that significant amounts of sulfate are released by DBT.

UV Spectrophotometry

Attempts were made to determine if the degradation of DBT to 2,2'-dihydroxybiphenyl (o,o'-biphenol) could be followed using UV spectrophotometry. DBT was dissolved in tert-butanol (0.5% to 0.0005% w/v) and scanned from 350 to 250 nm. Similar scans were

done on biphenol solutions in tert-butanol. It was determined that although there was overlap of the two spectra, the DBT and biphenol spectra were dissimilar enough that decreases in the DBT concentration could be monitored. Increases in biphenol would have to be greater than about 10% to be detected. Following this premise, it should be possible to grow cultures in 21C media with DBT, extract the DBT, and determine decreases when inoculated media are compared to uninoculated controls.

TABLE I

Presumptive 4S or "Kodama" Activity of Nine Krawiec(LeHigh University) Bacterial Strains Tested at UM

Strain	Activity[1]			
	0.1% DBT		3.0% DBT	
	F (time)	Color (time)	F (time)	Color (time)
A4	N	Y (18 h)	N	Y (18 h)
B24	N	Y (18 h)	N	N
C1	Y (15 m)	N	N	Y (18 h)
C2	Y (15 m)	Y (18 h)	N	Y (18 h)
C18	Y (15 m)	Y (18 h)	N	Y (30 m)
D20	Y (18 h)	Y (18 h)	N	N
E1	Y (18 h)	Y (18 h)	N	Y (6 h)
F14	N	Y (18 h)	N	N
F23	Y (2 h)	N	N	N

[1] N=none detectable; Y=detectable; times in min. or hours (all colored products were orange). Strain C18 gave the most vivid fluorescence.

TELEFAX TRANSMISSION

Date: 4-7-89

To: Brace Boyden, Interim Project Manager
Department of Chemical Engineering
Louisiana Tech University
Ruston, LA
Telephone: 318-257-2885

From: Bailey Ward, Principal Investigator
Carol J. Stevens, Research Associate

The University of Mississippi
Laboratory of Environmental and Applied Microbiology
Department of Biology
University, MS
Telephone: 601-232-7203

Re: Monthly Status Report, Biodesulfurization Project, Month of March, 1989.

A. Studies on the Lehigh University Isolates

Continued tests revealed that the fluorescence assay is sensitive to medium pH (using the 21C yeast extract medium described below). Best results were obtained at around pH 7, with low activity below 6.5, and with loss of detectable fluorescence at pH 7.5 and higher. The Hutner's vitamin-free stock solution changed from straw yellow to almost colorless when pH was adjusted to above about pH 6.5 and darkened at above pH 7.5. We have abandoned the use of glucose and the pure vitamin mix described earlier for 21C. We now mix the three major salts with 0.1% yeast extract, adjust the solution pH to 7.0, add agar at 1.5%, then autoclave. After the solution has cooled to about 55° C, we add Hutner's solution (adjusted to pH 7.0). The medium supports good growth of many isolates and gives a low background fluorescence.

We have observed an interesting phenomenon for the LeHigh isolate C-18. To build up product concentration for extraction and assay of possible 4S products, we repeatedly sprayed C-18 colonies with 0.1% DBT in ether; i.e., serial light applications were applied over a period of about 90 minutes. After about three applications, with concurrent fluorescence around the colonies, a distinct clear zone (clear of DBT crystals) developed around the colony at about the location of the fluorescence. Colored (Kodama-type ?) products collected only on the colony surface. We interpret this observation to mean that the cells were producing extracellular enzyme(s) that converted the crystalline DBT to colorless, noncrystalline (at least partially water soluble), fluorescing product(s).

B. Search for New Isolates

The P.I. collected a variety of crude-oil samples (fresh and weathered mixed with soil, decayed vegetation, or water in containment moats) from leaks and spills around pipelines and working or abandoned wells in oil fields in southern Mississippi and Alabama.

Several new isolates that exhibit DBT-dependent fluorescence were derived from the crude-oil samples. At least one of the new isolates appears to be a Bacillus sp, designated CIT-2, that produces fluorescence within about 30 min of DBT application, but does not produce colored compounds after up to 72 hours of incubation. The lack of colored DBT-degradation products will facilitate sulfate assays read at near 400 nm. Attempts to measure sulfate dynamics in cultures of strain C-18 (and other LeHigh isolates that produce colored compounds) were hampered by absorbance of the DBT-degradation products.

Research efforts now will focus on continued screening for new isolates, characterization of the new 4S+ strains, and on GC assays of new isolates to determine their ability to catalyze one or more of the proposed 4S pathway. Assays on sulfate rejection from DBT also will be made.

TELEFAX TRANSMISSION

Date: 5-8-89

To: Brace Boyden, Interim Project Manager
Department of Chemical Engineering
Louisiana Tech University
Ruston, LA
Telephone: 318-257-2885

LTU Fax # 318-257-3356
UM Fax # 601-232-5453

From: Bailey Ward, Principal Investigator
Carol J. Stevens, Research Associate

The University of Mississippi
Laboratory of Environmental and Applied Microbiology
Department of Biology
University, MS
Telephone: 601-232-7203

Re: Monthly Status Report, Biodesulfurization Project, Month of April, 1989.

Continued studies on the UM and LeHigh "4S+" isolates revealed that considerable variations in the fluorescence assay occur when attempts are made to obtain uniform and repeatable results. For reasons still unknown, the isolates (including the LeHigh C-18 strain) now do not yield good evidence of DBT-dependent fluorescence. For example, ether alone sometimes results in fluorescent products, perhaps due to cell lysis or stimulation of release of fluorescing substances from the cells. There also are indications that some of the LeHigh cultures contain more than one organism. We are investigating methods to obtain consistent, believable results.

We now have screened (for DBT-dependent fluorescence) about three dozen samples of coal or petroleum materials. The Louisiana Tech group provided five samples collected from sludgepits, petroleum storage areas, coal mines, and the like. The UM samples were from coal yards, refined petroleum-contaminated soils, and crude oils. Only two samples of fresh crude oil mixed with water or soil yielded organisms that gave evidence of DBT-dependent fluorescence, although we cannot obtain consistent results on subcultures of the isolates. The inconsistencies in the fluorescence screening method indicate a possibility that potential "4S+" isolates are being overlooked during routine screening.

We attempted unsuccessfully (via GC analysis of organic solvent extracts) to detect metabolism or transformation of DBT in liquid batch cultures or agar medium cultures of the UM and the LeHigh isolates. We also were not able to detect release of sulfate from DBT in liquid batch cultures of organisms (UM or LeHigh)

The University of Mississippi

Department of Biology
College of Liberal Arts
University, MS 38677
(601) 232-7203

TELEFAX TRANSMISSION

Date: 7-10-89

To: Brace Boyden, Interim Project Manager (LTU Fax # 318-257-2562)
Department of Chemical Engineering (UM Fax # 601-232-5453)
Louisiana Tech University
Ruston, LA
Telephone: 318-257-2885

From: Bailey Ward, Principal Investigator
Carol J. Stevens, Postdoctoral Research Associate

The University of Mississippi
Environmental and Applied Microbiology Laboratories
Department of Biology
University, MS
Telephone: 601-232-7203

SUMMARY

We tested several solvents for improved efficiency of extraction of reference "4S" compounds from microbial culture media. Ethyl acetate proved to give acceptably efficient and consistent recovery of DBT, DBT-sulfone, phenylphenol, biphenyl, and biphenol. We now extract the culture medium first with ethyl acetate, evaporate the solvent, then redisolve the crystals in acetonitrile, which gives best results for GC analysis. The new extraction procedure revealed that our several different lots of DBT (Aldrich Chemical) contain DBT-sulfone and biphenyl, at about 0.5% and 0.4% respectively, although different lots contain different amounts of the contaminants.

We applied the new assay procedure to several tests for "4S" activity of pure cultures and mixed cultures (of unknown composition) obtained from environmental sources. Some cultures degraded DBT and DBT-sulfone when these were added to the cultures in pure form. We have not yet obtained direct evidence of 4S pathway metabolism. The assay procedure and results of the first trials are described on the following pages.

EXTRACTION OF LIQUID CULTURES

I. Procedure

a. 1.0 ml of filter sterilized DBT (12.5 mg/ml acetonitrile) was added to a sterile flask. This was dried to a residue.

b. 25 ml of medium were added to each flask (final DBT conc = 0.05%).

c. The flasks received a 10% inoculum and were then incubated at 30°C, 150 rpm for 48 h.

d. After incubation, 12.5 ml ethyl acetate were added to each flask. The flask was tightly capped and mixed by inversion ~50 times.

e. When the layers separated, the organic layer was saved.

f. Steps d and e were repeated. The two organic layers were combined (final volume : 25 ml) and allowed to dry to a residue.

g. The residue was resuspended in 1.0 ml acetonitrile.

h. An internal standard (0.5 ml of 0.1% thianthrene w/v acetonitrile) was added.

i. The samples were analyzed via GC : HP-5 column with helium as the carrier gas (15 ml/min), FID detector at 280°C, oven temperature starting at 100°C, final temperature 225°C, 10°C increase/min.

II. Assays for 4S metabolism

Cultures: Lehigh strains, C18 and C1, UM strain Cit2B. These were chosen because C18 routinely produces fluorescence and orange compounds when sprayed with DBT, C1 originally produced fluorescence but no orange compounds, and Cit2B fluctuates in its ability to produce fluorescence. Mixed inocula: Tinsley (crude oil sample), CIT (crude oil sample), and ECUTA (crude oil sample), TS (Alabama "tar sands" sample).

a. Organisms were incubated for 48 h in 21C medium (0.5% benzoate, pH 7.0) containing either 45 mM DBT or 38 mM DBTS (DBT sulfone), a final solution of 0.05% w/v. The cultures were extracted with ethyl acetate and analyzed via GC for products of the 4S pathway.

b. Raw samples from 4 sources were incubated in 25 ml of 21C medium (0.5% benzoate, pH 7.0) into which 12.5 mg of non-sterile DBT had been added. After the DBT had visibly disappeared from the Tinsley sample, the cultures were subcultured into similar media. When the DBT had again disappeared, 2.0 ml were removed from each for subculturing and the remainder extracted with ethyl acetate and analyzed via GC. Colonies were isolated from the subculture and tested for fluorescence via the Lehigh spray plate method and for DBT utilization via the TTC assay.

III. Results

a. When grown in liquid medium containing DBT, C18 produced an orange color; neither C1 nor Cit2B produced the orange color. No color was present with any of the cultures when DBTS was added to the medium. When grown with DBT, C18 showed a 32% decrease in DBT, while C1 and Cit2B showed no decrease. No change in other 4S compounds was detected. When grown with DBTS, C18 showed a 36% decrease and C1 an 18% decrease in the DBTS concentration but no changes in other 4S compounds were detected. It appears, therefore, that in liquid medium C18 will utilize the Kodama pathway to degrade DBT. Although no color change occurred, it is also possible that C18 and C1 utilized the Kodama pathway to degrade DBTS. Cit2B does not appear to utilize DBT or DBTS at all, and the flucuating fluorescence may be due to something other than the production of 4S products.

b. Of the samples tested, Tinsley most readily used the DBT supplied. The subcultures turned orange, and GC analysis indicated a 25% decrease in DBT conc. None of the isolates from the subcultures produced fluorescence when sprayed with DBT, or were positive for the TTC assay; however, this may be due to one of two things. Either the organisms responsible for the degradation of DBT were not isolated, or the organisms need to be in a mixed consortium to synergistically utilize DBT. Both of these possibilities are being examined further.

Work in progress and planned. Further tests are underway or planned to provide details of metabolism of "4S" compounds by selected pure cultures and crude inocula. Some cultures are being challenged with precleaned coal (from LTU) for later analysis of organic carbon content (at LTU). The ASTM sulfate assay is being reevaluated for effectiveness; the question is "can we expect to be able to measure sulfate ion release from coal or DBT at the concentrations of each that seem to be manageable in growing bacterial cultures?.

ASSAY FOR 4S ACTIVITY

One ml filter-sterilized 4S substrate (12.5 mg/ml acetonitrile)

↓

Dried to residue in 125 ml flask

↓

25 ml growth medium (4S substrate = 0.05% w/v)

↓

Inoculated from growing cultures
(plus uninoculated controls)

↓

Incubated at 30°C, 150 rpm shaker bath

↓

Two extractions with 12.5 ml ethyl acetate = 25 ml pooled

↓

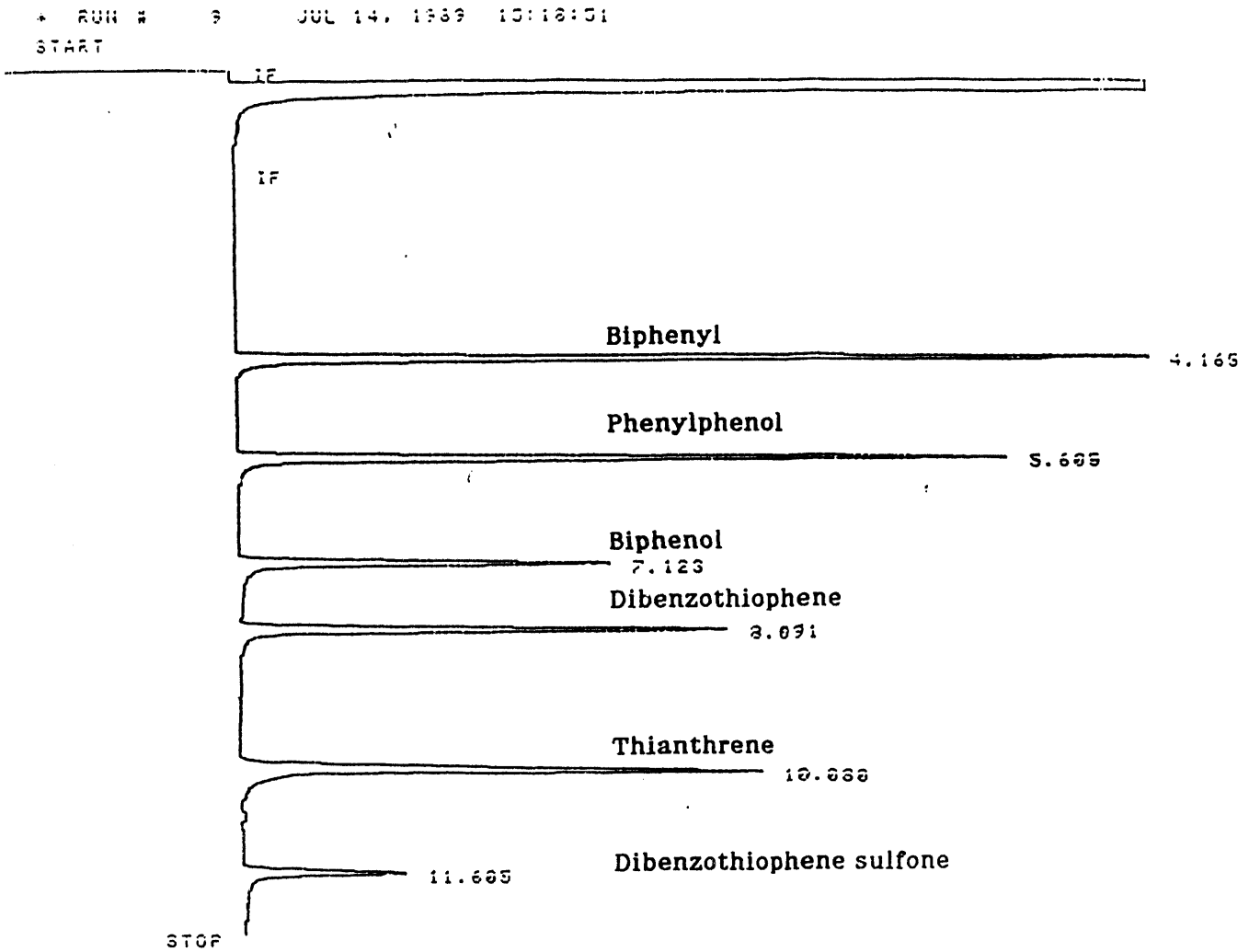
Dried to residue

↓

Resuspended in 1.0 ml acetonitrile
plus internal standard of 0.5ml of 0.1% thianthrene in acetonitrile

↓

GC analysis



Standards in acetonitrile (12.5mg/ml); HP 5890A GC, HP 3396A integrator, HP-5 column, He at 15 ml/min, Inlet at 250°C, FID at 280°C, 100°C initial, 10°C/min to 225°C.

APPENDIX II

SUMMARY OF DBT SPRAY-PLATE ASSAYS

LEHIGH CULTURES AND KODAMA PRODUCTS

GC/MASS SPECTROMETRY

SULFUR-STRESS ASSAY

Quarterly Progress Report, Biodesulfurization Project

Report Period: December 15, 1989 - March 15, 1990
(Submitted: March 12, 1990)

To: Dr. Gary Zumwalt, Interim Project Manager
Department of Petroleum Engineering and Geosciences
Louisiana Tech University
Ruston, LA 71272-0046
Telephone: 318-257-2885 Fax 318-257-2562

From: Bailey Ward, Ph,D.: Principal Investigator
Joe E. Lepo, Ph.D.: Research Associate
Frank Purdy, Graduate Research Assistant

The University of Mississippi
Laboratory of Environmental Microbiology
Department of Biology
University, MS 38677
Telephone: 601-232-7203 Fax 601-232-7577

TASK-4 MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

A. History of Screening Assays

During the past year, we have recovered from natural sources (crude oils and refined fuels mixed with soil or water) several individual or mixed cultures that gave one or more indications of the proposed "4S" pathway. About one hundred samples taken from a variety of locations were screened. The 4S indications were obtained by UV fluorescence via the Lehigh-type, spray-plate assay, or by capillary gas chromatography of extracts of growing cultures plus DBT or DBT-sulfone. Because of uncertainties about interference by colored "Kodama"-type products of DBT degradation, and by inability to obtain repeatable results, the spray-plate assays were suspended sometime about midsummer, 1989. The report by Monticello et al. (Appl. Environ. Microbiol. Vol. 49 (4), p. 756, 1985) states that a yellow product of DBT degradation has a molecular ion of 178 and fluoresces blue under UV illumination. Another major product, orange in color, fluoresced purple under UV. These are primarily water-soluble compounds. We suspected that organisms that produced Kodama-type products would show blue/purple UV fluorescence due to the colored products, thus severely limiting the value of the assay for screening for 4S products (phenylphenol or biphenol) which also fluoresce blue/purple under UV. Moreover, it would seem that the Kodama products would be more likely to diffuse into the agar medium around the colonies, thus amplifying their interference, inasmuch as the Lehigh assay relies on fluorescence around the colony perimeter. We note also that we did not observe fluorescence <u>on</u> a colony, even when fluorescence was strong around the colony, even when much colored product covered the colony. Some colonies were autofluorescent, and some produced fluorescence around the colonies when sprayed only with ether. Moreover, such factors as medium composition, pH, age of culture, DBT concentration, and some other unknown factors of laboratory personnel techniques seemed also to affect the spray-plate assay. However, we acknowledge that the assay should be useful for 4S organisms that do not produce interfering fluorescing compounds, and might also be applied to these latter organisms if one can differentiate between fluorescence of 4S and colored products.

In our initial spray-plate assays (early in 1989) of nine of Dr. Steve Krawiec's (Lehigh) isolates, we observed UV fluorescence of only one culture (F23) that did not produce conspicuous colored products. Our main interest then was to use a known "fluorescer" (presumed then to be 4S-positive) to serve as a reference as we screened for new isolates. We chose Lehigh strain C18 because it gave vivid and reliable DBT-

dependent fluorescence; we note, however, that C18 also produces considerable amounts of colored product from DBT. It was only later that we began to question the interference of the colored products (as related by B. Ward at the August 1989 workshop at the PETC Contractors' Review meeting).
We then focused on GC assays to screen for 4S-positive cultures. In brief, our assay consisted of incubating crude or purified cultures in shake-flask, liquid medium in the presence of DBT, followed by extraction in organic solvent (usually ethyl acetate), evaporation, then redissolving in acetonitrile for GC injection. A cocktail of pure compounds (biphenol, phenylphenol, biphenyl, DBT, and DBT-sulfone) along with an internal standard (phenanthrene) served to establish reference standards. At the time that we were testing the Lehigh isolates for UV fluorescence, we also attempted to detect 4S products using the GC assay both by extracting directly from the agar-based, UV-fluorescing colonies, and by extracting from liquid-culture media. We were not able to detect any evidence of 4S products by any Lehigh isolate. However, we suspect now that our techniques then were not properly tuned, and we believe that retrials will give different (better) results.

During the Autumn of 1989, we focused on refinement of techniques, development of reliable GC assays sensitive enough to detect small amounts of bioconversion of DBT to 4S products, initial trials on a low-to-no sulfur bioassay (with DBT as sole sulfur source), and on isolation of new cultures that exhibited 4S activity (via GC assay). We discovered that we were getting "ghost" peaks of 4S compounds due to condensation and bleeding off in the injection port, and that cultures that we thought were 4S-positive indeed gave no activity when retested under proper assay conditions. We subsequently obtained several individual or mixed cultures that produced weak to strong peaks that had retention times (RT) equal to or near those of DBT-sulfone, phenylphenol, and biphenyl. These cultures were derived from fresh crude oils from either the Tinsley (Mississippi), Ecuta (Mississippi), or Citronelle (Alabama) oil fields, or from weathered refined fuels (kerosene, oils) mixed with soil and coal particles (Smith Oil Company, Oxford, Mississippi).

B. Recent Screening Assays

During the present report period, we first focused on studies of two cultures, one a mix of two seemingly different organisms (the "Ecuta" culture), the other a Bacillus-type isolate (designated "SOC-3" from a Smith Oil sample).

The Ecuta culture gave indications of 4S activity in that three (one weak, one moderate, one strong) peaks were seen in the RT range between that of biphenyl and biphenol; another strong peak occurred identical with the RT for DBT-sulfone. The Ecuta and Tinsley cultures also produce orange-colored, water-soluble products that we take to represent Kodama products. Some color also extracted into the ethyl acetate solvent used to recover possible 4S compounds, and in the acetonitrile solution used for injection into the GC (these were especially obvious in the concentrated extract used for GC/MS analysis described in a following section). We presume therefore that Kodama products also exist in the solvent extract. We chose ethyl acetate (after tests on several solvents) because it gave good recovery of most "4S" compounds (polar to nonpolar) in a cocktail of standards extracted from cell suspensions and cell-free culture medium. For example, DBT, phenylphenol, biphenol, and DBT-sulfone were extracted in ethyl acetate from cell suspension at 79.9%, 74.5%, 82.5%, and 76.5% respectively. More recent tests indicate somewhat better extraction percentages (from cell suspensions) with dichloromethane (93.5%, 83.2%, 93.3%, and 79.3%, respectively for the same compounds. Biphenyl was extracted at 39.0% and 47.6% in ethyl acetate and dichloromethane, respectively. Except for DBT-sulfone (83.9%) efficiences in chloroform were less than those for ethlyl acetate.

The SOC-3 isolate is of interest because it readily removes DBT from culture suspension under either aerobic or anaerobic conditions. Moreover, SOC-3 does not produce colored Kodama-type products. However, we have not been able to detect 4S compounds using our standard GC analyses. Under anaerobic conditions, SOC-3 will readily metabolize either DBT or biphenyl, which is a possible product of DBT desulfurization in the absence of oxygen. However, no products were detected. We are continuing to investigate the possibility that SOC-3 takes DBT through biphenyl (without accumulation) with concomitant rejection of sulfate.

The aforementioned Ecuta extracts were analyzed by GC/MS, first during about September 1989 using an old instrument in the UM Chemistry Department. The resulting spectra indicated a sulfur-free product that we were calling DBT-sulfone. However, the mass spectra did not match our GC spectra as to retention times and number of peaks, so we were unable to determine that the sulfur-free product indeed was the one that we were calling DBT-sulfone. The other peaks near phenolphenol and biphenol appeared (by MS analysis) to contain sulfur. We prepared new samples of the Ecuta products, confirmed the presence of peaks for unknown products (including the "DBT-sulfone") via GC, and reanalyzed (during January 1990) using a new HP GC/MS system, equipped with a software-controlled identification package (with NBS library of spectra). Mass spectra were interpreted by faculty in the UM dept. of Chemistry. Figure 1 represents a gas chromatogram of the Ecuta extract along with a standard reference cocktail. Figure 2 consists of the mass spectrum of the same extract along with individual spectra for labeled peaks 1-3, and 6 (ion masses 150, 164, 178, and 200, respectively). We used the convention of comparing the peak (relative abundance) of the parent compound to that at plus two mass units, which if at 4% or greater of parent peak indicates the presence of sulfur atom (method prescribed by Dr. Norman Heimer, Professor of Chemistry, UM).

No identity now can be provided for the compounds of peaks 1 and 2 (< 30% match in the NBS library). The compound of peak 3 has an ion mass of 178, the molecular weight of 3-hydroxy-2-formylbenzothiophene, a colored Kodama product of DBT degradation, and Dr. Heimer acknowledged that the same compound could yield the observed mass spectrum (Fig. 2, peak 3 spectrum).

Analysis of the individual mass spectrum of the compound that had the same RT as pure DBT-sulfone (peak 6) revealed a possibility for the presence of two compounds, an abundant one with a molecular weight of 200, the same as that of DBT-5-oxide, and a small amount of another with a molecular weight of 216, that of DBT-sulfone. Although we have not yet obtained a mass spectrum for pure DBT-5-oxide or for DBT-sulfone (the GC/MS was not available for our use in time to include the data for this report), our spectrum for peak 6 matches that for DBT-5-oxide reported by van Afferden et al. (ACS, Div. Fuel Chem. Preprint Vol. 34(4): 561-572 [1988]) with respect to ion mass fragmentation. DBT-5-oxide is a product reported to be produced by "Kodama" bacteria. We do not know if the ion mass at 216 represents the major parent compound (DBT-sulfone ?). Professor Iain Campbell (personal communication) suggested the possibility for formation of small amounts of DBT-sulfone from DBT-5-oxide via GC, whereas the reverse would not be the case. We have obtained DBT-5-oxide from P.R. Dugan and colleagues at INEL. The pure DBT-5-oxide has a GC Rt equal to that of pure DBT-sulfone. During the next few weeks, we will gather more GC/MS data in attempts to resolve questions about Kodama vs 4S products.

We now believe that the GC peaks of the Ecuta culture extracts represent Kodama products or other derivatives of DBT with intact sulfur atom. It appears that a variety of sulfur-containing products of DBT are generated by microbial activity, and that some of these might give false-positive indications of 4S compounds when extracts are analyzed by GC. We must note that we have analyzed via GC/MS only the Ecuta culture; we plan to retest (via GC/MS and other methods) several isolates, including some of the Lehigh strains, to determine the link between possible 4S activity and Kodama activity. Our position for now is that routine GC analyses yield only presumptive evidence of 4S activity, just as does the UV fluorescence plate assay.

We (in earlier reports) described that which we believed to be production of DBT-sulfone from DBT by several of our own as well as some of the Lehigh isolates. We based our belief on the presence of a GC peak with the same RT as DBT-sulfone. We note that all such isolates produced colored products, and it is possible that for these other isolates it is DBT-5-oxide rather than DBT-sulfone that is detected via GC. Moreover, we know now that the weak indications of production of phenylphenol, biphenol, or biphenyl for some cultures (as reported earlier) probably was the result of ghost peaks of residual, condensed pure compounds used to generate reference chromatograms. We must retest all such cultures by use of GC/MS (or by TLC/GC as described in the following section) before we can make definitive statements as to 4S activity.

C. New Approaches

During the past few weeks, we have returned to studies of Lehigh isolates, as well as some of our own. Our aim is to reassess the spray-plate assay and to retest (via GC) the cultures for production of 4S products. We especially want to address the issue of Kodama vs. 4S products as these relate to spray-plate fluorescence and detection of 4S products via GC and GC/MS. We do not feel that routine screening and assays for 4S activity are warranted until we are confident that our assays will indeed detect and monitor true 4S activity. Of special interest is a renewed investigation using the Lehigh strains C18, A4/B24 (suspected to be the same strain), and F23. C18 has received much attention in other laboratories (Lehigh University and North Dakota, for example) where the spray-plate fluorescence activity is used as a putative 4S genetic marker for gene cloning.

Resting-cell Suspensions

We have reconsidered our practice of incubating developing cells in the presence of DBT. Although we have not seen evidence that DBT itself is inhibitory to growth (at least for the organisms tested), we are aware that Kodama products are inhibitory to some organisms (Monticello, et al., cited preceding). It is probable that those cultures (most of ours) that produce colored products are growth-inhibited. It also is possible that 4S products can be inhibitory, or that the same products can be metabolized by growing cultures. For generation, detection, and subsequent analysis of 4S products, it is desirable to maximize cell-to-substrate (DBT) ratio, especially in consideration of the observations (J. Kilbane, IGT) that desulfurization of DBT requires cell/substrate contact. We suggest also that addition of surfactants might enhance activity on hydrophobic compounds. Acidification of the culture suspension prior to extraction might also improve recovery of phenolic compounds. Our goal is to arrive at reaction conditions for maximal 4S activity.

We are turning to the use of resting-cell suspensions for screening assays. We will test effects of acidification on extraction efficiency of phenylphenol, biphenol, and effects of different concentrations of synthetic surfactants (or microbially produced, as these become available) on DBT bioconversions.

We have tested one method whereby we spray DBT (in ether) onto the bottom of a glass culture dish, evaporate, then overlay the uniformly dispersed DBT film with a harvested cell suspension. The cells were grown up in complete nutrient medium, harvested by centrifugation, resuspended in a sulfur-free basal medium, layered (about 10 ml in 100 mm diameter Petri plates) over the DBT film and incubated at 30° C for 24 h. Following acidification with two drops of 6N HCl, the suspensions were extracted with ethyl acetate which then was evaporated; the residue was redissolved in acetonitrile for GC analysis. We tested the Lehigh strains A4, D20, C18, and F23, as well as a few of our own isolates. The F23 strain was the only one in previous tests that did not produce conspicuous colored products, but which did fluoresce via the spray-plate assay. We also retested A4, C18, F23, and our SOC-3 for DBT-dependent fluorescence via the spray-plate assay. Controls consisted of Cells only and for C18, cells also sprayed with ether only (no fluorescence).

Results.

1. When extracts of the resting-cell preparations were analyzed by GC, only one strain yielded a peak with a 4S retention time, and that was strain C18 with a peak at the DBT-sulfone/DBT-5-oxide RT. Strain A4 produced several peaks but none matched a RT for a 4S product. C18, D20, and A4 produced colored products. Although the extracts were tinted with yellow/orange color, these apparently were too dilute to be detected. We do not now know the identity of the C18 peak at DBT-sulfone/5-oxide, but we will apply GC/MS and TLC analyses in an attempt at identification.

2. Only one strain, C18, produced UV fluorescence via the spray-plate assay. Within < 5 min after application of DBT, the C18 colony and its immediate perimeter fluoresced bright blue. At the same time, yellow/orange pigments appeared in the fluorescing zones. After about 12 hours, both color and fluorescence had disappeared. And after 12 hours, no other strain showed fluorescence or colored products. We note that we never have seen either F23 or SOC-3 produce colored products from DBT. However, A4 usually does produce colored DBT products, but in this spray-plate assay it did not, and it also

did not fluoresce. Each culture was resprayed with the result that C18 again produced fluorescence and colored products, whereas no other culture produced either.

We note that the GC analysis of C18 extracts indicated either DBT-sulfone or DBT-5-oxide, and that of the two, only DBT-sulfone fluoresces blue under 254 nm illumination. We ask... Is the C18 spray-plate fluorescence due only to the colored products, or only to DBT-sulfone, or to both, or to neither? If the fluorescence is due to phenylphenol or biphenol, then why haven't we been able to detect these 4S products via GC analysis? (refer to C18 tests using TLC described in the next section). We will continue work to answer these questions for C18 and for other isolates. We will attempt to extract the C18 products from fluorescing agar plates for analysis via GC or GC/MS. The described method will be used both for screening of crude cultures and individual isolates, and for quantitative assays when known amounts of organosulfur substrates are added.

TLC/GC

We also have been exploring another method for screening for 4S activity. During the past several weeks, we have experimented with thin layer chromatography (TLC) as a means by which we can differentiate between Kodama (and other) products and 4S compounds, and to recover the latter in relatively "clean" form for GC or GC/MS analysis. Tests with a variety of solvent mixtures and solid phases yielded preparations with which we can separate and visualize a variety of compounds of interest to our assays, including both colored and 4S products.

We tested the Ecuta extract (same preparation used for the aforementioned GC/MS data) using TLC, and observed that the colored products stayed with or near the solvent front, and fluoresced blue; we saw no evidence of DBT-sulfone, phenylphenol, or biphenol. We were unable to distinguish between DBT and DBT-5-oxide, which in pure forms had Rf values of 0.70 and 0.72, respectively. With a diffferent solvent system, the colored compounds stayed behind the front and fluoresced blue.

We also tested via TLC the C18 extract (described above under Resting Cells, Results) that via GC showed a peak at the RT for DBT-sulfone/DBt-5-oxide, and also produced colored products. The colored products separated, with conspicuous blue fluorescence. We could not detect any 4S compound other than the DBT substrate. We will continue to explore use of TLC to separate the compounds of interest and to provide a means by which the individual substrates and products can be recovered independently for GC or GC/MS analysis.

Bioassays: Growth on DBT or DBT-sulfone as sole source of sulfur

Rejection of sulfur in the form of sulfate (or sulfite) accompanies the desulfurization of DBT via the proposed 4S pathway. Microbial cultures incorporate sulfur (usually via sulfate) into cellular biomass as they grow. A complete nutrient culture medium provides an excess of sulfur (commonly as magnesium sulfate). If 4S activity occurs independently of the availability of free sulfur for growth, then the 4S-generated sulfate would enter the general pool of medium sulfate. Under conditions where sulfate would not be growth-limiting, it might be possible to measure the increased sulfate concentration in the growth medium relative to control cultures minus DBT, presuming of course that sufficient DBT desulfurization occurred. Under sulfur-limiting growth conditions, 4S-generated sulfur probably would be consumed as produced, and it is unlikely that sulfate release could be detected by analysis of the culture medium. However, one would expect to be able to measure increase in cellular biomass, relative to DBT-free controls, as a consequence of DBT desulfurization under sulfur-limiting growth conditions. Moreover, in a sulfur-limiting environment, selection of DBT-desulfurizing strains might be favored. Sulfur is required in relatively small quantities by most microorganisms, therefore growth on organosulfur compounds as sole source of sulfur probably will result in only small amounts of organosulfur desulfurization, with attendant production of desulfurized product that must be detected and quantified to substantiate 4S activity. We are testing media formulations and experimental procedures for using microbial growth in low-to-no sulfur media for both screening and confirmation of desulfurization of model organosulfur compounds. Earlier and preliminary trials indicated that several complications must be resolved before the assay can be applied reliably.

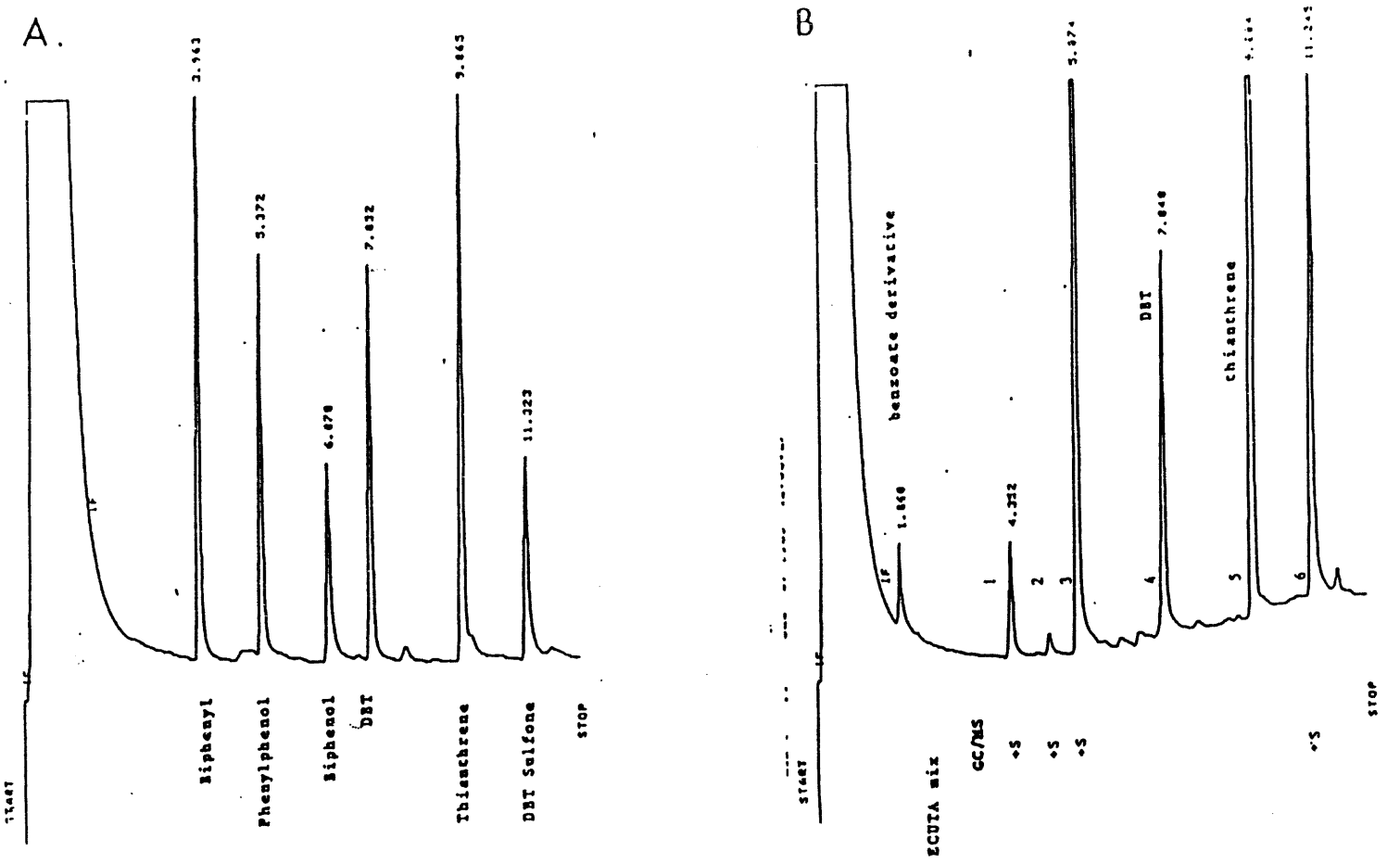


Figure 1 - Gas chromatograms of (A) reference cocktail of pure standards and (B) ethyl acetate extract of the Ecuta culture grown with DBT. Peak numbers correspond to numbers of GC/MS peaks in Figure 2.

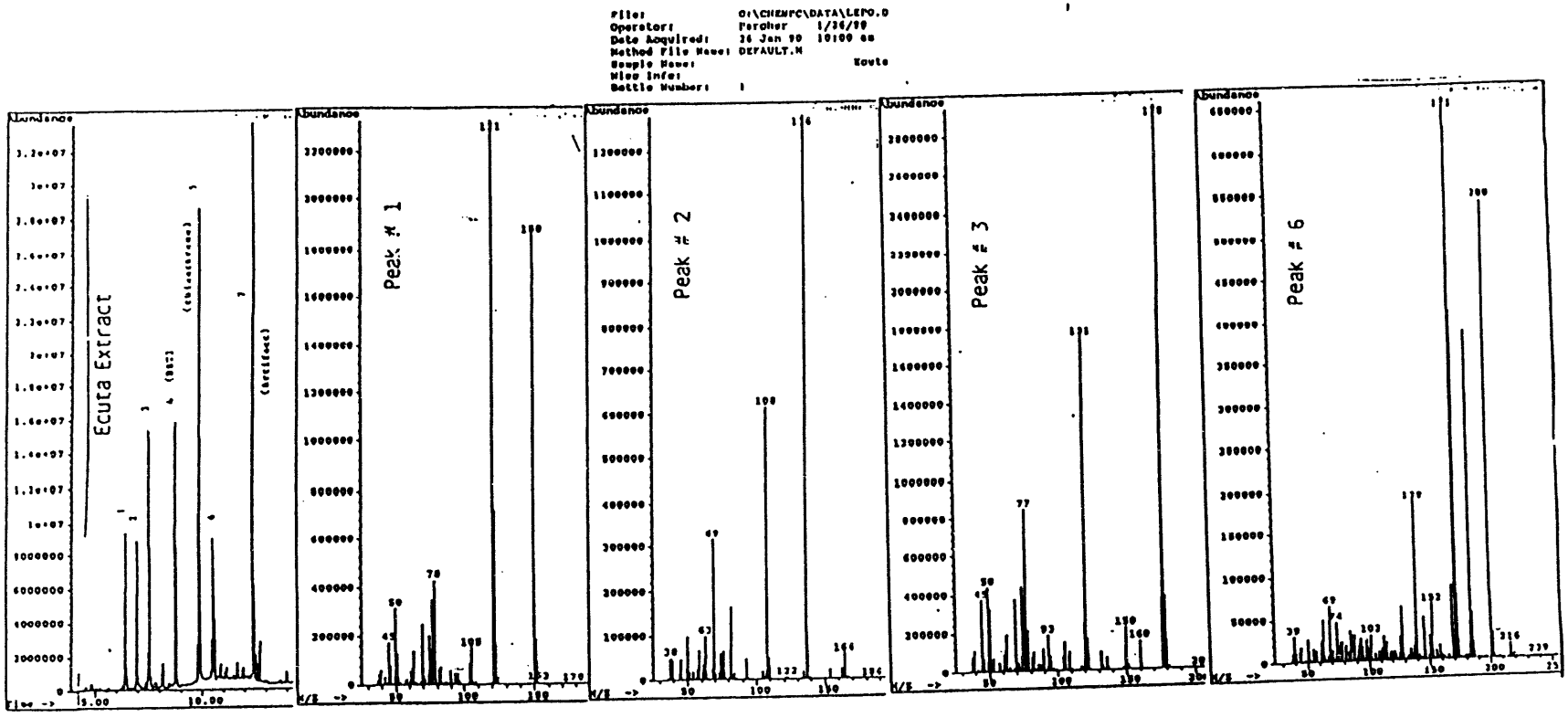


Figure 2 - GC/ mass spectra corresponding to Ecuta extract peaks for Fig. 1.

APPENDIX III

SOLVENT EXTRACTION EFFICIENCY

THIN-LAYER CHROMATOGRAPHY

GC/MS ANALYSIS

Monthly Progress Report, Biodesulfurization Project

(Submitted: 12 April 1990)

To: Dr. Gary Zumwalt, Interim Project Manager
Department of Petroleum Engineering and Geosciences
Louisiana Tech University
Ruston, LA 71272-0046
Telephone: (318) 257-2885 Fax: (318) 257-2562

From: Bailey Ward, Ph.D.: Principal Investigator
Joe E. Lepo, Ph.D.: Research Associate
Frank Purdy, Graduate Research Assistant

The University of Mississippi
Laboratory of Environmental Microbiology
Department of Biology
University, MS 38677
Telephone: (601) 232-7203 Fax: (601) 232-7577

TASK 4-MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

A. Screening Assays

We are continuing to refine and improve the reliability of the resting cell assay in which diethyl-ether solutions of dibenzothiophene (DBT) or DBT sulfone are sprayed onto Petri plates; the plates with a thin film of crystalline compound are then overlaid with suspensions of the resting cells and incubated for 24 hours. Ethyl acetate extracts (acidified by the addition of 10 drops 6N HCl) of these suspensions are being checked by GC for degradation products with particular attention being paid toward identification of putative "4S" compounds.

Tabulated below are percents of DBT sulfone utilized by resting cell suspensions various bacterial strains in our culture collection.

Strain	% DBTS Removed
SL1	21
SL2	39
SL3	57*
SL4	43
C18	50*
F23	46*
B24	61*
SOC3	62*
Ecuta	23
Tinsley	56*

* These values differed significantly from the amount of DBT sulfone extracted from control plates (i.e., plates that had not been incubated with cell suspensions) at P >= 0.05 according to ANOVA.

To determine whether the extraction efficiency of DBT or metabolic breakdown products might be improved by the disruption of cells, we subjected cell suspensions that had been incubated 24 hours on DBT sulfone plates to nitrogen-decompression in a "Parr Cell Disruption Bomb".

Then effect of Parr Bomb treatment on the extraction efficiency of DBT sulfone was determined. Cell suspensions (approximately 5 ml) were held at 2000 psi of nitrogen in the Parr Bomb for 10 minutes after which the suspensions were brought to atmospheric pressure.

C18 control	C18 Normal Extr.	C18 Parr Extr.
2.73 mg	1.04 mg	0.97 mg
1.72 mg	1.17 mg	1.25 mg
2.06 mg	1.35 mg	2.05 mg

Means were 2.09 mg for the control, 1.18 mg for the normal extraction procedure and 1.12 mg for the Parr bombed cells; average utilization of DBTS by the cells of the two treatments was calculated at 43 and 46 percent, respectively.

Microscopic examination of Parr bombed suspensions showed that over 90% of the cells had lost their rod morphology. However, we found no significant difference in the extraction efficiency nor the DBTS utilization between Parr-bombed suspensions and those that were not.

Thus we conclude that disruption of the cells is a superfluous addition to the procedure.

B. Thin-Layer Chromatography

The unambiguous and facile identification of intermediates in desulfurization or degradation of DBT is crucial to an understanding of microbial action on DBT. We have achieved the separation of several of these critical compounds using thin-layer chromatography (TLC) on silica gel plates.

DBT, DBT 5 oxide, and DBT sulfone were separable using a solvent system composed of hexane: n-butanol: glacial acetic acid: water in ratio of 20:20:5:1 by volume. Biphenol, 2-phenylphenol, and biphenyl were separable using chloroform: acetic acid in ratio of 95:5.

Compounds of interest:

List of compounds (1 mg/ml in acetonitrile)

1. DBT (dibenzothiaphene)
2. DBT5Ox (DBT 5 oxide) 10 ul/spot
3. DBTS (DBT sulfone)
4. BPOH (biphenol)
5. 2PP (2-phenylphenol)
6. BP (biphenyl)

Most of the compounds could be readily distinguished from the others by a combination of Rf values and fluorescence qualities. Silica gel plates were activated at 160°C for 1 hour or more. Chromatograms were developed for 1 hour, allowed to air dry and the compounds were visualized under short wave UV (254 nm).

Below are the results of a typical run using the hexane: butanol: acetic acid: water solvent system.

Compound	Rf	Appearance
1	0.94	gray shadow
2	0.82	dark *
3	0.92	bright blue fluorescence
4	0.99	dark, focused spot
5	0.99	dark
6	0.99	barely visible

* The DBT 5 oxide (compound 2) showed two other spots at Rfs of 0.92 and 0.96. The appearance and Rf values of these secondary spots, and our GC/MS analysis of this preparation leads us to conclude that it contains DBT and DBT sulfone as contaminants.

Below are the results of a typical run using the chloroform: acetic acid solvent system.

Compound	Rf	Appearance
1	0.70	bright blue fluorescence
2	0.70	bright blue fluorescence
3	0.70	bright blue fluorescence
4	0.85	dark, slight ghost spots
5	0.90	dark, gray
6	0.99	dark, diffused

Note: Compounds 1, 2, 3 are behaving as identical in appearance and Rf; these have been run repeatedly from several sources with this solvent with the same result.

C. Gas chromatography/mass spectrometry

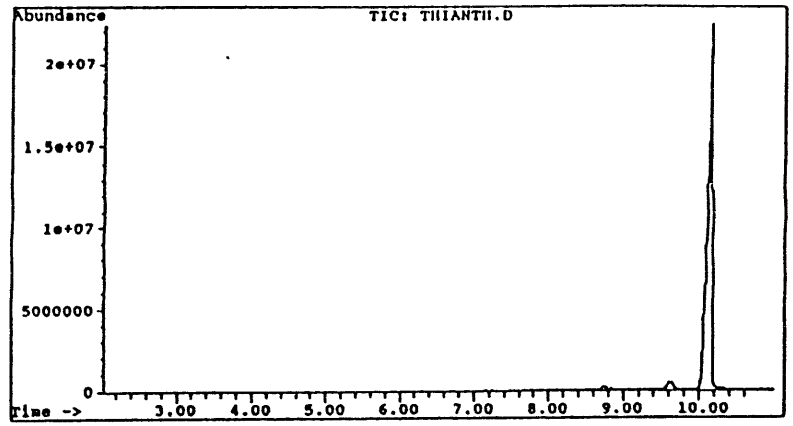
"Positive" identifications of most of the critical compounds have been achieved using GS/MS analysis; the Hewlett-Packard software that includes the "NBS49K" library yielded identities between 90 and 99 percent match for DBT, thianthrene, biphenol, phenylphenol, and biphenyl. DBT sulfone and DBT 5 oxide continue to present problems for us: They have essentially identical retention times during chromatography, they may degrade to DBT prior to MS analysis, and the DBT 5 oxide standard probably has contaminants of DBT and DBT sulfone. Because DBT, DBT sulfone, and DBT 5 oxide can be separated by TLC methods it seems likely that we can clean up our DBT 5 oxide standard. Its mass spectral analysis can then be included into a custom library that we are building for our standard compounds against which unknown peaks generated from microbial activity on model compounds or depyritized coal can be compared.

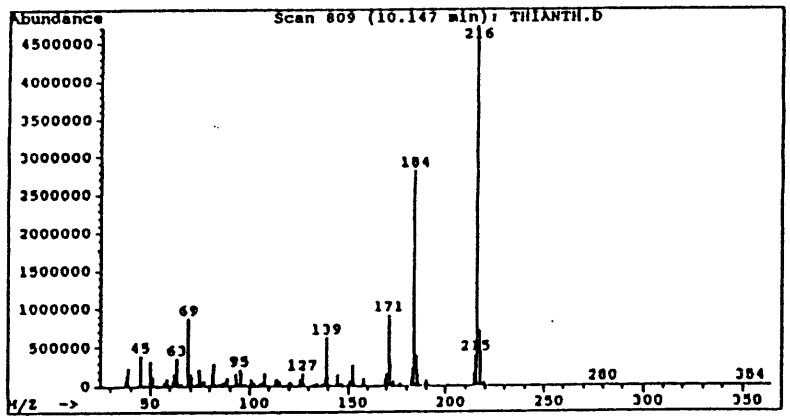
The appended chromatograms and spectra for thianthrene and biphenol are typical of these analyses.

D. All strains in the culture collection have been preserved at -80^{o}C in glycerol to ensure genetic stability and replicability of experiments in which they are used.

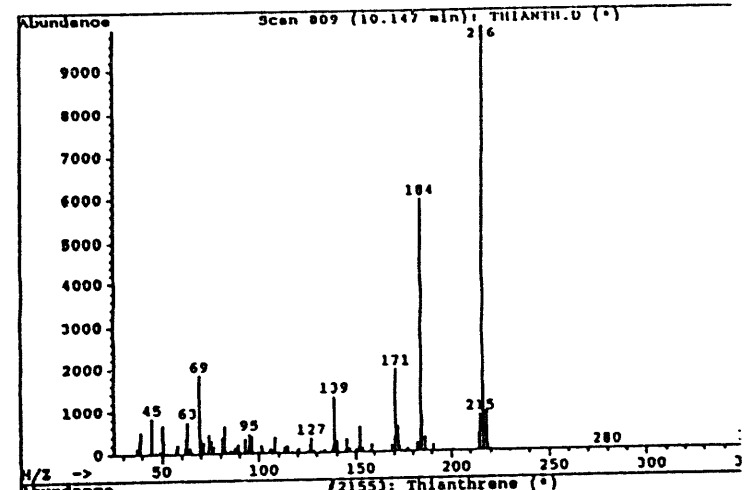
E. A highly-specific and sensitive enzymatic assay for sulfate is being developed.

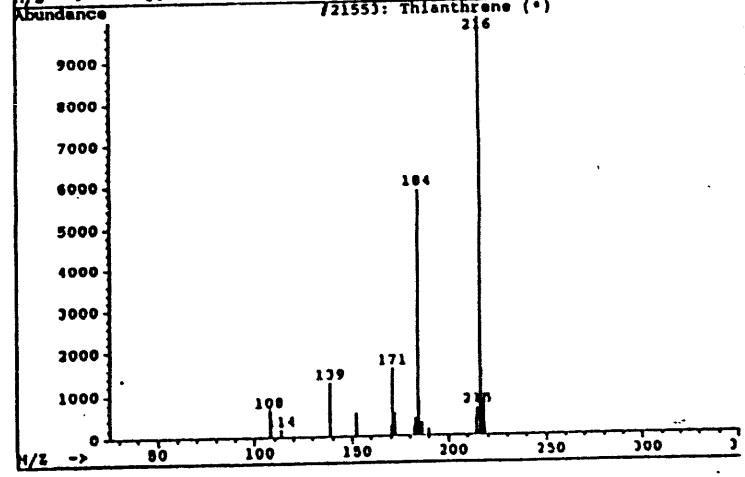
File: D:\MSDATA\THIANTH.D
Operator: LEPO
Date Acquired: 22 Mar 90 3:20 pm
Method File Name: DEFAULT.M
Sample Name: THIANTHRENE STANDARD
Misc Info:
Bottle Number: 1



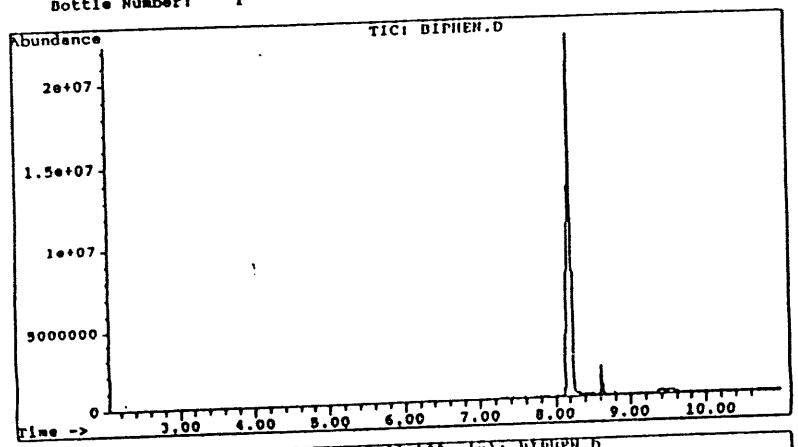


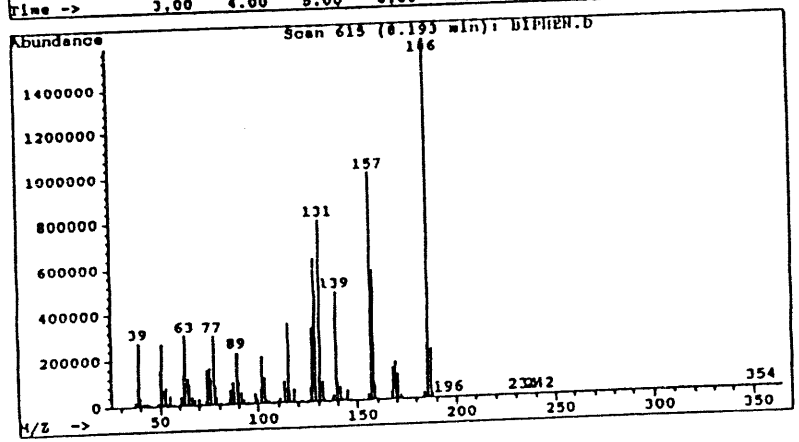
Library Searched : C:\DATABASE\NBS49K.L
Quality : 97
ID : Thianthrene



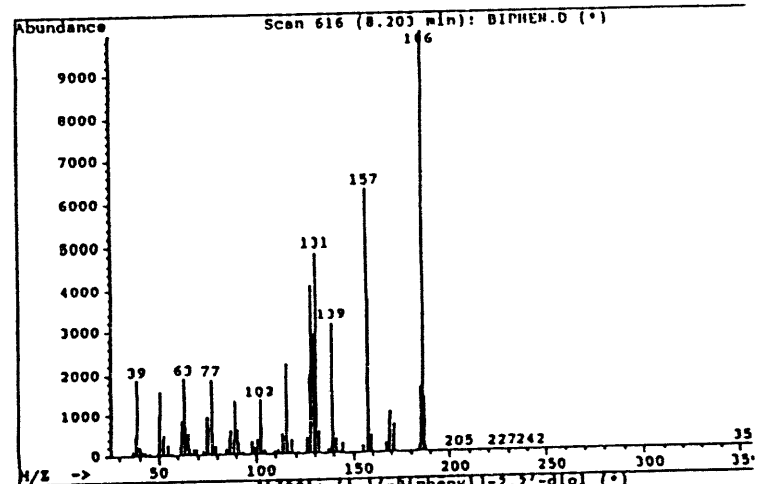


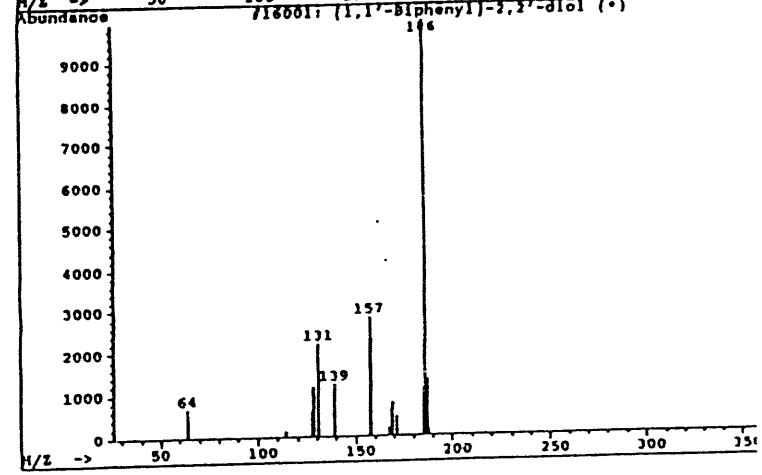
File: D:\MSDATA\BIPHEN.D
Operator: LEPO
Date Acquired: 22 Mar 90 10:01 am
Method File Name: DEFAULT.M
Sample Name: DIPHENOL STANDARD
Misc Info:
Bottle Number: 1





Library Searched : C:\DATABASE\NBS49K.L
Quality : 91
ID : [1,1'-Biphenyl]-2,2'-diol





APPENDIX IV

BIOACTIVITY ON DBT-SULFONE

SPRAY-PLATE ASSAY

SHAKE-FLASK ASSAYS

CELL-SLURRY ASSAYS

ASTM SULFATE ASSAYS

Quarterly Progress Report, Biodesulfurization Project

Report Period: 15 March -- 15 June 1990
(Submitted 15 ~~March~~ June 1990)

To: Dr. Bill Elmore, Project Manager
Department of Chemical Engineering
P. O. Box 10348 TS
Louisiana Tech University
Ruston, LA 71272
Telephone: (318) 257-2885 Fax: (318) 257-2562

From: Bailey Ward, Ph.D.: Principal Investigator
Joe Eugene Lepo, Ph.D.: Research Associate
Frank Purdy, Graduate Research Assistant

The University of Mississippi
Laboratory of Environmental Microbiology
Department of Biology
University, MS 38677
Telephone: (601) 232-7203 Fax: (601) 232-5144

TASK 4-MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

INTRODUCTION

Microorganisms offer a potential for the selective removal of organic sulfur from coals. Ideally, the sulfur atoms should be removed from the coal complex with minimal loss of coal organic carbon. Whole cells or cell-free enzymes may be employed to selectively catalyze the cleavage of carbon-sulfur bonds, with the attendant release of sulfate ions.

In this work, dibenzothiophene (DBT) is employed as a model compound with structural similarity to organic sulfides believed to exist in coals. A desulfurization sequence referred to as the "4S" pathway has been proposed (1) in which the sulfide is oxidized to DBT-sulfoxide, DBT-sulfone, and ultimately to the sulfur-free parent compound plus free sulfate ion. Figure 1 illustrates an abbreviated version of the proposed reaction sequence.

Our objectives for the work reported here are to isolate from natural environments bacteria that exhibit metabolic activity on either DBT or DBT-sulfone, with evidence of desulfurization activity reflected as production of sulfur-free products (e.g., the 4S products, 2,2′-biphenol or 2-phenylphenol) and possibly free sulfate ion.

EXPERIMENTAL

Cultures

All of the bacterial cultures tested for the present report were isolated from fresh or weathered crude oils, refined petroleum spills, or activated sludge. Strains A4, B24, C18, and F23 were isolated by Professor Steven Krawiec, Lehigh University, and were obtained with his permission from Dr. Pat Dugan's group at the Idaho National Engineering Laboratory. The Lehigh strains are *Pseudomonas* spp. that reportedly produce biphenol and DBT-sulfone from DBT (1). The other cultures were isolated by our group at Mississippi. SOC-3 is a *Bacillus* sp. isolated from a weathered oil spill at the Smith Oil Co., Oxford, Miss. Strains designated SL1, SL2, SL3 and SL4 were isolated from activated sludge from

the University of Mississippi waste water treatment facility; SL1 is a *Bacillus* sp.; the remaining "SL" strains are most likely pseudomonads. The cultures designated ECUT and TIN were consortia of two or three unidentified bacteria that were obtained from the Mississippi oil fields, Ecuta and Tinsley, respectively.

Screening\Detection Assays

We used several methods to screen for possible DBT or DBT-sulfone desulfurization activity via 4S reactions:

1) Spray-plate assay (developed by Professor Steven Krawiec, Lehigh University). Discrete colonies on the defined agar medium "21C" (2) were sprayed lightly with DBT in ethyl ether (0.1% w/v), which immediately evaporated leaving a fine coating of crystalline DBT. The proposed 4S products biphenol or phenylphenol fluoresce blue or purple (pure compounds) respectively under shortwave (254 nm) ultraviolet radiation. The proposed intermediate, DBT-sulfone, also fluoresces bright blue, whereas DBT does not fluoresce; thus the assay is potentially useful only when DBT is the initial substrate. Incubated cultures were monitored for presumptive 4S activity by appearance of fluorescing products in the vicinity of the sprayed colonies. Presumptively positive cultures were extracted directly for chromatographic analysis or retested by one or more of the methods described below.

2) Shake-flask Cultures. DBT or DBT-sulfone (12.5 mg) in acetone was evaporated from culture flasks, after which 25 ml of 21C medium (+ 0.5 mg/ml benzoate) was inoculated with pure or mixed cultures or with crude inocula from collection sites. After incubation for up to two weeks, three replicate suspensions were acidified with HCl and extracted with ethyl acetate. The evaporated extract was redissolved in acetonitrile for analysis by thin-layer chromatography (TLC), gas chromatography (GC), or GC in combination with mass spectrometry (GC/MS) to quantify substrate removal and to detect possible 4S compounds. Controls consisted of medium plus substrate only and cell cultures minus substrate.

3) Cell Slurry Assays. The objective was to use a short-term assay whereby cell/substrate ratios were maximized. Cell suspensions were grown to density in one-tenth strength Bacto tryptic soy broth, harvested by centrifugation, resuspended in a sulfur-free defined mineral medium containing 0.5 mg/ml sodium benzoate at pH 7.0; the O.D. at 660 nm was adjusted to 0.45 - 0.55. Ethereal solutions of DBT or DBT-sulfone were sprayed onto 100 mm diameter culture dishes, then evaporated to leave a finely dis-

persed crystalline coating of about 2.0 mg DBT. Ten milliliters of cell suspension then was layered over the substrate. After incubation (24 h, 30°C), the preparations were acidified with HCl, then extracted in two volumes ethyl acetate (pooled) and treated as described earlier for shake-flask cultures. Three replicates of each treatment were compared with medium plus substrate controls. Fragmented cell suspensions were prepared with a Parr cell disruption bomb at 2000 psi nitrogen for ca. 10 min.

4) Cell-free extract experiments. We have been preparing cell-free extracts of several cultures grown on tryptic soy broth and on defined media with the intention of allowing such extracts to act on DBT and DBT-sulfone. This approach has the potential of providing direct exposure of the substrates to high concentrations of intracellular enzymes (30 - 70 mg protein/ml will be used), and diminishes our concerns about transport and contact--factors that may limit the efficiency of processing. We will examine reaction products for putative "4S" intermediates by our standard procedures (TLC, GC, GC/MS).

5.) Bioassays for sulfur utilization. A low-sulfur, defined broth medium (21C) was constituted with glycerol as the sole carbon source and supplemented with saturating solutions of DBTS (166 micromolar). Inoculants can consist of washed cell suspensions of pure cultures or of consortia of cells. Serial transfer of bacteria to fresh tubes of this medium establishes a sulfur limitation of growth; control tubes with added $MgSO_4$ will support growth indefinitely. Growth can be monitored by optical density at 660 nm or visually.

Monitoring of Desulfurization Activity

1) Production of biphenol or phenylphenol (or both). Desulfurization of DBT or DBT-sulfone via 4S reactions is evidenced by release of sulfur free metabolites of the parent compounds. We used TLC, capillary GC or GC/MS to monitor bioconversions of DBT and DBT-sulfone. These methods are detailed and typical analytical results are presented in the preceding two monthly progress reports (April, May 1990).

2) Sulfate Assays. The ASTM D516 turbidimetric method was used for sulfate assays of some cultures that exhibited superior biodegradation of DBT in shake-flask cultures. The ASTM method has limitations in sensitivity and is subject to confounding interferences in biological materials and culture media. A highly-specific and sensitive enzymatic assay for sulfate is being

developed; the theoretical basis and status of this assay is discussed in detail in the Monthly Report for May. We are also developing an ion chromatographic method for sulfate analysis that shows considerable promise in being sensitive, specific, and free from interferences.

RESULTS AND DISCUSSION

Spray-plate Assays. We observed considerable variability in results obtained with the spray-plate, UV fluorescence assay. No tested culture consistently produced DBT-dependent fluorescence during repeated trials under the same conditions. Cultures that produced fluorescing products also produced yellow or orange products coexisting with the fluorescing zones around colony perimeters. A pathway of DBT degradation leading to sulfur-retaining, mostly water-soluble colored products has been described in detail (4), and some of the products fluoresce blue under 254 nm irradiation. DBT-sulfoxide also is a product of the alternate pathway (1), but DBT-sulfoxide does not fluoresce.

In our hands, colonies of Lehigh Strain C18 demonstrated DBT-dependent fluorescence and simultaneous production of colored products; however, GC analysis of extracts these colonies did not reveal 4S products. More recently we have compared a line of C18 obtained from Dr. Kevin Young (University of North Dakota) with our laboratory C18 (obtained as described in "Cultures" above). Inocula for the spray plate assays were obtained in two manners: Each line of C18 was first grown for two days on tryptic soy agar plates; the same organisms were used directly from cryostorage vials (-80 ^{o}C). All treatments and lines of C18 produced zones of colored, diffusing material around the colonies that had been sprayed with DBT. Dichloromethane extractions of these zones were subjected to GC and GC/MS analysis. Peaks with retention times near that of biphenol were evident; GC/MS analyses were equivocal; further GC/MS work is in progress. TLC of these extracts also produced spots with Rf values corresponding to biphenyl and DBTS.

Shake-flask Cultures. After several days of incubation, orange to amber-colored products were obvious in cultures with DBT, but no colored products appeared in DBT-sulfone cultures. For some cultures, GC analysis of ethyl acetate extracts revealed prominent peaks at the retention time for DBT-sulfone and DBT-sulfoxide, with other peaks near the retention times for phenylphenol and biphenol. Figure 2 presents a representative analysis for

the Ecuta consortium of two bacteria. Further analysis via GC/MS revealed that all unknown products contained sulfur. Peak 6 appeared to be DBT-sulfoxide. TLC separations showed only DBT-sulfoxide; no 4S products were detected, and the only UV blue-fluorescing spots were the products that were orange to red-colored under white light. A compound of molecular ion 178, with an ion spectrum that could result from 3-hydroxy-2-formylbenzothiophene, a major colored product of DBT biodegradation (3,4,5,6) comprised peak 3.

Ethyl acetate extracts of Strain C18 grown with DBT showed only a prominent GC peak at the retention time for DBT-sulfone/sulfoxide. Analysis by TLC and GC/MS indicated only the presence of DBT-sulfoxide. Water-soluble, colored products fluoresced blue under UV irradiation.

Only one culture tested, SOC-3 (a *Bacillus* sp.) removed DBT from suspension without producing colored compounds (and did not produce fluorescing products); no 4S products were detected.

It is apparent that the DBT-dependent production of sulfur-retaining, UV-fluorescing products by cultures being tested for 4S-mediated desulfurization of DBT can interfere with detection of possible 4S products. Routine gas chromatographic or UV-fluorescence indications of 4S products from DBT should be confirmed by more exacting methods such as mass spectrometry.

We were not able to detect (using the ASTM method) increase in sulfate in extracted shake-flask cultures grown in the presence of either DBT or DBT-sulfone. For these tests, the complete growth medium contained sulfate. In a sulfur-limited environment, metabolically active cells probably will scavenge any sulfur removed from organosulfur compounds, or from coal; thus, liberated sulfate would not be detectable, unless the rate of sulfur release exceeded that of sulfur uptake. In a sulfur-sufficient environment, sulfur liberated from model compounds or coals by co-metabolic reactions might accumulate to a detectable level relative to cultures growing in the absence of the organosulfur source. For potential applications to coal biodesulfurization, it would be desirable to use biological systems that do not require coal organic sulfur for growth or metabolic activity, or coal organic carbon for energy. A desired situation would be one whereby coal organic sulfur is liberated via co-metabolic reactions.

Cell Slurry Assays. The monthly report for April details refinements of our cell-slurry assay for the utilization of DBT and DBT sulfone. In addition, we established that disruption of the cell slurries by nitrogen decompression did not significantly enhance the extraction efficiency of DBT sulfone.

Preliminary tests revealed that bioconversions of DBT-sulfone did not generate colored products; thus this substrate was used for further investigations. Our rationale was that a culture capable of desulfurizing DBT via 4S reactions also would be capable of desulfurizing DBT-sulfone. In developing the assay, we compared extractions of whole vs. fragmented cells, and effects of benzoate and sulfate on removal of DBT-sulfone. No significant differences in DBT-sulfone removal occurred between sets of extracted whole and fragmented cells of strain C18. Moreover, repeated extractions with ethyl acetate did not recover additional substrate. We conclude that the microbial activity on DBT-sulfone represents biodegradation of the substrate. Table I illustrates that the presence of benzoate enhanced DBT-sulfone removal, whereas activity appeared to be insensitive to sulfate, under the assay conditions.

TABLE I

EFFECTS OF BENZOATE AND SULFATE ON CELL-SLURRY ASSAY[a]

Conditions:	- Benz + SO_4	- Benz - SO_4	+ Benz + SO_4	+ Benz - SO_4
% DBT-sulfone Removed:	33	34	47	50

[a] Benzoate at 0.5 mg/ml; SO_4 at 0.1% (w/v); values are means of three replicates, relative to controls.

A time-course assay of DBT-sulfone degradation vs. benzoate utilization in presented in Figure 3. These data may mean that DBT-sulfone degradation proceeded via cometabolism, with benzoate serving as the primary organic carbon source.

Figure 4 exhibits the results of cell-slurry assays on a variety of cultures using the sulfate-free defined medium plus benzoate. Strains are described in "Cultures" under the Experimental por-

tion of this paper. Although all cultures degraded DBT-sulfone to some extent, in no case did we detect products of any kind.

REFERENCES

1. Krawiec, S. Bacterial desulfurization of thiophenes. Developments in Industrial Microbiology. In Press, 1990.

2. Guirard, B. V., and E. E. Snell. Biochemical factors in growth. In Manual of Methods for General Bacteriology (G. Gerhardt, ed.), American Society for Microbiology, 1981, Washington, D.C.

3. Kilbane, J. J. 1989. Desulfurization of coal: the microbial solution. TIBTECH, **Vol.** 7 (Apr. 1989), 97-101.

4. Kodama, K., K. Umehara, K Shimizu, S. Nakatani, Y. Minoda, and K. Yamada. 1973. Identification of microbial products from dibenzothiophene and its proposed oxidation pathway. Agr. Biol. Chem. **37**:45-50.

5. Monticello, D. J., D. Bakker, and W. R. Finnerty. 1985. Plasmid-mediated degradation of dibenzothiophene by *Pseudomonas* species. Appl. Environ. Microbiol. **49**:756-760.

6. Laborde, A. L., and D. T. Gibson. 1977. Metabolism of dibenzothiophene by a *Beijerinkia* species. Appl. Environ. Microbiol. **34**:783-790.

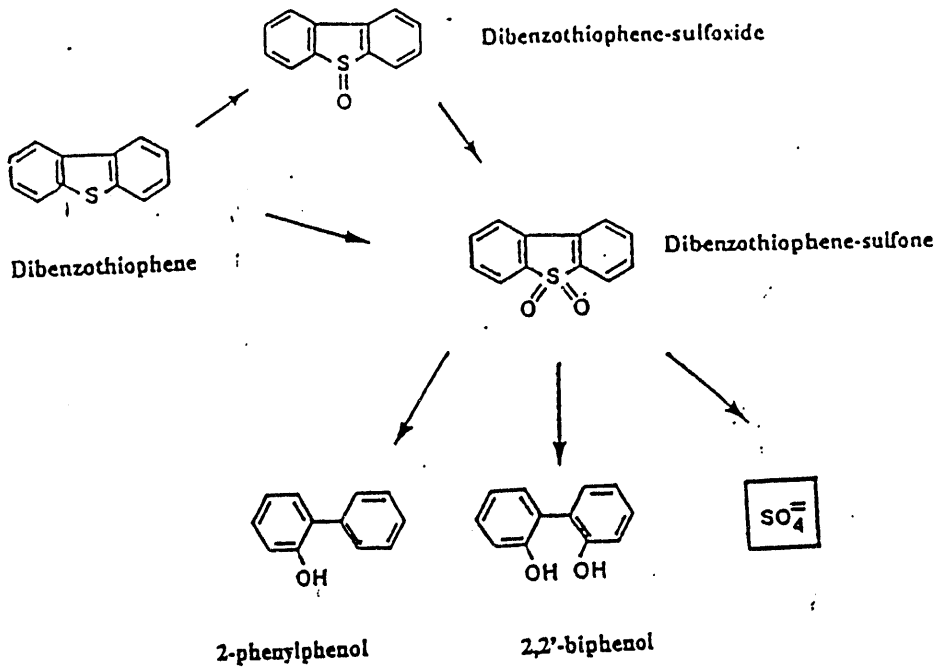


Figure 1 - Representation of proposed "4S" reactions leading to desulfurization of dibenzothiophene.

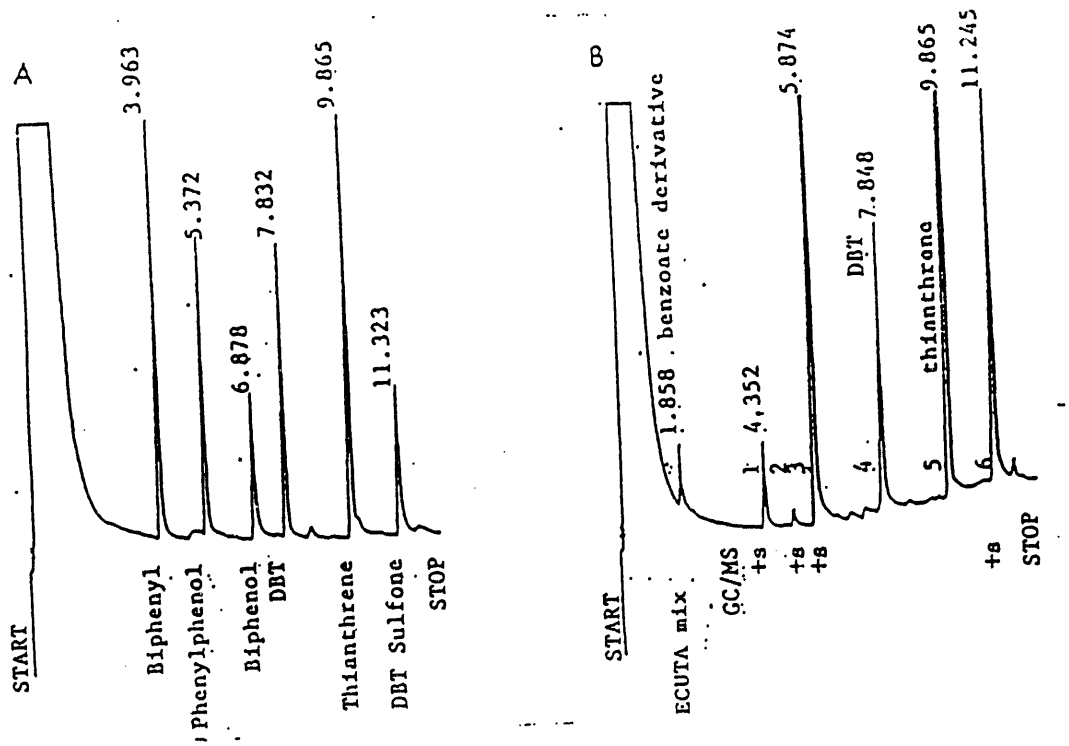


Figure 2 - Gas chromatographic analysis of ethyl acetate extract of the ECUTA culture grown in shake flasks with DBT (12.5 mg/25 ml) for two weeks. A. Cocktail of pure compounds. B. ECUTA extract (thianthrene = internal standard). All DBT-derived products contained sulfur (GC/MS analysis).

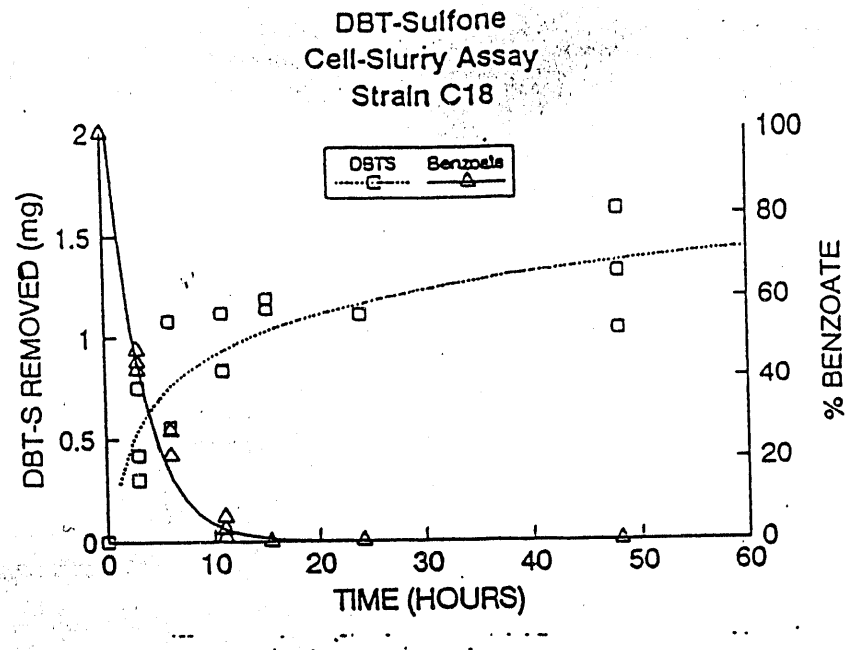


Figure 3 - Time-course, cell-slurry assay of DBT-sulfone degradation by Lehigh Strain C18. Replicate cell suspensions plus or minus DBT-S were incubated at 30°C for 24 hours, then extracted and analyzed by gas chromatography.

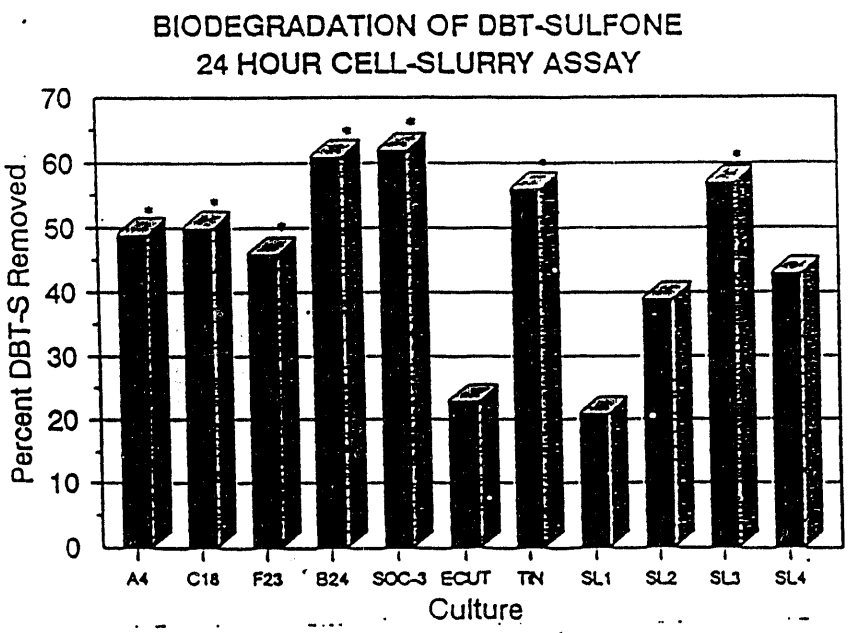


Figure 4 - Biodegradation of DBT-sulfone by various cultures; strain descriptions and sources are found above in the Experimental section, "Cultures". Conditions are as described in text for cell-slurry assay. Analysis by gas chromatography. Starred bars represent significance at 0.05% level (ANOVA, n = 3) relative to controls. No degradation products were detected.

APPENDIX V

THE UMX CONSORTIUM

THE SULFUR-STRESS ASSAY

ION CHROMATOGRAPHIC SULFATE ASSAY

TESTS ON DEPYRITIZED COAL

FOCUS ON UMX3 AND UMX9

CELL-FREE EXTRACTS

TASK 4--MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

Components of the UMX Consortia. The consortia derived from a filtrate sent us by John Kilbane have yielded a number of organisms with key characteristics listed below and in Table 1; only isolates capable of a positive bioassay are described (see monthly reports for July and August 1990 for a more complete description of origin of these isolates).

A positive bioassay depends on growth in a defined broth medium containing dibenzothiophene sulfone (DBTS) in saturated solution (0.166 mM) as the sole sulfur source; glycerol and glucose serve as carbon sources. Comparisons of growth of the organisms and consortia are made with the same medium containing 0.2 mM $MgSO_4$ and medium without added sulfur. Utilization of the organosulfur source was considered positive if the isolated organism or consortium demonstrated vigorous growth on DBTS but not in the no-sulfur medium for at least five consecutive transfers.

The UMXa consortium was obtained directly from Kilbane's filtrate as received. The UMXb consortium and its isolates were obtained after the filtrate was subcultured to tryptic soy broth.

A description of the isolated organisms follows:

UMX1: Typical *Pseudomonas fluorescens*; short gram negative rod, bright yellow fluorescent pigment; profuse growth on S1; grows poorly if at all with DBT or DBTS as sole source of sulfur.

UMX2: A fluorescent pseudomonad, but elaborating a brown (melanin-colored) pigment in addition to the siderophore pigment. It is also unable to grow with all the characteristics of UMX2

UMX3: A large gram variable coccoid bacterium that shows quite a bit of gram and morphological variability. Produces slightly pink mucoid colonies on TSA. This organism appears in both of the consortia.

UMX4: Off white to brown colonies on TSA, non-pseudomonad, short, gram-negative rod (almost a coccus). This organism is clean and does not grow at all on DBT or DBTS as sole sulfur source.

UMX5: Large gram positive rod; looks like *Bacillus* sp. but spores have never been observed. Seems to be carrying UMX3 with it and thus far we've not separated it cleanly from that organism.

UMX6: Large gram variable fusiform rod; shows variegated staining with gram stain. This organism as well has not been cleanly separated from UMX3.

UMX9: Large, but short gram positive rod; pink or light orange, waxy colonies that appear grainy on agar surfaces and flocculate from homogeneous suspension in static broth culture.

TABLE 1. Characteristics for UMX Consortia

UMXa CONSORTIUM a

Strain	Morphology	Growth DBT/DBTS[1]	Possible ID
UMX3	gm var, coccus	good	*Rhodococcus* sp.
UMX9	gm +, short rod	good	??

UMXb CONSORTIUM b

Strain	Morphology	Growth DBT/DBTS	Possible ID
UMX1	gm-, short rod	poor	*Ps. fluorescens*
UMX2	gm-, short rod	poor	*Pseudomonas* sp.
UMX3	gm var, coccus good		*Rhodococcus* sp.
UMX9	gm +, short rod	good	??

[1]DBT=dibenzothiophene; DBTS=dibenzothiophene sulfone.

Sulfate Concentration During Cell Slurry Interaction with Dibenzothiophene Sulfone (DBTS). We monitored the concentration of sulfate in minimal medium in the presence and absence of DBTS for certain isolates and the UMX consortium, Cell slurries were made in minimal medium containing 0.2 mM Mg_2SO_4, and with glycerol and glucose as carbon sources; the slurry was layered over plates sprayed with DBTS and the sulfate remaining was determined by ion chromatography (see APPENDIX) after 24 hours incubation. The results are shown in Table 2.

TABLE 2. Sulfate Concentration After Cell Slurry Assay

	ppm Sulfate/Sulfite[1]	
Sample	+ DBTS	Control, w/o DBTS
MM + 0.2 mM Mg_2SO_4	20.2	20.2
E. coli WT[2]	11.7	9.8
C18	8.8	7.3
B24	14.6	13.2
SL3	7.6	not done
UMX1	11.1	9.8
UMX2	10.4	not done
UMX consortium[3]	not done	1.6

1. Sulfate was assayed by ion chromatography; the details of the method, standard curves and typical chromatograms are appended to this report.

2. *Escherichia coli* strain K12, wild type, which does not utilize any of the model organosulfur compounds as a source of sulfate.

3. The UMX consortium (described above) was carried for 3 transfers in the minimal broth medium with DBTS as the sole source of sulfur. This minimal medium has no detectable sulfate unless it is added.

In all cultures--including those that showed no activity on the organosulfur compounds (e.g., *Escherichia coli*)--about 50% of the sulfate was taken up from the medium; in all cases, the slurries exposed to DBTS had about 10% more sulfate remaining than the control slurries.

Thus--in the presence of 0.2 mM SO_4--we could find no release of appreciable amounts of sulfate from organic sources. Furthermore, sulfate released into media from organic sources seems negligible and should be interpreted with caution--unless the magnitude of the release is much greater than we observed.

On the other hand, when the same medium was used during a cell slurry assay, but without added sulfate ion, low levels (< 1 ppm) of organosulfate-derived sulfate could be reliably detected. The organisms/consortia (e.g. UMX) that release sulfate do so only when supplied with DBTS; control organisms (e.g., *E. coli*) do not release sulfate into the medium with or without DBTS.

Utilization of Dibenzothiophene Sulfone. We followed the disappearance of DBTS in broth minimal medium cultures of various isolates and consortia after they had sustained growth on DBTS as the sole source of sulfur for three or more transfers. Table 3, below, tabulates some of these results.

TABLE 3. Utilization of Dibenzothiophene Sulfone

CULTURE	[DBTS]	% REMOVED
medium	0.164 mM	0
E. coli (K-12)	0.164 mM	0
Rhodococcus (Huntsville)	0.164 mM	0
UMXa (7 da)	0.087	47.5
UMXa (30 da)	0.000	100.0
UMX3 (7 da)	0.156	6.0
UMX9 (3 da)	0.156	6.0
UMX9 (6 da)	0.104	37.3
VANX[1] (20 da)	0.138	16.8
HUNTX[1] (20 da)	0.152	8.5

[1]VANX is a consortium obtained from Vanderbilt via Dr. Bailey Ward, consisting of two bacteria, a gram positive micrococcus and a gram negative rod; HUNTX is a consortium obtained from the University of Alabama, Huntsville, consisting of or more than two uncharacterized bacteria.

None of the isolates, including UMX3, when grown on DBT or DBTS, released sulfate into the medium under these conditions; the UMXa consortium and UMX9 release phenylphenol derived from the model organosulfur compounds.

Coal culture of desulfurizing bacteria

In order to determine whether any of the consortia and isolates that grew on or released products from organosulfur compounds would reduce organosulfur content of coal we prepared the sulfate-free minimal medium (pH 7.0) with depyritized coal (10 g/L); 20 ppm sulfate was detectable.

One-liter quantities of this medium were inoculated with *Escherichia coli* wild type to deplete available sulfate, while leaving the organosulfur intact. Although there was initial bacterial growth in these cultures, the sulfate decreased to 11.5 ppm and then rebounded to 17.7 ppm. Thus, the *E. coli* preculture of the coal medium did not give the desired result.

An alternative method proved successful. Samples of depyritized coal (10 g each) were suspended in 100 ml 6N HCl and stirred for 1 hour; the coal could be removed from the acid by filtration onto Whatman no. 1 paper on a Buchner funnel; after three washes, 200 mL each with deionized water, the coal was resuspended in 80 ml of water and the pH adjusted to pH 7.0 and the volume brought to 100 ml.

Medium made from coal treated in this manner typically contained 1 to 2 ppm sulfate.

We have inoculated such media with several isolates and consortia that degrade the model compounds and will monitor these cultures for bacterial growth and release of sulfate.

Time course studies of UMXa consortium; UMX3 and UMX9

Tubes of minimal medium with glycerol as the carbon source and 0.166 mM DBTS as the sole sulfur source were inoculated with the UMXa consortium, or the isolates UMX3 or UMX9. The inocula had been precultured on the same medium. Growth was monitored by optical density; the disappearance of DBTS was monitored by UV absorption as previously described, and the appearance of phenylphenol was monitored by HPLC.

Figure 1 depicts the kinetics of the parameters when the inoculum is the UMXa consortium.

Figure 2 and Figure 3 show the growth of the isolate and the disappearance kinetics of DBTS for isolates UMX3 and UMX9, respectively. UMX3 did not produce detectable organic products from DBTS. UMX9 did produce phenylphenol, but we have not completed the analysis for the levels of this product.

AIIM

Association for Information and Image Management

1100 Wayne Avenue, Suite 1100
Silver Spring, Maryland 20910
301/587-8202

Centimeter

Inches

MANUFACTURED TO AIIM STANDARDS
BY APPLIED IMAGE, INC.

3 of 3

APPENDIX

Ion chromatographic assay for sulfate

We are now routinely utilizing an ion chromatographic method for sulfate analysis that is specific, sensitive to one-half part per million sulfate (ca. 5 micromolar), and free from interferences in our minimal media. It employs a Dionex Model 2000i instrument equipped with an HPIC-AS4A column and a conductivity detector.

Crucial parameters for setup (taken from literature supplied with the instrument) are as follows.

Eluant: 1.80 mM Na_2CO_3, 1.70 mM Na_2HCO_3; 2.0 mL/min

Regenerant: 25 mN H_2SO_4; 3 mL/min

Background conductivity is typically 17 microsiemens

Sulfate standards ($MgSO_4$) were prepared in *Nanopure* deionized water (> 17 megohm). A standard curve was generated by plotting peak height versus parts per million sulfate. Figure 4 shows this relationship is linear from below 1.0 ppm to about 50 ppm. Figure 5 is a chromatograph of a mixture of standard anions.

Figure 6 is a chromatogram of our minimal medium supplemented with 0.2 mM $MgSO_4$ (19.2 ppm). Sulfate consistently eluted at 7.0 - 7.1 minutes after injection regardless of the mixture in which it was supplied.

FIGURE LEGENDS

Figure 1. Utilization of DBTS by and growth of UMXa consortium described in the text; kinetics of phenylphenol production.

Figure 2. Utilization of DBTS by and growth of UMX3 isolate described in the text.

Figure 3. Utilization of DBTS by and growth of UMX9 isolate described in the text.

Figure 4. Relationship of sulfate concentration to recorder peak height during anion chromatography of sulfate standards.

Figure 5. Anion chromatograph of anion standard mix; peaks are: 1. fluoride, 2. chloride, 3. nitrate, 4. phosphate, 5. sulfate.

Figure 6. Anion chromatograph of the minimal medium supplemented with 0.2 mM $MgSO_4$ (19.2 ppm sulfate).

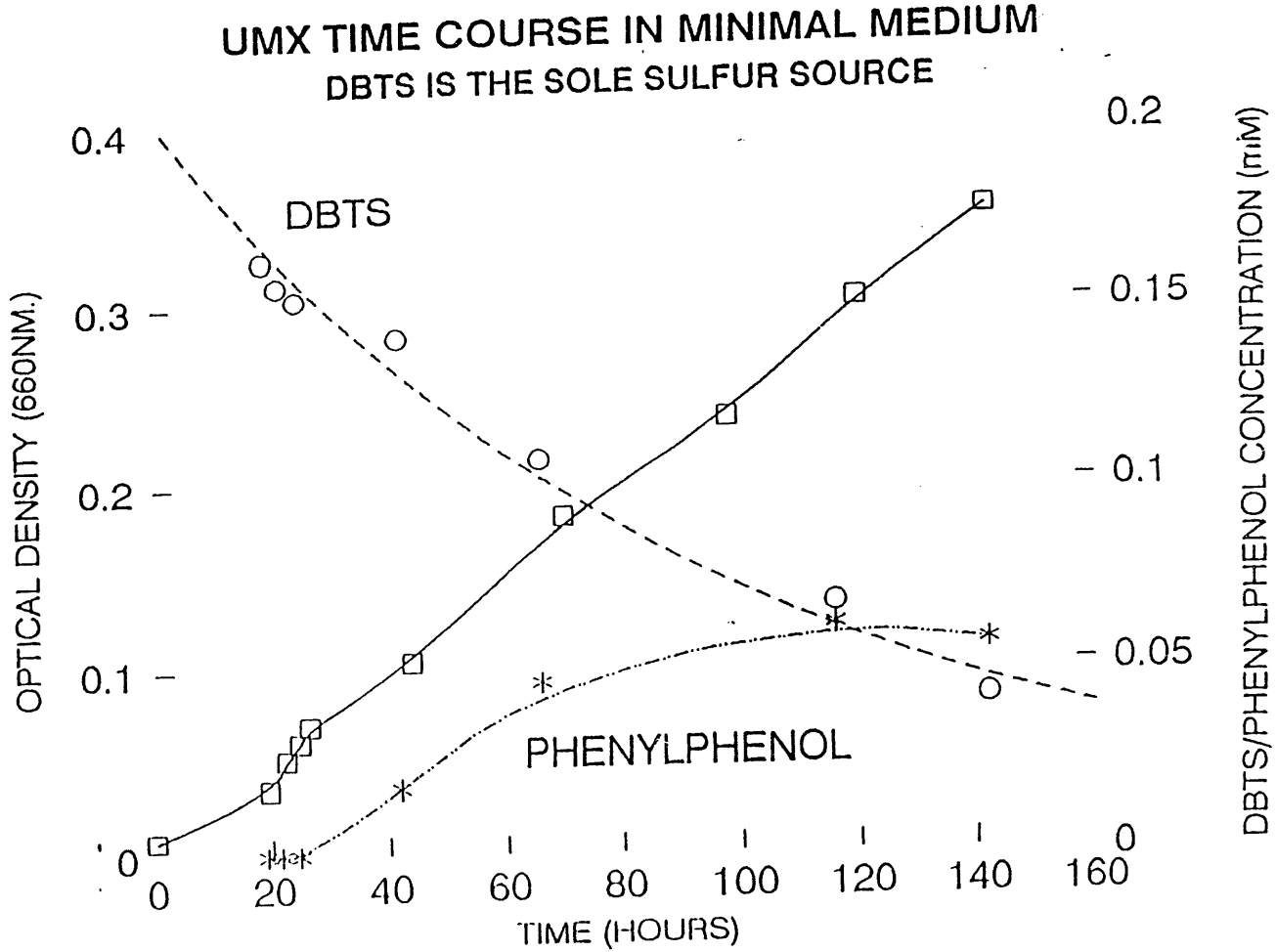

UMX TIME COURSE IN MINIMAL MEDIUM
DBTS IS THE SOLE SULFUR SOURCE
DBTS
PHENYLPHENOL
OPTICAL DENSITY (660NM.)
DBTS/PHENYLPHENOL CONCENTRATION (mM)
TIME (HOURS)
0.4
0.3
0.2
0.1
0
0.2
0.15
0.1
0.05
0
0
20
40
60
80
100
120
140
160

Figure 1

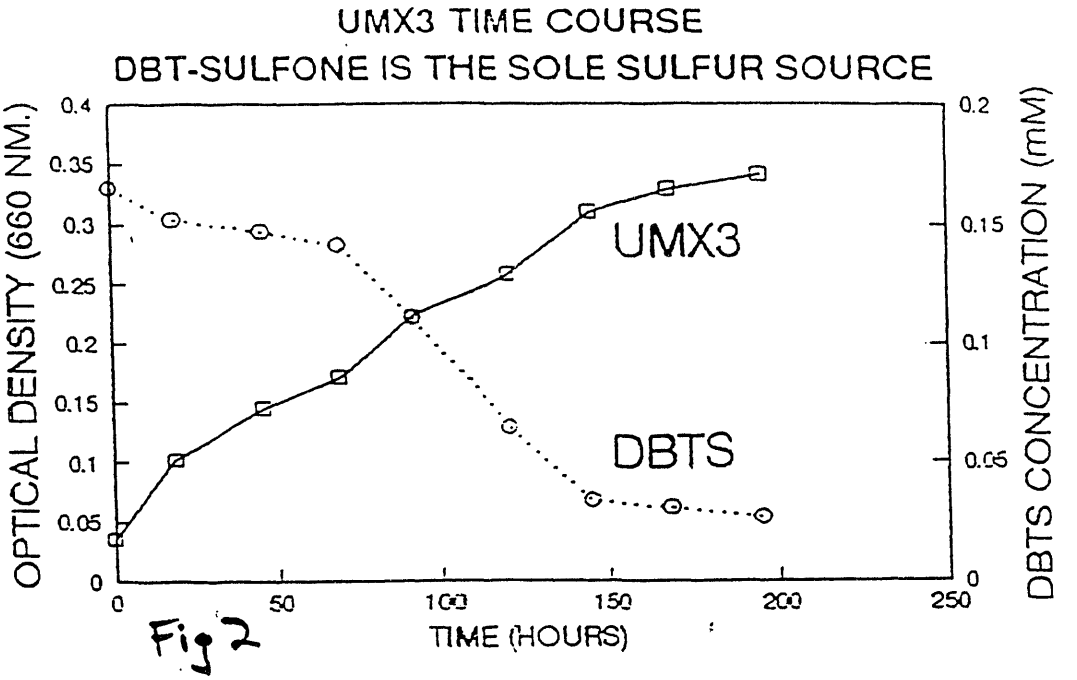

UMX3 TIME COURSE
DBT-SULFONE IS THE SOLE SULFUR SOURCE
OPTICAL DENSITY (660 NM.)
DBTS CONCENTRATION (mM)
UMX3
DBTS
TIME (HOURS)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
50
100
150
200
250

Fig 2

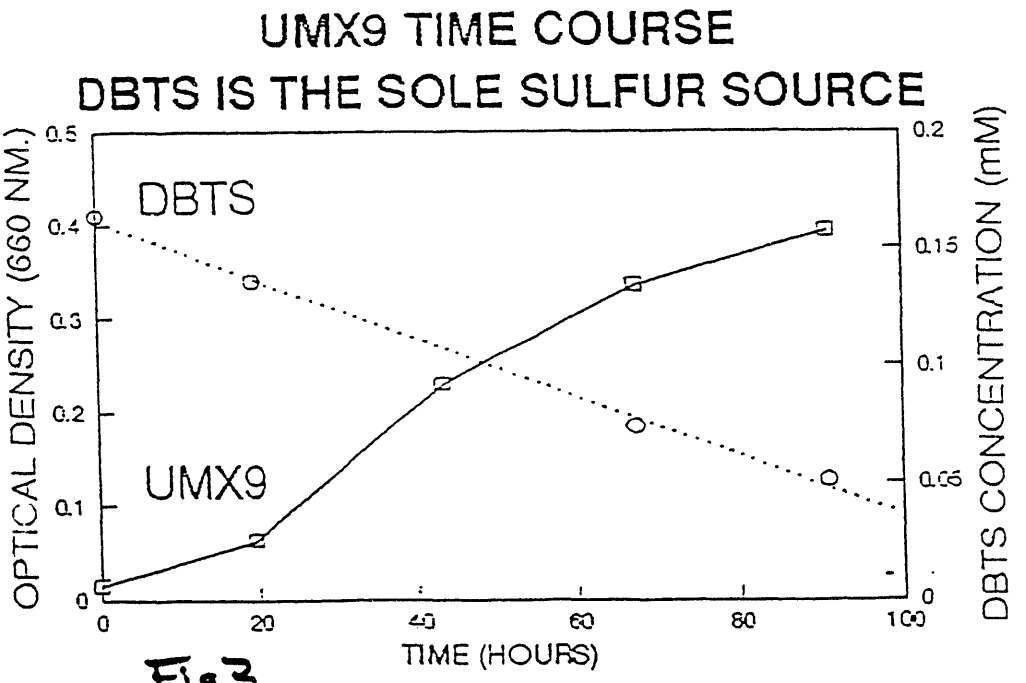

UMX9 TIME COURSE
DBTS IS THE SOLE SULFUR SOURCE
OPTICAL DENSITY (660 NM.)
DBTS CONCENTRATION (mM)
DBTS
UMX9
TIME (HOURS)
0
0.1
0.2
0.3
0.4
0.5
0.15
20
40
60
80
100

Fig 3

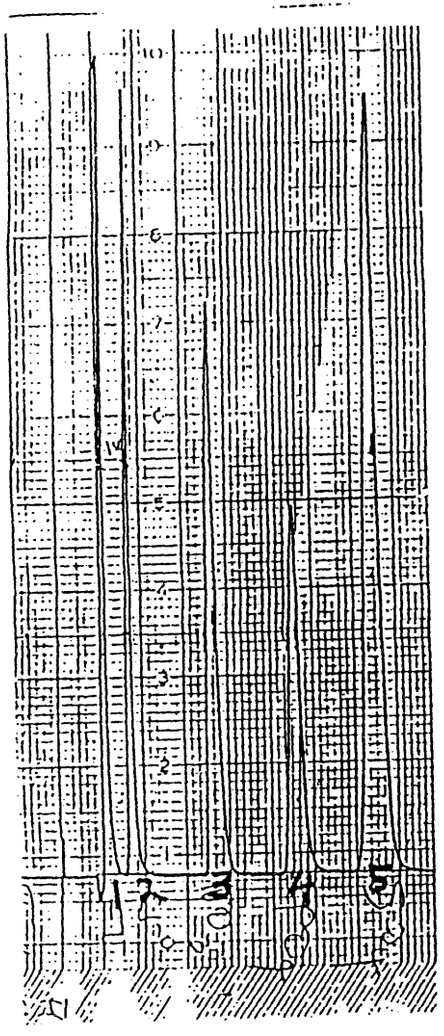


Fig 5

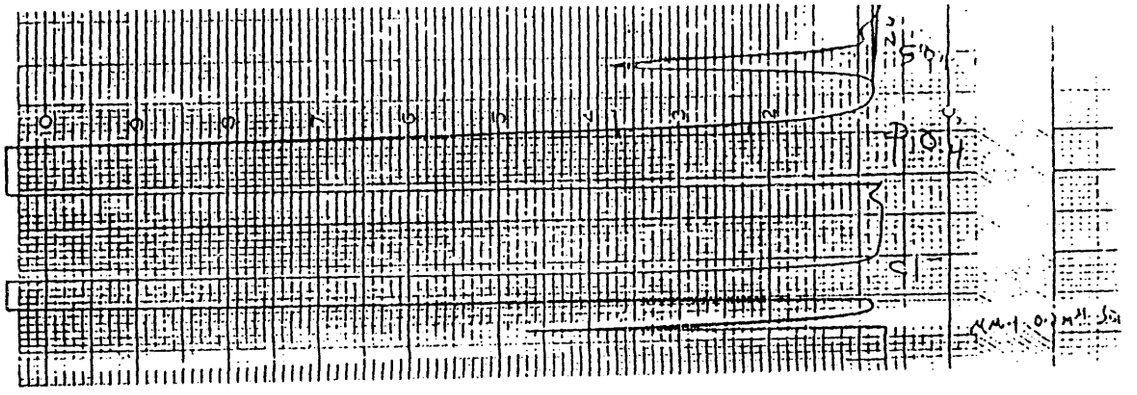


Fig 6

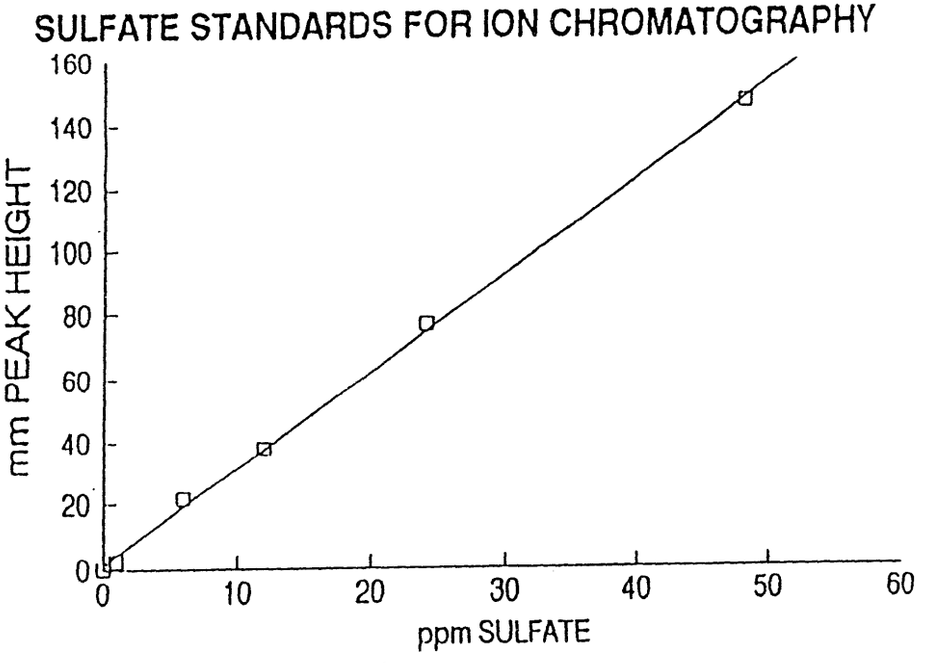


Fig 4

TASK 4--MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

Utilization of Dibenzothiophene Sulfone

Neither UMX3 nor UMX9 (isolates from the UMX consortia that are described in the preceding quarterly progress report; 15 September 1990) were able to use dibenzothiophene sulfone (DBTS; at 0.166 mM) as the sole carbon source. However, as has been previously reported, either isolate is capable of using DBTS as the sole source of sulfur; under such growth conditions, UMX9 produces phenylphenol as a degradation product; we have been unable to identify any degradation product of DBTS as a result of similar growth by UMX3.

Experiments are in progress to determine the capability of these two strains to degrade DBTS in the presence of 0.2 mM Mg_2SO_4 with glucose or glycerol as the carbon sources.

Coal culture of desulfurizing bacteria

Preliminary growth experiments with UMX3 and UMX9 inoculated into sulfate-free minimal medium with glycerol and glucose as the carbon sources and depyritized coal (10 g/L) as the sole source of available sulfur have been completed. (The coal was washed to remove excess sulfate as described in the preceding quarterly progress report; 15 September 1990.)

Because medium made with acid-washed coal typically contained 1 to 2 ppm sulfate, we also ran a control culture without coal but containing 2 ppm sulfate. The cultures were static at room temperature (25°C) in 2.8 liter low-form flasks; each flask was swirled twice daily to mix the coal slurry with the medium.

We followed growth (by viable cell count), and the concentration of free sulfate (by ion chromatography) in the medium over a course of 16 days. The minimal medium without added sulfate did not support growth of either UMX3 or UMX9. The control with 2 ppm sulfate added supported an increase from 5×10^5 CFU/mL at inoculation to approximately 2×10^6 CFU/mL attained at 5 days; no significant increase occurred over the next 11 days. The UMX3 culture supplied with coal increased from the initial 5×10^5 CFU/mL to a maximum density of 8.7×10^8 CFU/mL over the 16 days. Over the same time period, the UMX9 culture supplied with coal increased

from 4.2×10^5 CFU/mL to a maximum of 3.8×10^8 CFU/mL; there was noticeable clumping and the formation of small waxy colonies on the surface of the broth in these cultures.

Viable cell count may not be a reliable way of checking accumulated biomass in these experiments; preliminary attempts at colorimetric protein determination were confounded by interferences from culture components with the assay method.

Sulfate concentrations of the uninoculated coal + minimal medium control stayed at 2 ppm throughout the experiment. The 2 ppm of sulfate in the control culture without sulfate was no longer detectable within 2 days of inoculation. Sulfate concentration of the medium containing coal that was inoculated with UMX3 increased at least twofold within 5 days of inoculation; the analogous UMX9 culture did not show a similar increase in sulfate.

The coal that had been acted on by either UMX3 or UMX9 showed a noticeable physical deterioration at the conclusion of the experiment. In contrast, coal that simply soaked in uninoculated medium still settled out rapidly as a fine granular mass. A high proportion of the coal exposed to the microbes remained in suspension indefinitely. However, by centrifugation for 20 minutes at 17,700 x g, removed particles from suspension.

Similar experiments have been initiated using other microbial strains and a more valid index of biomass accumulation is being sought.

Summary

Two isolates of the UMX consortia described in the preceding quarterly progress report have been further characterized regarding their physiologic capabilities in the presence of dibenzothiophene sulfone. Preliminary experiments in which these isolates have been cultured on a minimal medium containing depyritized coal as the sole source of sulfur showed enhanced growth; the increase in colony forming units per mL in cultures of these organisms that were supplemented with depyritized coal seems to be related to the utilization of organically-combined sulfur. Refinements of these experiments are being considered; among them are more valid determinations of any increase in biomass that is dependent on sulfur derived from coal.

14 November 1990

TASK 4--MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

Utilization of Dibenzothiophene Sulfone

Although both UMX3 and UMX9 are able to use dibenzothiophene sulfone (DBTS) as a sole source of sulfur, neither of the bacteria (isolates from the UMX consortia that are described in the preceding quarterly progress report; 15 September 1990) were able to use DBTS as the sole carbon source.

When sulfate was present in the medium (0.166 mM), and glycerol and glucose were available as carbon sources, the utilization of DBTS by UMX3 was noticeably less than when sulfate was not present; a similar effect on the utilization of DBTS by UMX9 grown under the same conditions was apparent, although not as dramatic.

Strain UMX3 was grown with DBTS as the sole sulfur source and the following as carbon substrates: benzoate, glycerol, glucose. Benzoate supported the highest rate of growth and the highest final cell yield, followed by glycerol and glucose. DBTS utilization with glucose as the sole carbon substrate ceased after 48 hours, whereas DBTS degradation with glycerol as the sole carbon source continued at a steady rate for up to 120 hours. (DBTS degradation in the presence of benzoate was not measured because of its interference with the DBTS assay.)

Strain UMX9 was grown under the conditions described in the preceding paragraph: DBTS was the sole sulfur source and either benzoate, glycerol or glucose were carbon sources. As with UMX3, benzoate, followed by glucose and glycerol supported the highest growth rate. In contrast to UMX3, the rate of degradation of DBTS with both glucose and glycerol was the same.

Thus, UMX3 would not be able to survive with glucose as the sole carbon source and DBTS as the sole sulfur source, whereas UMX9 should be able to do so.

14 November 1990

Cell-free extracts of UMX9

We attempted to use methylene blue as a spectrophotometric assay for potential cleavage of carbon-sulfur bonds by cell-free extracts of UMX9. The bacteria were grown in minimal medium with DBTS as the sulfur source, and glycerol and glucose as carbon sources; after the cells were harvested by centrifugation, they were washed in 0.1 M phosphate (pH 7.0) and passed through a French Press three times at 20,000 psi. The French cell exudate was centrifuged 20,000 x g for 20 min, and the supernatant fluid (approx. 5 mg protein/ml) served as the cell-free extract.

Two hundred microliters of the CFE was incubated with 0.10 mM methylene blue and the degradation and/or reduction of the dye was followed by monitoring the decrease in absorbance in the area between 500 to 700 nm. There was a 30% change in initial absorbance; however, when nitric acid was added to reoxidize methylene blue, and the pH and dilution factors were compensated for, there was no apparent decrease in optical absorbance that could be related solely to carbon-sulfur cleavage. Thus, the change in absorbance could be entirely accounted for by the reduction of the dye by normal intracellular metabolites and metabolic events.

These experiments suffered from having a low protein concentration in extracts; furthermore, there is doubt as to whether methylene blue can serve as the sole sulfur source for the bacteria. However, we have grown UMX3 and UMX9 on methylene blue-containing, "sulfur-free" agar plates and achieved decolorization of the dye in the locality of the bacterial colonies.

Coal culture of desulfurizing bacteria

A second series of growth experiments wherein both UMX3 and UMX9 were inoculated into sulfate-free minimal medium with glycerol and glucose as the carbon sources and depyritized coal (10 g/L) as the sole source of available sulfur have been completed. (The coal was washed to remove excess sulfate as described in the preceding quarterly progress report; 15 September 1990.)

As described last month, the medium formulated with acid-washed coal typically contained 1 to 2 ppm sulfate; we also ran a control cultures without coal but containing 2 ppm sulfate. The cultures were static at room temperature (25°C), 1 L in 2.8 liter low-form flasks; each flask was swirled twice daily to mix the coal slurry with the medium.

We followed growth (by viable cell count), and the concentration of free sulfate (by ion chromatography) in the medium over a course of 20 days. The minimal medium without

added sulfate would not support growth of either UMX3 or UMX9. The control with 2 ppm sulfate added supported an increase from 5 x 10^5 CFU/mL at inoculation to a final population density of approximately 1.0 x 10^7 CFU/mL after 20 days for either UMX3 or UMX9. The UMX3 culture supplied with coal increased from the initial 5 x 10^5 CFU/mL to final density of 2.8 x 10^9 CFU/mL. Over the same time period, the UMX9 culture supplied with coal increased from 5 x 10^5 CFU/mL to a maximum of 2.5 x 10^8 CFU/mL; as before, there was noticeable clumping and the formation of small waxy colonies on the surface of the broth in the UMX9 cultures.

Sulfate concentrations are currently being determined for this experiment.

Additional cell-free extract work.

We have grown in static culture for 7 days, a 24 liter volume of UMX9 on minimal medium with glycerol as the carbon source and saturating concentrations of DBTS and dibenzothiophene as potential sulfur sources. The cells have been harvested by Sharples centrifugation and are being used to prepare high-protein cell-free extract for further enzymological work using the model compounds and possible catabolic intermediates as substrates.

Summary

Additional work on the catabolic potential of UMX3 and UMX9 in the presence of DBTS is described. Further work on these isolates grown on depyritized coal as the sole source of sulfur has been done; the coal can apparently supply organically-derived sulfur for these bacteria; additional data on the release of sulfate derived from coal into the medium are being collected from these experiments.

APPENDIX VI

ASSAYS ON UMX3 AND UMX9

- Utilization of DBT-sulfone
- Sulfate Effects on Biodesulfurization
- Organic Carbon Effects

ASSAYS ON IGT S-8

- Confirmation of 2-hydroxybiphenyl

TAXONOMIC STUDIES

- MIDI Analysis

TESTS OF UMX3 AND UMX9 ON COAL

TASK 4--MICROBIAL ACTIVITY ON MODEL COAL ORGANOSULFUR COMPOUNDS

Utilization of Dibenzothiophene Sulfone.

We attempted to grow strains UMX3 or UMX9 (isolates from the UMX consortia that are described in the preceding quarterly progress report; 15 September 1990) on dibenzothiophene sulfone (DBTS; at 0.166 mM) as the sole carbon source. Neither UMX3 nor UMX9 were able to use DBTS as the sole carbon source.

Either isolate, as we have previously reported, is capable of using DBTS as the sole source of sulfur; under such growth conditions, UMX9 produces phenylphenol (2-hydroxybiphenyl) as a degradation product; we have been unable to identify any degradation product of DBTS as a result of similar growth by UMX3.

When we supplied sulfate (0.166 mM) in a medium containing glycerol and glucose as carbon sources, the utilization of DBTS by UMX3 was noticeably less than when sulfate was not present; a similar effect on the utilization of DBTS by UMX9 grown under the same conditions was apparent, although not as dramatic.

In order to investigate the effect of alternate carbon sources on the utilization of organically-combined sulfur, we grew strains UMX3 and UMX9 with DBTS as the sole sulfur source with one of the following as carbon substrates: benzoate, glycerol, glucose. Growth was monitored by optical

density and the disappearance of DBTS was monitored by UV-absorption as we have previously described. Figure 1 depicts these data for UMX9, Figure 2 for strain UMX3.

For both strains, benzoate supported the highest rate of growth and the highest final cell yield, followed by glycerol and then by glucose.

For strain UMX3, DBTS utilization with glucose as the sole carbon substrate ceased after 48 hours, whereas DBTS degradation with glycerol as the sole carbon source continued at a steady rate for up to 120 hours. (DBTS degradation in the presence of benzoate was not measured because of its interference with the UV-absorbance assay for DBTS.)

In contrast to UMX3, the rate of degradation of DBTS by UMX9 with either glucose and glycerol as carbon sources was the same. As with UMX3, benzoate, followed by glucose and glycerol supported the highest growth rate.

Thus, UMX3 would not be able to survive with glucose as the sole carbon source and DBTS as the sole sulfur source, whereas UMX9 should be able to do so. These data are consistent with our observations that UMX3 cannot utilize glucose as a sole source of carbon and energy (see Table 1)

Comparison of UMX3, UMX9 and IGTS8

Dr. John Kilbane of the Institute of Gas Technology has provided us with a culture of IGTS8 to which we have compared the strains designated UMX3 and UMX9.

TABLE 1. Comparison of *Rhodococcus*-like strains.

Strain	IGTS8	UMX3	UMX9
Cell Morph	all strains were gram-variable coccoid rods		
Colony Morph	slimy	slimy	rough/waxy
Colony color	light pink	light pink	orange/pink
Glucose[1]	-	-	++
DBT/DBTS[1]	+++	+++	+++
Product from DBTS	2OHBP[2]	none[3]	2OHBP[2]

1. Ability to use as the sole source of carbon.
2. 2-hydroxybiphenyl
3. We were unable to detect any aromatic breakdown products traceable to DBTS for this strain.

Confirmation of phenylphenol as a product of DBTS degradation by IGTS8.

We grew strain IGTS8 in minimal medium with DBTS and thianthrene as the sulfur sources (total culture volume, 9 mL). The culture was extracted twice with 5 mL dichloromethane

and passed through a phase-separating paper; Figure 3 is a gas chromatogram of that extract; the peak labeled 2-OHBP was identified by GS/MS analysis as 2-hydroxybiphenyl (Figure 4 is the mass spectrum from that peak; Figure 5 is a comparison of the spectrum with the library spectrum for 2-hydroxybiphenyl). Thus, we have confirmed that the IGTS8 strain of *Rhodococcus rhodochrous* obtained from Dr. John Kilbane is capable of releasing detectable quantities of phenylphenol into the medium as a product of DBTS degradation. No other metabolites of DBTS could be identified. Other major peaks on the chromatogram appear to be thianthrene derivatives and have not been identified.

"Microbial ID"--fatty acid analysis of UMX3, UMX9 and IGTS8

We supplied cultures of the three strains to Dr. Al Mikel at University of Alabama, Huntsville to be evaluated by the "Microbial ID" method, which is based on the gas-chromatographic analysis of the methyl-esters of the bacterial fatty acids. The data from these analyses are presented in Table 2, Table 3, Table 4, and Table 5 that are appended to this report.

Table 2 shows the fatty acid content of a Huntsville isolate of *Rhodococcus rhodochrous* with a match of 0.395 to the database profile for *R. rhodochrous*. Table 3 shows similar data for strain IGTS8; this strain had a match of 0.173 to the database profile. Table 4 shows similar data for strain UMX3, which had a match of 0.221 to the database profile. Strain UMX9 data are shown in Table 5; there was no match identified for this strain.

FIGURE LEGENDS

Figure 1. Growth of UMX3 on DBTS as the sole sulfur source with either benzoate, or glycerol, or glucose as carbon sources.

Figure 2. Growth of UMX9 on DBTS as the sole sulfur source with either benzoate, or glycerol, or glucose as carbon sources.

Figure 3. Gas chromatogram of a dichloromethane extract of the broth culture of the IGTS8 strain of *Rhodococcus rhodochrous* (obtained from Dr. John Kilbane). Details of growth conditions and medium composition are given in the text. The peak at 6.382 min is 2-hydroxybiphenyl (2-OHBP); the peak at 9.084 is dibenzothiophene (DBT). Most other peaks are unidentified breakdown products of thianthrene.

Figure 4. Frame a: Gas chromatogram of a dichloromethane extract of the broth culture of the IGTS8 strain of *Rhodococcus rhodochrous* (obtained from Dr. John Kilbane), employing a mass-170.00 filter to focus on the 2-hydroxybiphenyl peak. Frame b: The mass spectrum of the peak in frame a.

Figure 5. The mass spectrum of the mass 170.00 peak from Figure 4 compared with the library spectrum of 2-hydroxybiphenyl.

Figure 6. Growth on coal of UMX3 and UMX9 compared to their growth on minimal medium with 2 ppm sulfate. Frames a and b are data from the two independent experiments.

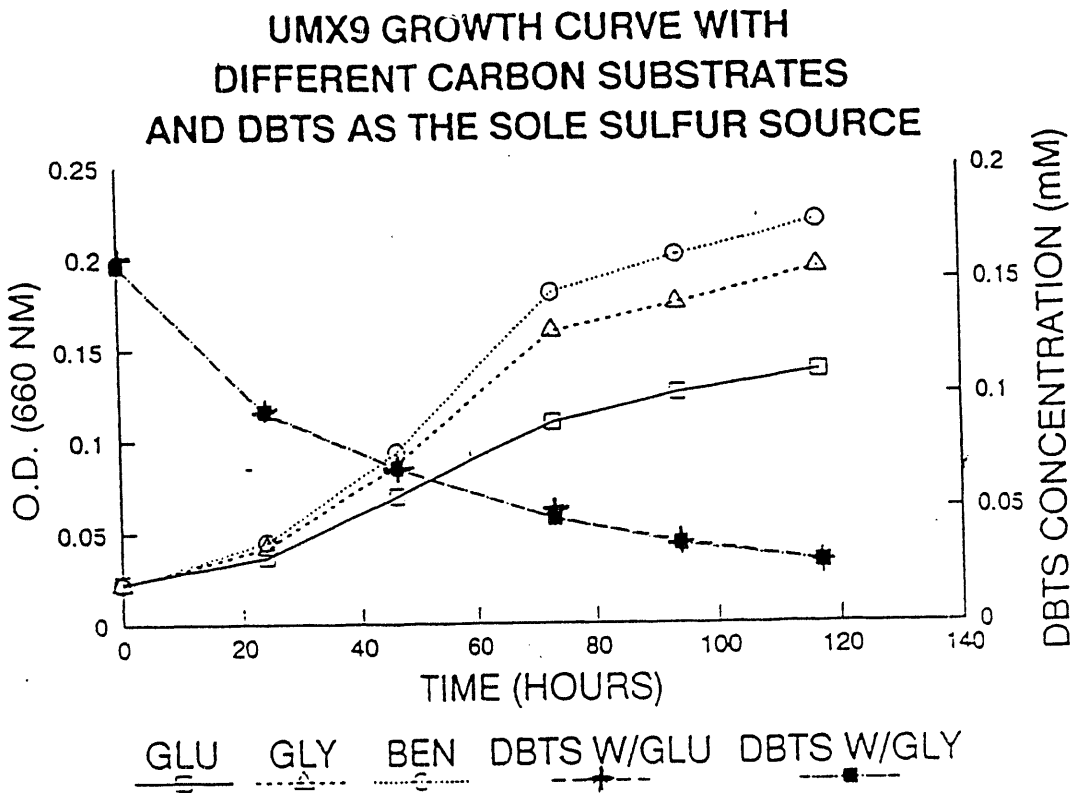


FIGURE 1

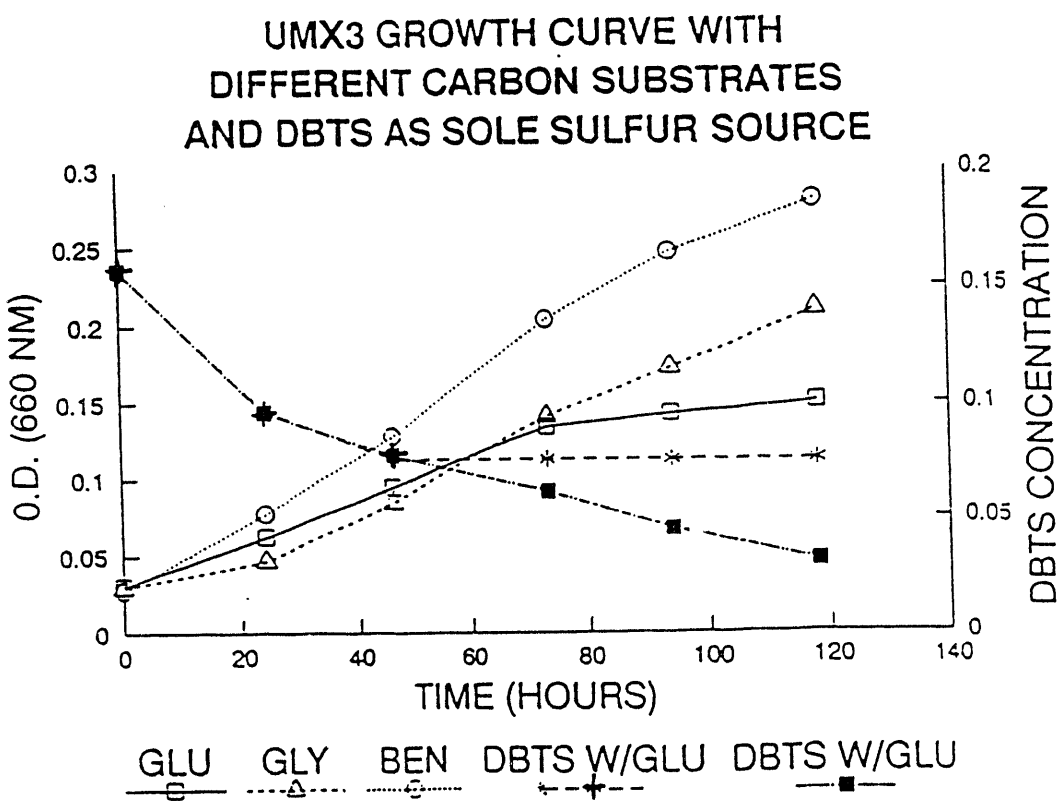
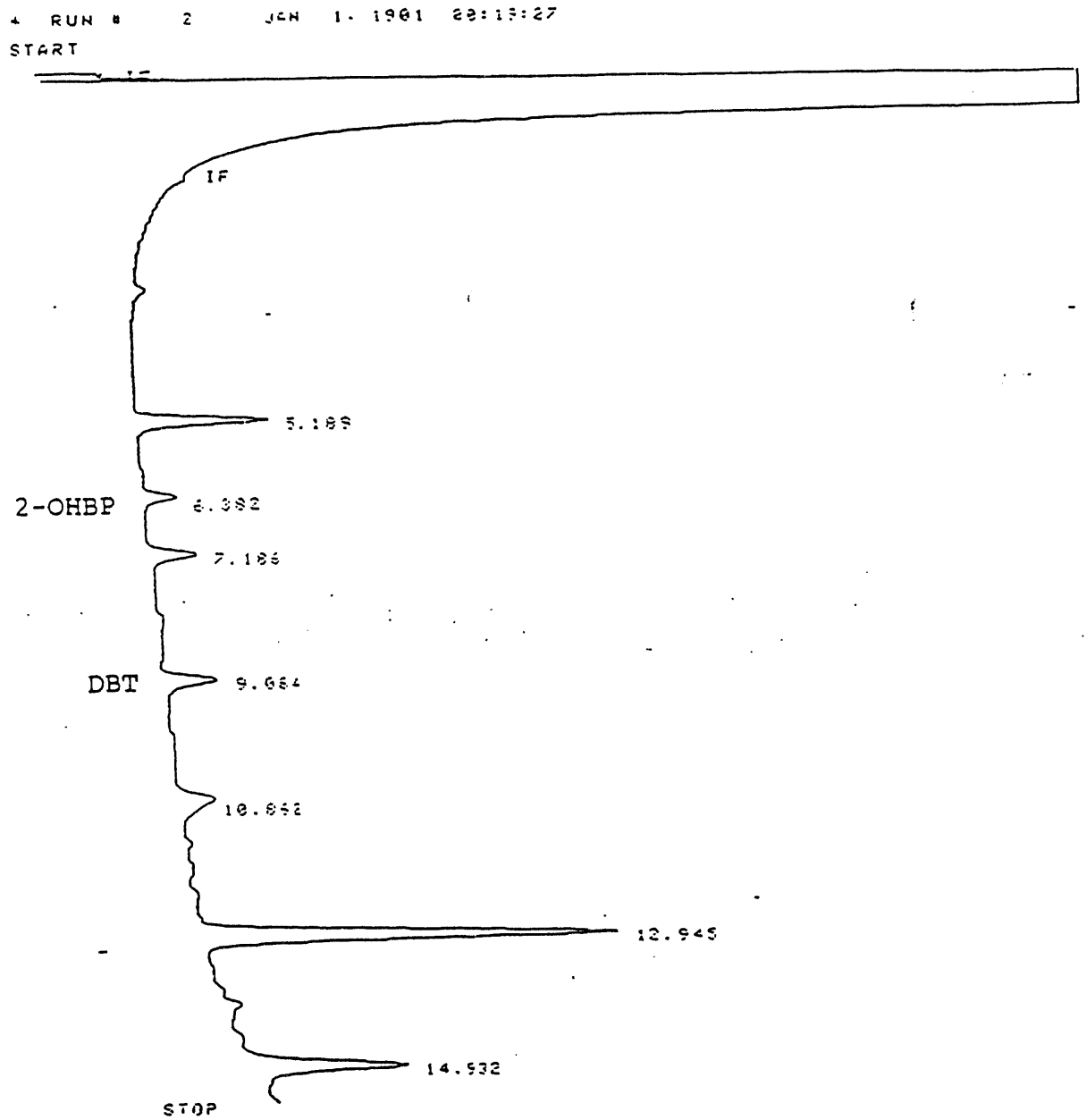


FIGURE 2

FIGURE 3



RUN# 2 JAN 1, 1901 00:19:27

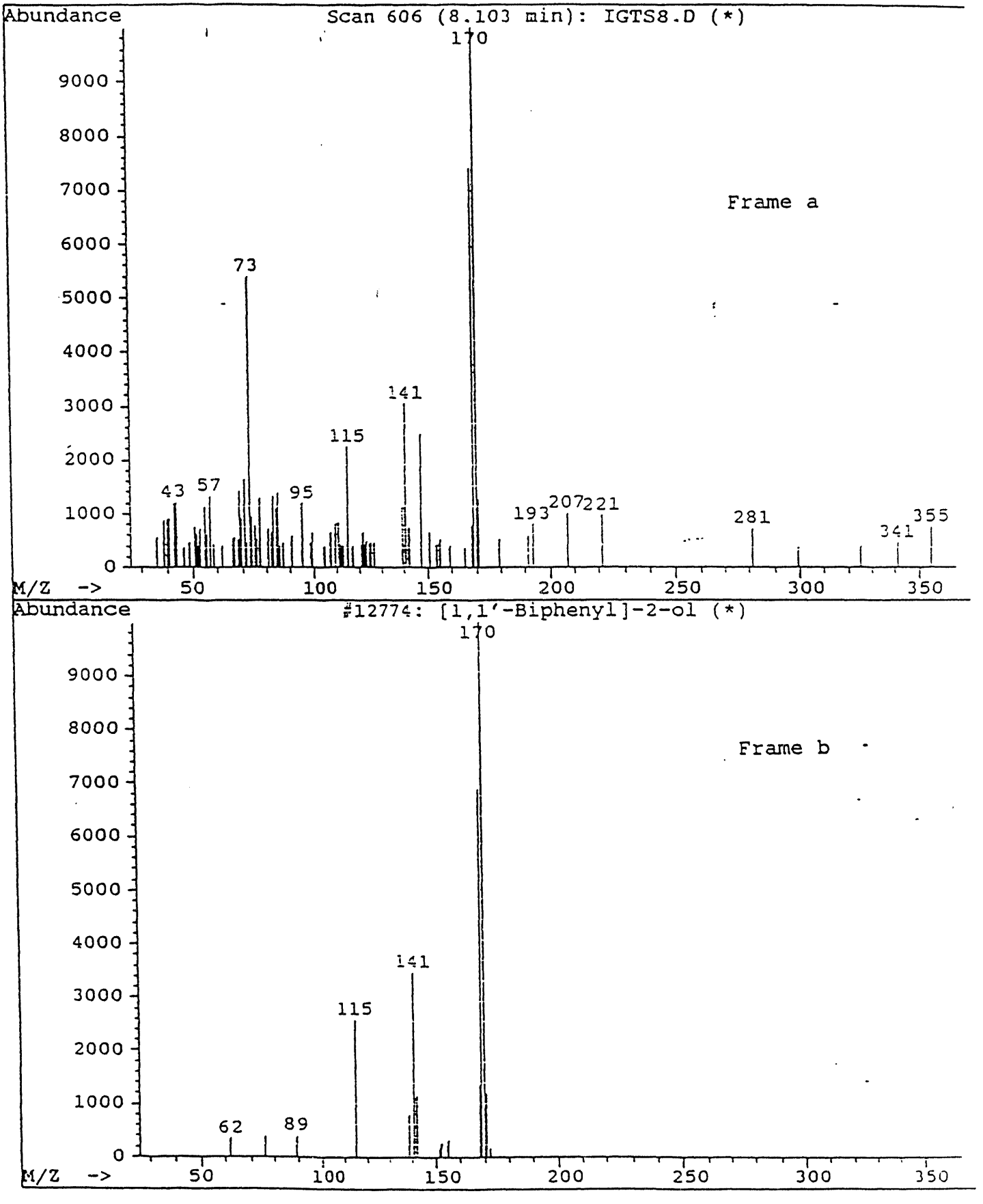
AREA%				
5.254	192178	BB	.132	5.19256
6.382	43089	BB	.136	1.16423
7.247	88727	PB	.156	2.39734
9.884	35979	PB	.172	6.48882
10.862	39919	BP	.267	7.19548
12.945	269823	BB	.162	48.51816
14.932	113479	PB	.183	20.46595

TOTAL AREA= 554477

RUL FACTOR=1.0000E-00

Library Searched : C:\DATABASE\NBS49K.L
Quality : 68
ID : [1,1'-Biphenyl]-2-ol

FIGURE 4



There was substantial agreement between the strains with regard both to qualitative and quantitative fatty acid content; however, the strains differed in some fatty acids.

Based on the data shown in Table 1, as well as the fatty acid analyses, we believe that strain UMX3 is likely the same as IGTS8: like IGTS8, it is unable to grow on glucose as the sole carbon source; moreover, it is morphologically indistinguishable from IGTS8 and exhibits similar growth kinetics on minimal medium with DBTS as the sulfur source. However, we find it disconcerting that we have been unable to detect any release of phenylphenol in the medium following growth on DBTS by strain UMX3. Strain UMX9 produces phenylphenol when grown on DBTS as the sole source of sulfur; but UMX9 grows on glucose as the sole carbon source and has a markedly different colonial morphology.

We are comparing the abilities of UMX3, UMX9 and IGTS8 to grow at the sole expense of 40 additional carbon sources; this study should shed additional light on the question of the taxonomic relationship between these strains.

Coal culture of desulfurizing bacteria

In the past quarter, we did two experiments to determine whether the UMX3 and UMX9 isolates could obtain their sulfur from the organically-combined sulfur in coal. We added biodepyritized coal (prepared by Louisiana Tech University, sample # 1003, A & B; 10 g/L) as the sole source of available sulfur to a sulfate-free minimal medium (pH 7.0); such media contained 20 ppm sulfate (detectable by ion chromatography), an unacceptably-high amount.

Our attempts to deplete this free sulfate from the medium by allowing growth of wild type *Escherichia coli*, were not successful. An alternative washing method was employed: Samples of depyritized coal (10 g each) were suspended in 100 mL 6N HCl and stirred for 1 hour; the coal could be removed from the acid by filtration onto Whatman no. 1 paper on a Buchner funnel; after three washes of 200 mL each with deionized water, the coal was resuspended in 80 mL of water, the pH adjusted to pH 7.0 and the volume brought to 100 mL. Medium made from coal treated in this manner typically contained 1 to 2 ppm sulfate.

Two series of growth experiments were completed with UMX3 and UMX9 inoculated into sulfate-free minimal medium containing glycerol and glucose as the carbon sources and washed, depyritized coal (10 g/L) as the sole source of available sulfur.

To assess the level of growth of the bacteria that would be supported by residual sulfate in the coal cultures, we also ran control cultures without coal but containing 2 ppm sulfate. All cultures were static at room temperature (25°C). One-liter volumes of media were contained in 2.8 liter low-form flasks; each flask was swirled twice daily to mix the coal slurry with the medium.

We followed growth (by viable cell count), and the concentration of free sulfate (by ion chromatography) in the medium over a course of 16 days for the first experiment and 19 days for the second. The minimal medium without added sulfate did not support growth of either UMX3 or UMX9.

The final pH values for each culture condition were:

Uninoculated Minimal Medium	pH 7.0
Uninoculated coal control	pH 6.8
UMX3 grown on MM + 2 ppm SO_4	pH 6.5
UMX9 grown on MM + 2 ppm SO_4	pH 6.8
UMX3 grown on MM + coal	pH 5.7
UMX9 grown on MM + coal	pH 6.9

We have no explanation for the substantial drop in pH that occurred during the growth of UMX3 in the coal culture.

Figure 6 (a and b) depicts the growth of UMX3 and UMX9 on coal as the sulfur source; "control" lines were generated from the cultures in minimal medium with 2 ppm sulfate added. Uninoculated coal-containing medium maintained a free sulfate concentration of around 2 ppm throughout the course of the experiments.

In all cases, the control cultures with 2 ppm sulfate added supported populations that were 1.5 to 3 logs below those growing on coal. The UMX9 bacteria formed small waxy colonies on the surface of the broth; UMX3 seemed to be more evenly distributed throughout the medium.

Although our use of viable cell count as an index of biomass accumulation in these experiments suffers some limitations, it is probably an index of the <u>minimal</u> growth of the bacteria on the coal: The bacteria in the coal cultures were visibly clumped, and one would expect that colonies of bacteria might form on the coal particles; both of these factors tend to lower counts of colony forming units. Our preliminary attempts at colorimetric protein determination

in these experiments were confounded by interferences from culture components with the assay method.

The coal that had been acted on by either UMX3 or UMX9 showed a noticeable physical deterioration when we examined it at the conclusion of the experiment: there was a dramatic increase in fines, so that a high proportion of the coal exposed to the microbes remained in suspension indefinitely. In contrast, coal that simply soaked in uninoculated medium still settled out rapidly as a fine granular mass. However, centrifugation for 20 minutes at 17,700 x g, removed particles from suspension.

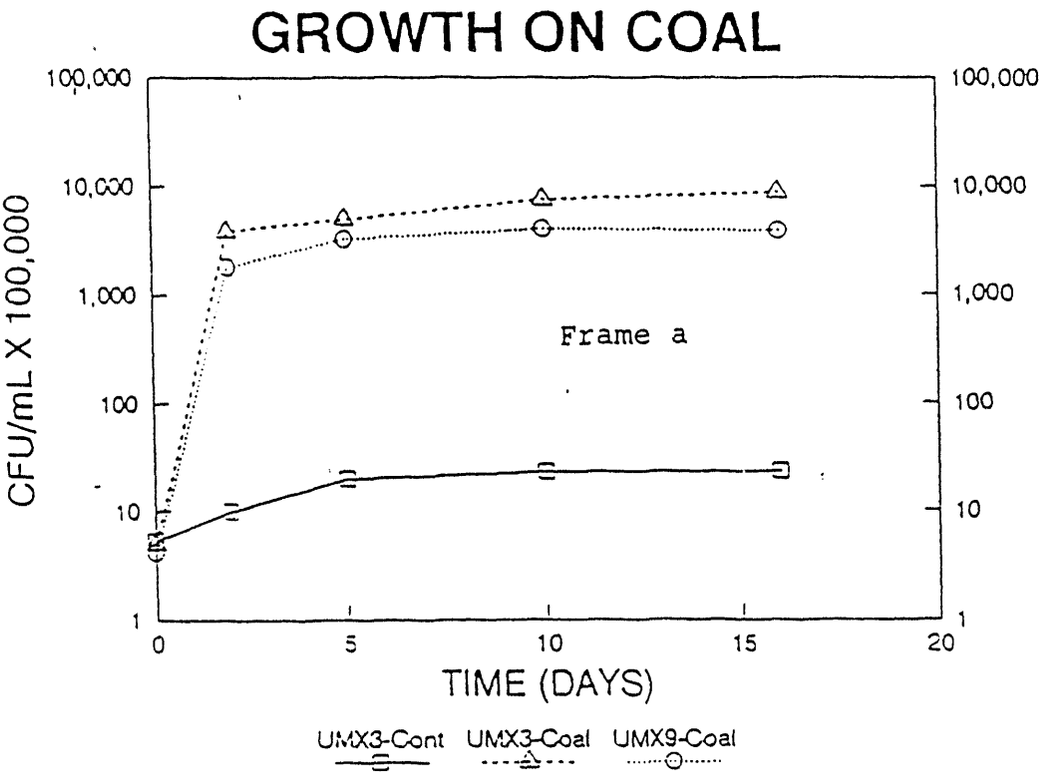
Sulfate concentrations during the course of the two experiments. In both experiments, sulfate in the uninoculated coal-minimal-medium-control stayed at 1.5 to 2 ppm throughout the experiment; and, the 2 ppm of sulfate in the control culture (without coal) was no longer detectable 2 days after inoculation.

In the first experiment, sulfate concentration of the medium containing coal that was inoculated with UMX3 has increased at least twofold (to 5 ppm) in the 5-day post-inoculation sample but then declined to 1 to 2 ppm; the analogous UMX9 culture did not show a similar increase in sulfate. In the second experiment, we did not observe this small increase in free sulfate in either the UMX3 or the UMX9 coal culture. Most likely, the sample vial for the 5-day data point in the first experiment was contaminated with sulfate: points on either side of the 5-day sample showed only 1 to 2 ppm sulfate.

Thus, in these experiments we show growth of either strain UMX3 or strain UMX9 on the organically derived sulfur in coal that apparently serves as the sole source of sulfur. However, in neither of these experiments did we see release of substantial amounts of sulfate into the medium. It could be that the low levels of free sulfate we see in the coal cultures is all that can be expected because it is taken up almost as rapidly as it is released. Furthermore, the sulfur from the coal may be either directly incorporated into cell biomass, or else released as some combined form other than sulfate.

We are currently removing the bacterial biomass from the coal harvested from these experiments. We will send the coal to Louisiana Technical University for sulfur analyses (sulfur : silicon ratio) to assess changes in organic sulfur content of the coal effected by growth of the microbes.

FIGURE 6


GROWTH ON COAL
CFU/mL X 100,000
100,000
10,000
1,000
100
10
1
0
5
10
15
20
TIME (DAYS)
Frame a
UMX3-Cont
UMX3-Coal
UMX9-Coal

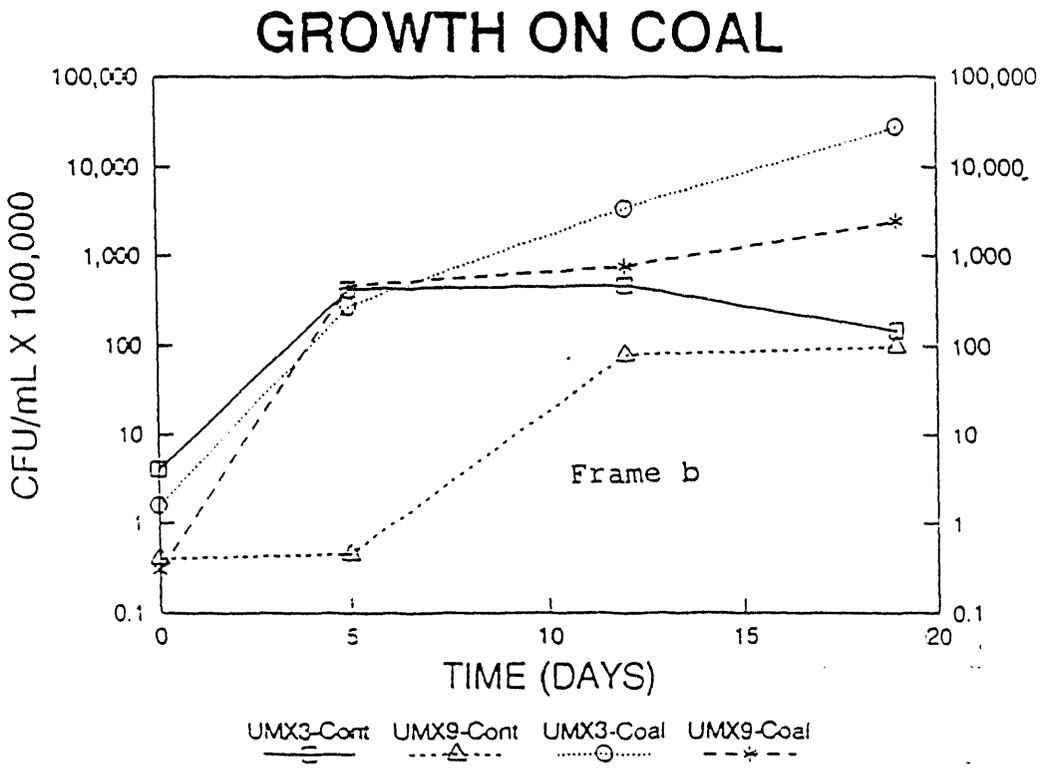

GROWTH ON COAL
CFU/mL X 100,000
100,000
10,000
1,000
100
10
1
0.1
0
5
10
15
20
TIME (DAYS)
Frame b
UMX3-Cont
UMX9-Cont
UMX3-Coal
UMX9-Coal

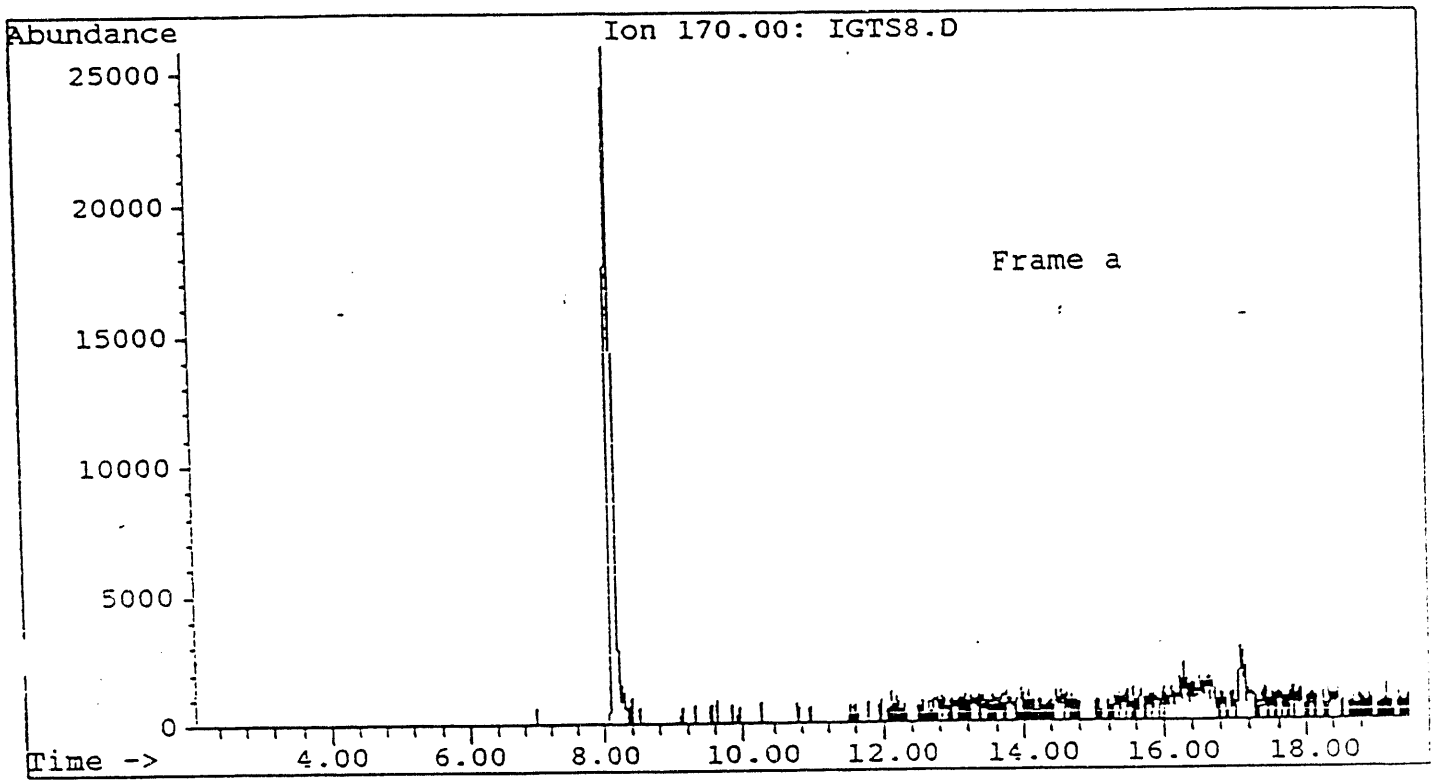
Table 5. Results of the CH Assay

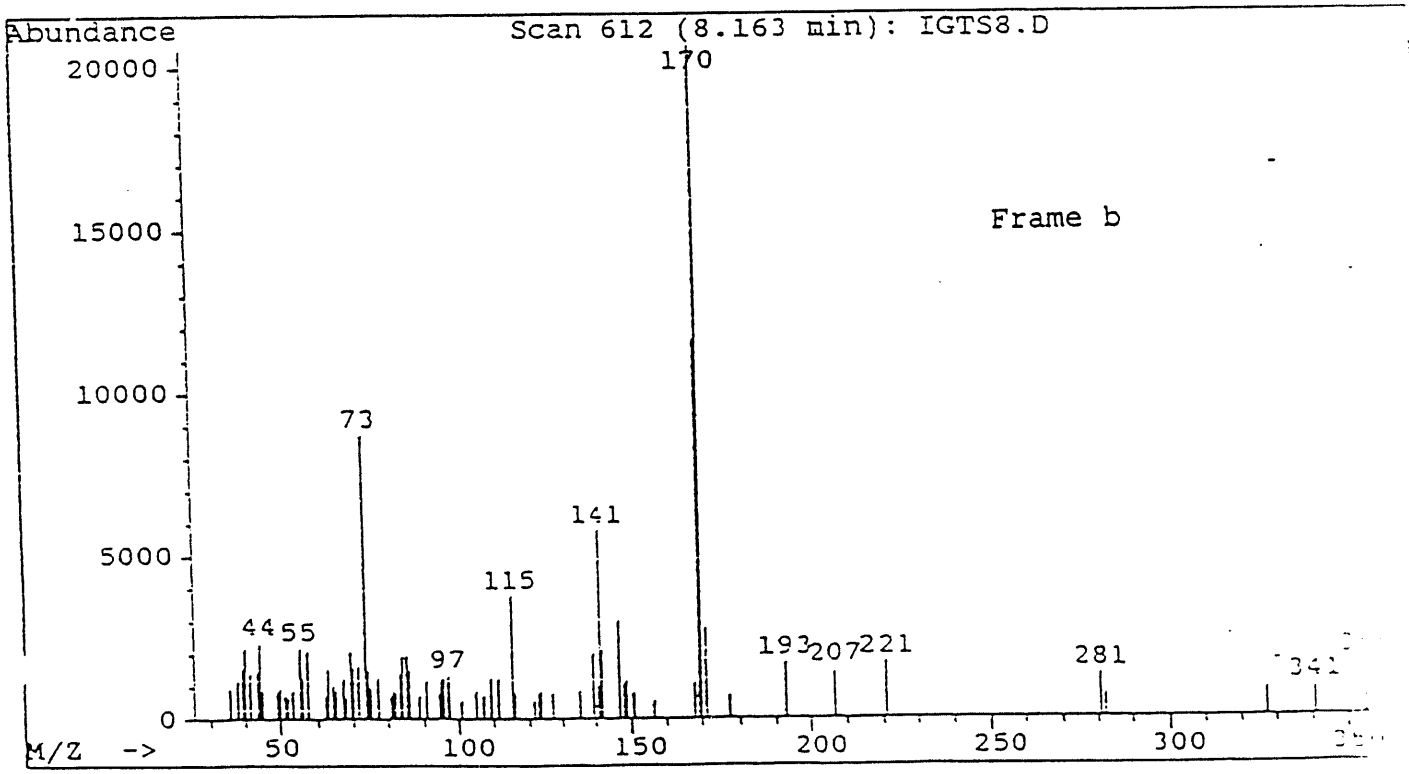
Carbohydrate[1]	UMX3	IGTS8	UMX9
Glycerol	Yes	Yes	Yes
Ribose	Yes	Yes	Yes
D-Glucose	No	No	Yes
D-Fructose	Yes	Yes	Yes
Inositol	Yes	Yes	Yes
Mannitol	Yes	Yes	Yes
Sorbitol	Yes	Yes	Yes
N-Acetyl-glucosamine	Yes	Yes	Yes
Esculin	Yes	Yes	Yes
Saccharose	Yes	Yes	Yes
Trehalose	Yes	Yes	Yes
D-Arabitol	Yes	Yes	Yes
Gluconate	Yes	Yes	Yes
L-Arabinose	No	Yes	No

1. Carbohydrate utilization was tested by the API CH test kit. The strains were cultivated for 48 hours in organic sulfur medium with magnesium sulfate as the sole sulfur source. Cellls were aseptically harvested, centrifuged (15,000 g for 20 min), and washed with 0.01 N phosphate buffer. Cells were then centrifuged again and resuspended in organic sulfur medium with no carbon source. The O.D. of the strains was adjusted to 0.016 before inoculation into the API test wells. The length of the assay was 8 days and yes denotes growthh in the test well.

File: D:\BIODATA\IGTS8.D
Operator:
Date Acquired: 30 Nov 90 10:13 am
Method File Name: DEFAULT.M
Sample Name:
Misc Info:
Bottle Number: 1

FIGURE 5





MIS System Software 3.2 (Rev: 3.2) (Sw s/n: 3026A90125) (Hw s/n: 2344A10204) DATA:F90921551 26-NOV-90 09:40:57

ID: 90112124 ?REF-UNK-UNK3 Date of run: 22-NOV-90 05:02:54
Bottle: 26 SAMPLE [ACROBC50]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.613	191067520	0.028	. . .	7.042	SOLVENT PEAK		< min rt	
6.709	4592	0.044	0.983	13.999	14:0	3.87	ECL deviates -0.001	Reference 0.001
8.165	1948	0.053	0.963	15.001	15:0	1.53	ECL deviates 0.001	Reference 0.001
9.395	1576	0.052	0.951	15.774	16:1 B	1.29	ECL deviates -0.000	
9.464	2200	0.041	0.950	15.817	16:1 CIS 9	1.79	ECL deviates 0.000	
9.525	12312	0.050	0.950	15.855	Sum In Feature 4 . .	10.03	ECL deviates -0.001	16:1 TRANS 9/15\2OH
9.757	37624	0.047	0.948	16.001	16:0	30.59	ECL deviates 0.001	Reference 0.000
11.001	2024	0.064	0.940	16.791	17:1 B	1.63	ECL deviates -0.001	
11.431	1576	0.061	0.939	17.000	17:0	1.27	ECL deviates 0.000	Reference -0.001
12.750	34072	0.052	0.934	17.787	18:1 CIS 9	27.29	ECL deviates -0.002	
13.150	3760	0.056	0.933	17.999	18:0	3.01	ECL deviates -0.001	Reference -0.001
13.828	15272	0.052	0.932	18.392	TBSA 10Methi=0 . . .	12.21	ECL deviates 0.000	
14.875	4528	0.062	0.931	18.998	19:0	3.61	ECL deviates -0.002	Reference 0.001
16.593	2352	0.092	0.930	20.001	20:0	1.88	ECL deviates 0.001	Reference 0.000
17.915	2056	0.093	. . .	20.772	. . . . .		> max rt	
18.022	1744	0.063	. . .	20.835	. . . . .		> max rt	
18.292	8168	0.057	. . .	20.992	. . . . .		> max rt	
*******	12312	. . . . .		. . .	SUMMED FEATURE 4 . .	10.03	15:0 ISO 2OH/16:1w5	16:1 TRANS 9/15\2OH

Solvent Ar	Total Area	Named Area	% Named	Total Amt	Nbr Ref	ECL Deviation	Ref ECL Shift
191067520	125736	125736	100.00	[illegible]	7	0.001	0.000

TSBA [Rev 3.30] Rhodococcus 0.221
R. rhodochrous 0.221
AICC [Rev 1.03] * NO MATCH *
CLIN [Rev 3.30] * NO MATCH *

Table 4. MIX 3

ID: 90112125 ?REF-UNK-IGT58   Date of run: 22-NOV-90 05:03:23
Bottle: 28   SAMPLE [AEROSESO]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.612	189260800	0.028	. . .	7.036	SOLVENT PEAK	. . .	< min rt	
6.707	2640	0.045	0.983	14.000	14:0	4.26?	ECL deviates -0.000	Reference -0.001
9.163	929	0.049	0.963	15.000	15:0	1.55	ECL deviates 0.000	Reference -0.001
9.393	816	0.045	0.951	15.773	16:1 ?	1.27	ECL deviates -0.001	
9.464	976	0.041	0.950	15.818	16:1 CIS 9	1.52	ECL deviates 0.001	
9.525	6384	0.048	0.950	15.856	Sum In Feature 4 . .	9.95?	ECL deviates -0.000	16:1 TRANS 9/15:2OH
9.755	20936	0.047	0.948	16.000	16:0	32.40?	ECL deviates 0.000	Reference -0.001
11.081	1000	0.055	0.940	16.792	17:1 ?	1.54?	ECL deviates 0.000	
12.749	21872	0.051	0.934	17.767	18:1 CIS 9	32.32?	ECL deviates -0.002	
13.148	1832	0.055	0.933	17.999	18:0	2.81?	ECL deviates -0.001	Reference -0.002
13.827	5723	0.056	0.932	18.393	10Me18:0 TBSA . . .	8.76?	ECL deviates 0.001	
14.874	2289	0.065	0.931	19.000	19:0	3.53?	ECL deviates 0.000	Reference 0.001
18.290	5504	0.063	. . .	21.002	. . . .	. . .	> max rt	
*******	6384	. . .	. . .	. . .	SUMMED FEATURE 4 . .	9.95?	15:0 ISO 2OH/16:1t9	16:1 TRANS 9/15:2OH

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	ECL Deviation	Ref ECL Shift
189260800	64592	64592	100.00	60962?	5	0.001?	0.001

TSBA [Rev 3.30] Rhodococcus 0.172
R. corallinus 0.172
R. rhodochrous 0.172
R. fascians . . . . [illegible]
Gordona . . . . 0.107 (Rhodococcus-bronchialis)
G. bronchialis . . . . 0.107 (Rhodococcus-bronchialis)
AICC [Rev 1.0] * NO MATCH *
CLIN [Rev 3.30] * NO MATCH *

64592

Table 3 IGT58

Huntsville Rhodococcus

Table 2. Huntsville Rhodococcus

Microbial Identification System (Rev: 3.0) (Su s/n: 2812R30001) (Hu s/n: 2411FH1135) 02-APR-90 09:50:06

ID: 119063 UNK-SOIL-RMA-390-118-09 Date of run: 03-APR-90 21:03:21
Bottle: 64 SAMPLE [AEROBIC50]

RT	Area	Ar/Ht	Respon	ECL	Name	%	Comment 1	Comment 2
1.476	29258000	0.058	. . .	7.051	SOLVENT PEAK	. . .	(min rt	
6.882	1986	0.051	0.991	14.000	14:0	2.65	ECL deviates 0.000	Reference 0.001
8.394	2262	0.042	0.966	15.000	15:0	2.99	ECL deviates 0.000	Reference 0.000
9.726	3435	0.055	0.949	15.812	16:1 CIS 9	4.46	ECL deviates -0.005	
9.796	9621	0.047	0.948	15.854	Sum In feature 4 . .	12.49	ECL deviates -0.002	16:1 TBSAS 9/15i2OH
10.053	21337	0.045	0.946	15.999	16:0	27.62	ECL deviates -0.001	Reference -0.002
11.396	4770	0.052	0.933	16.789	17:1 B	6.09	ECL deviates -0.003	
11.749	1394	0.050	0.930	17.001	17:0	1.77	ECL deviates 0.001	Reference 0.000
12.465	1107	0.048	0.925	17.412	18:1 ISO F	1.40	ECL deviates 0.002	
13.084	23802	0.050	0.920	17.766	18:1 CIS 9	29.97	ECL deviates -0.003	
14.178	8410	0.049	0.913	18.393	TBSA 10Me18:0 . . .	10.51	ECL deviates 0.001	
********	9621	. . .	. . .	. . .	SUMMED FEATURE 4 . .	12.49	15:0 ISO 2OH/16:1 I	16:1 TBSAS 9/15i2OH

Solvent Ar	Total Area	Named Area	% Named	Total Peak	Nbr Ref	ECL Deviation	Ref ECL Shift
29258000	79125	78125	100.00	7807	4	0.002	0.001

ISBA [Rev 3.0] Rhodococcus 0.395
R. rhodochrous 0.395
R. fasciens . . . . . 0.214 (Corynebacterium fasciens)

Comparison with ISBA [Rev 3.0]: Rhodococcus-rhodochrous Distance: 4.046

	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
14:0.	---+---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
15:0.	---+---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
16:1 CIS 9.	---+x--	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
16:0.	.	.	.	.	.	-----+-----	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17:1 B.	---+---x	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
17:0.	--+--.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18:1 ISO F.	-+-- .	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18:1 CIS 9.	.	.	.	.	----------+----------x---------.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
18:0.	x--+---	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
TBSA 10Me18:0 . . .	----------+----------	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
20:0.	x+---.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SUMMED FEATURE 4. .	. -------x+-------	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SUMMED FEATURE 8. .	x+---.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

ID: 90112125 PREF-UNX-UMX9 Date of run: 22-NOV-90 05:33:14
Bottle: 27 SAMPLE (PERIOD50)

RT	Area	Ar/Mt	Respon	ECL	Name	%	Comment 1	Comment 2
1.613	189960320	0.029	...	7.047	SOLVENT PEAK		< min rt	
6.708	5480	0.048	0.983	14.000	14:0	4.45	ECL deviates -0.000	Reference 0.000
8.164	2168	0.049	0.963	15.000	15:0	1.72	ECL deviates 0.000	Reference 0.000
9.391	1376	0.050	0.951	15.771	16:1 B	1.08	ECL deviates -0.005	
9.460	1528	0.035	0.950	15.814	16:1 CIS 9	1.20	ECL deviates -0.005	
9.525	17448	0.049	0.950	15.855	Sum In Feature 4 . .	13.69	ECL deviates -0.001	16:1 TRANS 9/15:1CIS
9.756	38220	0.045	0.949	16.000	16:0	30.02	ECL deviates -0.000	Reference -0.001
11.080	2672	0.063	0.940	16.790	17:1 B	2.08	ECL deviates -0.002	
11.435	1304	0.051	0.939	17.001	17:0	1.01	ECL deviates 0.001	Reference 0.002
12.750	33976	0.051	0.934	17.765	18:1 CIS 9	26.22	ECL deviates -0.004	
13.155	1248	0.060	0.933	18.000	18:0	0.96	ECL deviates 0.000	Reference 0.002
13.825	13424	0.052	0.932	18.368	TBSA 10Me18:0 . . .	10.33	ECL deviates -0.004	
14.877	7464	0.059	0.931	18.997	19:0	5.74	ECL deviates -0.003	Reference 0.002
16.597	1944	0.062	0.930	20.001	20:0	1.49	ECL deviates 0.001	Reference 0.010
17.920	3192	0.094	...	20.773	. . . . .		> max rt	
18.024	3960	0.069	...	20.834	. . . . .		> max rt	
18.292	12550	0.055	...	20.990	. . . . .		> max rt	
18.945	1016	0.057	...	21.362	. . . . .		> max rt	
*******	17448	.....	...	...	SUMMED FEATURE 4 . .	13.69	15:0 ISO 2OH/15:1(13	16:1 TRANS 9/15:1CIS

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Mbr Ref	ECL Deviation	Ref ECL Shift
189960320	129552	129552	100.00	121046	7	0.002	0.004

TSBA (Rev 3.00 • NO MATCH •
ATOC (Rev 1.00 • NO MATCH •
CLIN (Rev 3.00 • NO MATCH •

Table 5 UMX9

APPENDIX VII

MATERIALS AND METHODS

MATERIALS AND METHODS
(adapted from text by R.F. Purdy)

Sulfur-Stress Assay. Glassware was acid-washed (6N HCL for 60 min) to remove residual sulfate, then rinsed at least three times in deionized water (Nanopure Type I, 17 megohm) and capped to prevent sulfur contamination from dust particles. Growth was quantified at 660 nm using a Milton Roy spectronic 501; optical path was 1.6 cm with the 20 ml culture tubes. An uninoculated tube containing growth medium served as the reference.

A modified 21C medium (Table I) in which chloride salts replaced sulfate salts was used for the maintenance of working cultures and testing of cultures on the sulfur-stress assay. Carbon substrates were 0.5 % w/v glucose and 0.5% v/v glycerol. Inorganic sulfur (ion chromatography) in the sulfur-stress medium did not exceed 1 ppm sulfate. Tryptic soy broth (12 g/l) was used to grow cells prior to testing in the sulfur-stress assay.

The sulfur-stress assay served both as a screening device to detect organisms capable of desulfurizing model coal organosulfur compounds and to characterize the physiology of the desulfurizing cultures. The assay was a modification of the Kilbane and Biegla sulfur bioavailability assay in which organisms were screened for ability to grow under a sulfur-stress environment where the only plentiful source of sulfur was an organosulfur compound. We used DBT-sulfone because of its greater water-solubility than DBT. A water-saturated solution contained 0.166 mM DBT-sulfone and gave an optically clear medium, needed for accurate measurements of O.D. of cell suspensions. Moreover, DBT-sulfone did not yield Kodama-type products.

Cultures were first grown in tryptic soy broth (12 g/l) for a period of 48-72 hours, then washed twice with 0.01 N phosphate buffer and resuspended in sulfur-stress medium for 24 hours, at which point the O.D. of the cultures was about 0.3. Inoculum size was 0.5 ml/10 ml medium in culture tubes. The usual assay consisted of three sets of tubes, each containing sulfur-stress medium: one set contained organic sulfur compound; a second set contained no sulfur source (negative control); a third set contained 0.16 mM magnesium sulfate (positive control). Initial screening tests also included a fourth set of tubes, containing sulfate plus DBT-sulfone to ascertain possible inhibitory effects of DBT-sulfone; such effects were lacking for UMX3, UMX9, and IGT S-8, thus we eliminated the fourth test set for subsequent trials. Positive biodesulfurization was indicated by growth on DBT-sulfone relative to the negative control.

To isolate bacteria from a consortium that tested positive on the sulfur-stress assay, 12 gm/l of noble agar was mixed with sulfur-stress medium containing DBT-sulfone. More organic sulfur was added by 1 ml of ether sprayed on the plate from a stock solution of 90% wt/vol DBTS. Separation of organisms that could not utilize DBTS as a sulfur source was by the spread-plate technique on 1.4 g/l TSB with 14 g/l of agar.

Cultures capable of growth in the sulfur-stress assay were maintained on the same medium for working stocks. Transfers of stocks were made at least weekly. Cultures were also maintained on TSA slants stored at -5 oC, and in glycerol at -80°C.

Instrumental Analysis. Quantification of 2-phenylphenol (2-hydroxybiphenyl) in the sulfur-stress assays was conducted by gas chromatography. Disappearance of DBT-sulfone in the resting cell assays was also monitored by gas chromatography. The gas chromatograph was a Hewlett Packard 5890 series equipped with a 530 series capillary column with a splitless injector. The carrier gas was helium (15 ml/min). Hydrogen and compressed air flow were 30 ml/min and 135 ml/min respectively. Detection of substrate was by flame ionization detector (FID). Injector temperature was 260 oC, and detector temperature was 280 oC. Temperature programming for the oven was 100 oC initially with ramped at 1 oC/min until the maximum oven temperature of 255 oC was reached. Figure 9 depicts the standard curves for DBT-sulfone and 2-phenylphenol as measured by gas chromatography.

Mass spectrometry, courtesy of the Ole Miss chemistry Department, was used to confirm identity of desulfurization products. We used a Hewlet-Packard MSD-5971 GCC/MS coupled with an HP software library of ion spectra plus a customized library of our own standard compounds. Faculty of the Chemistry Department assisted with operation and interpretation of spectra.

Sulfate analysis was accomplished by the use of anion chromatographic procedures. This method is sensitive and specific for sulfate at concentrations of 0.5 parts per million up to 1000 ppm. We used a Dionex Model 2000I equipped with a HPIC-AS4A column and a conductivity detector.

The disappearance of DBT-sulfone or 2-phenylphenol was monitored by UV-spectrometry using a Milton Roy Spectronic 1201. This system was coupled to a Zenith XT computer that ran the Spec Scan Application software ver 1.01 (Milton Roy Analytical Products Division, 820 Linden Avenue Rochester, New York 14625). One cm matched-quartz cuvettes were used.

Samples for gas chromatography and GC/MS were extracted according to the following protocol: acidification by the addition of two drops of 6N HCL, extraction with dichloromethane equal to one half the total volume of the samples, removal of aqueous portion by phase-separating paper (Whatman 1PS). The extraction procedure was performed twice: extracts were pooled, transferred to 50 ml jars, and allowed to evaporate completely. Samples then were resuspended in 1 ml of dichloromethane. To each sample, 0.5 ml of a 100% thiantherene solution was added as an internal standard.

The extraction procedures for UV-spectroscopy: a 1.5 ml sample was centrifuged for 10 minutes at 10,000 x g, after which the clear supernatant was analyzed.

Comparative Tests on UMX3, UNX9, and IGT S-8: Cultures were grown for at least three transfers in sulfur-stress medium with DBT-sulfone and thiantherene as the sulfur sources. On the third transfer, cells were grown with DBT-sulfone as a sole sulfur source for a period of at least 48 hours and not more then 72 hours. Experimental variables were pH, temperature, different carbon substrates, addition of inorganic sulfur, and different organic sulfur substrates. The O.D. of the starter inoculum was adjusted to about 0.3. Inoculation volume was 1 ml into 9 ml of medium unless otherwise noted. Growth was monitored at 24 hour intervals. The concentration of DBT-sulfone and 2-phenylphenol was monitored by UV spectrometry unless otherwise noted.

Carbohydrate utilization was tested by the use of rapid CH test kit (API analytab products. Division of Sherwood Medical. 200 express Street, Plainview, New York 11803.). Organisms to be tested were incubated in sulfur-stress medium (50 ml) with 0.2 mM magnesium sulfate and no DBT-sulfone for 48 hours. Protocol for CH test: cells were aseptically harvested by centrifugation (15,000 x g for 15 min), washed once with 100 ml 0.01 N phosphate buffer, centrifuged (as above) and then resuspended in 100 ml of medium (magnesium sulfate instead of DBT-sulfone) with no added carbohydrate source. The final O.D. in the 100 ml of medium was adjusted to 0.05. About 1 ml of inoculum was placed in each of the 49 test sites on the API strip. Carbon utilization as evidenced by visual confirmation of growth was monitored at 24 hour intervals for 8 days.

Tests for degradation/utilization of 2-phenolphenyl or DBT-sulfone were made by supplying each compound (at 0.166 mM) as a sole source of organic carbon. Assays for 2-phenylphenol also contained 0.16mM magnesiumm sulfate, whereas DBT-sulfone assays contained no other source of sulfur. Inocula were taken from the third transfer cultures grown on standard sulfur-stress media (glucose and glycerol as organic carbon sources). Growth was monitored by optical density and substrate disappearance was monitored by UV spectroscopy.

To determine if the desulfurization process was inhibited by inorganic sulfur, we added 0.166 mM magnesium sulfate to the sulfur-stress medium. Growth and DBT-sulfone disappearance were monitored by standard techniques.

The effect of glucose and glycerol (as carbon substrates) on desulfurization activity was investigated by growing cultures for 140 hours in ssulfur-stress medium medium with a single carbon substrate (10 mM) of either glucose or glycerol. Growth and DBTS disappearance were measured by standard techniques after 140 hours.

Resting-cell cultures were defined as cells that had entered the stationary phase of batch culture. Stationary phase for the cultures occured after growth for 165 hours in flasks (non-agitated) that contained 25 ml of sulfur-stress medium medium with DBT-sulfone and thiantherene. For the resting-cell assays: cells were harvested in stationary phase and centrifuged at 15,000 x g for 20 min; the cell pellet was washed with 0.01 N phosphate buffer, then centrifuged again at 15,000 x g for 20 min, and resuspended in sulfur-stress medium without any type of sulfur source. Cells were allowed to incubate for 24 hours to expend endogenous energy reserves. Ten ml of cells (0.45-0.55 O.D.) then was layered onto a plate that contained DBT-sulfone and incubated for for 24 hours at 30 oC and 70% humidity. The DBT-sulfone plates were

prepared by this method: 1 ml of ethereal solution of DBT-sulfone (2.5 mg/ml) was sprayed on a 100 mm Petri plate, and the ether was allowed to evaporate leaving fine dispersed crystals of DBT-sulfone on the plate. Plates were extracted after 24 hours and analyzed GC.

To test biodesulfurization activity on other organosulfur compounds, a variation of the standard assay was used, whereby ethanol was used to enhance solubility of the compounds. Ethanol also served as organic carbon source (all three cultures-UMX3,UMX9, and IGT S-8-grew on ethanol). Stock solutions of DBT, DBT-sulfone, DBT-5-Oxide, Diphenyl sulfide, 3-Aminophenyl sulfone, Methylene blue, 3-Carboxyl thiophene, Benzothiophene, Thionapthalene,and trithiane) were prepared by the following method: the sulfur substrate (1.66 mM) was added to 5% v/v ethanol and water. The sulfur-stress medium was prepared without DBT-sulfone or a carbon substrate. The medium was completed by the addition of one ml of organosulfur stock solution to 9 ml of assay medium. Final ethanol concentration in each tube was 0.5 % v/v. Final concentration of the organic sulfur substrate was 0.16 mM in each tube. Sulfur substrates were considered growth supportive in the event of growth (0.1 O.D.) on the third transfer.

Sulfur-stress medium was used with 3-aminophenyl sulfone at 0.5 mM. Utilization of 3-aminophenyl sulfone was monitored by HPLC, and growth was monitored after 165 hours by standard technique.

Fatty acid profiles (MIDI analysis) were conducted by Craig Richardson at the University of Alabama, Huntsville, on the cultures UMX3, UMX9 and IGTS8 to aid in taxonomic identification.

TABLE I - SULFUR-STRESS MEDIUM

Table 1. Modified 21C medium

Stock Solutions: (500 ml)	
KH_2PO_4	69.25 g
Na_2HPO_4	50.25 g
NH_4CL	50.00 g
Salts Mix: (L)	
$MgCL_2$	11.2 g
$CaCl_2-2H_2O$	5.0 g
$(NH_4)_6Mo_7-4H_2O$	10.0 mg
$FeCl_3-6H_2O$	200.0 mg
$MnCl_2-4H_2O$	50.0 mg
EDTA	500.0 mg
Trace Metals: (100 ml)	
EDTA	150.0 mg
$ZnCl_2$	71.25 mg
$CuCL_2$	20.6 mg
$NaB_4O_7-10H_2O$	17.7 mg
Organic Sulfur Source: (L)	
Dibenzothiophene sulfone	50.0 mg
Carbon Source: (100 ml)	
D-glucose	10.0 g
Glycerol	10.0 ml
Vitamin Stock: (50 ml)	
Biotin	0.5 mg
Nicotinic acid	50.0 mg
Thiamine HCL	25.0 mg

Preparation

1. Measure 500 ml of saturated organic sulfur stock (liquid only).
2. To the liquid portion of the organic sulfur stock, add 20 ml of each phosphate stock solution and 10 ml of ammonium chloride stock solution.
3. Add 20 ml of salts mix, 1 ml trace metals, and 10 ml of each carbon substrate.
4. Add liquid portion of organic stock solution to make 1 L of medium.
5. Adjust the pH of the medium to 7.
6. Filter sterilize.

Conf paper
cycled separately.
AS

DATE
FILMED
9/23/94
END