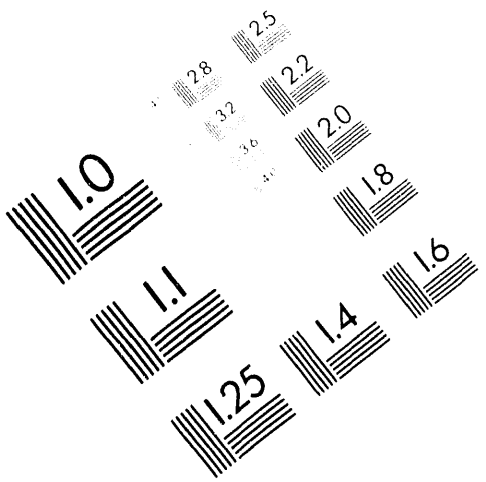

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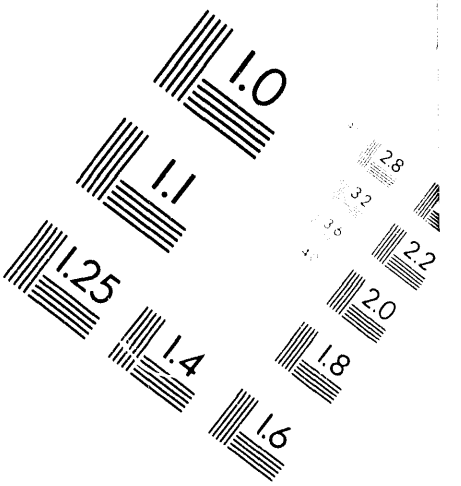

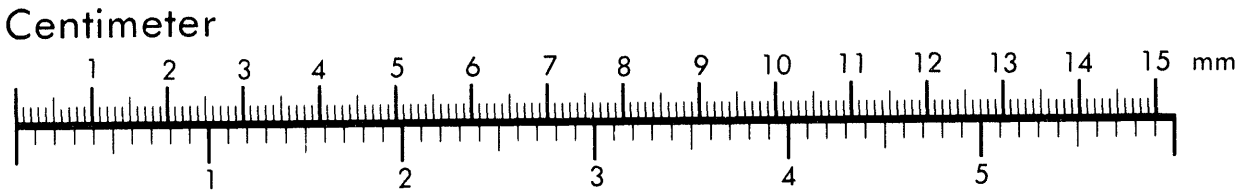

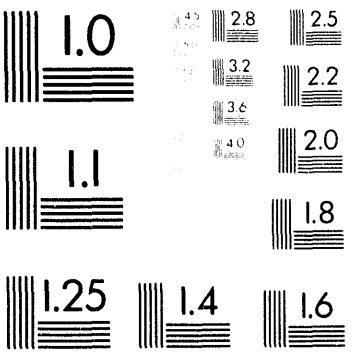

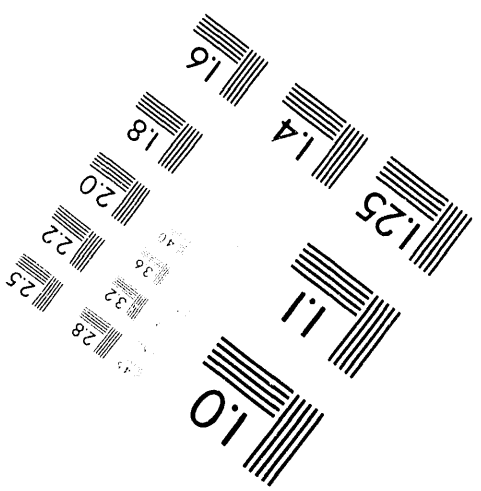

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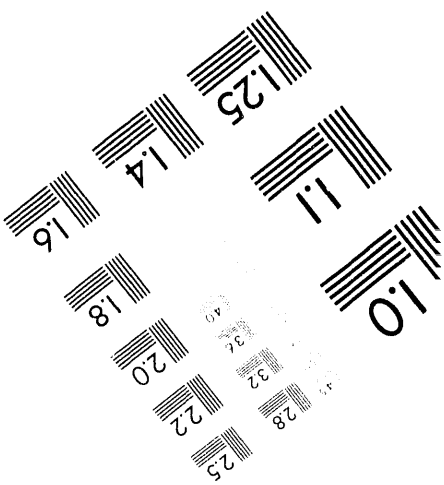


1 of 1

2

DOE/PC/92521--T146

TECHNICAL REPORT
December 1, 1993 through February 28, 1994

Project Title: **MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION**

DOE Grant Number:	DE-FC22-92PC92521(year 2)
ICCI Project Number:	93-1/5.4A-1P
Principal Investigator:	Dr. John J. Kilbane II, Institute of Gas Technology
Project Manager:	Dr. Ken Ho, ICCI

ABSTRACT

IGT has developed a microbial culture of *Rhodococcus rhodochrous*, designated as IGTS8, that is capable of specifically cleaving carbon-sulfur bonds in a range of organosulfur model compounds and is capable of removing organic sulfur from coal and petroleum without significantly sacrificing the calorific value of the fuel. Although IGTS8 possesses the ability to specifically remove organic sulfur from coal, a major research need is to develop improved strains of microorganisms that possess higher levels of desulfurization activity and therefore will permit more favorable biodesulfurization process conditions: faster rates, more complete removal, and smaller reactor size. Strain improvement is the single most important aspect to the development of a practical coal biodesulfurization process and accordingly is the focus of research in this project.

During this quarter we have constructed and successfully used a promoter probe vector using the β-galactosidase gene from *E. coli*. A chromosomal promoter library was constructed upstream from the β-galactosidase gene. Over 200 colonies were isolated that yielded β-galactosidase activity. One of these candidates designated pYGAL-A is the strongest *Rhodococcus* promoter isolated to date. Northern analysis of *Rhodococcus* gene expression has yielded some new and interesting results. Gel motility shift assays have been initiated to examine DNA binding proteins. Sequencing and transcription start site studies continue.

EXECUTIVE SUMMARY

The objective of this project is to develop derivatives of *Rhodococcus rhodochrous* IGTS8 that possess enhanced levels of desulfurization activity and to evaluate the ability of improved cultures to remove organic sulfur from coal.

The approach taken in this project to achieve that objective is to use genetic engineering/recombinant DNA techniques to produce strains of IGTS8 with improved desulfurization activity. IGTS8 has been shown to be capable of specifically cleaving carbon-sulfur bonds in a range of organosulfur model compounds and in coal. The challenge now is to develop a practical, economical coal biodesulfurization process. It is not only desirable, but absolutely essential, that improved derivatives of IGTS8 with greatly enhanced levels of desulfurization activity should be developed in order to allow briefer treatment times, more complete removal, smaller reactor volumes, and more favorable coal-to-bacterial culture ratios in the biodesulfurization of coal.

The focus of our research has shifted slightly this quarter from the past quarter in that in addition to promoter characterization we are also conducting experimentation to understand exactly how the desulfurization genes are being regulated. We intend to obtain microbial cultures with enhanced levels of desulfurization activity ultimately by replacing the promoter/regulatory sequences of the desulfurization genes with stronger promoters capable of achieving high levels of transcription/expression and by overcoming repression systems that may interfere with full desulfurization expression/activity.

We continue to uncover valuable information about genetic expression in *Rhodococcus*. We have isolated over 200 colonies that possess *Rhodococcus* promoters. One of these isolates is the strongest promoter known to *Rhodococcus* in our laboratory. We are in the process of sequencing and mapping transcriptional start sites of several of these candidates. We have conducted Northern analysis on the desulfurization genes. Northern analysis allows for extremely sensitive methodologies for the quantification of mRNA in a sample at a given time and for determination of the size of the mRNA. The amount of protein translated from the mRNA should be relatively equal to the transcript levels. We have found that this is not always the case. The mRNA levels of the *dsz* gene are higher than the coded protein observed. The three *dsz* genes appear to be transcribed as a single operon yet the observed levels of the three protein derived from open reading frame 2 (ORF 2) is substantially lower than proteins corresponding to ORF's 1 and 3. This suggests either a problem in the translation of ORF 2 or a problem in the recovery of ORF 2 protein from cells.

Northern Blot attempts to gauge the size of the *dsz* genes' transcript have yet to yield a distinct band even though distinct bands are observed for other RNA species. This suggests that the mRNA transcript of the *dsz* genes may be subject to rapid degradation. Gel motility shift assay have given some preliminary data on the presence of DNA binding proteins associated with the native desulfurization promoter.

Also during this past quarter several of the over 200 DNA fragments found to contain Rhodococcus promoters were characterized. A promoter probe vector designated pEBC26 was constructed that can replicate both in *E. coli* and in *Rhodococcus* hosts and possess a multiple cloning site upstream from a promoterless lac Z gene which encodes β-galactosidase. DNA fragments derived from the chromosome of *Rhodococcus rhodochrous* IGTS8 were ligated into pEBC26. The ligation mixture was electroporated into a Rhodococcus host and colonies that contained a cloned DNA fragment encoding a promoter were detected by observing the conversion of the chromogenic substrate for β-galactosidase, X-gal, from colorless to blue. DNA fragments that encode promoters were found to range in size from about 100 base pairs (bp) to over 2000 bp. About a dozen of the small DNA fragments encoding promoters have been subsequently subcloned into other *E. coli* cloning vectors to facilitate DNA sequence determinations and/or transcriptional start site determination studies. The DNA sequences of four of these promoter fragments have been determined bringing the total number of DNA sequences in our data files that are known or suspected to encode *Rhodococcus* promoters to ten. Two experimental approaches are being utilized to determine the transcriptional start site of these promoters: primer extension studies, and ribonuclease protection studies. Some initial technical difficulty encountered in these studies has been traced to problems of RNA purity and integrity so that currently transcription start site determination studies are proceeding well.

An examination of all of the DNA sequences known to encode *Rhodococcus* promoters using the computer program (PC GENE, Intelligenetics, Inc.) designed for this purpose was unable to specifically define the location of promoters within these DNA sequences. In general, the currently available computer programs are not very effective in identifying promoters. Even for promoters derived from *E. coli* where the database is immense, computer programs routinely fail to recognize some sequences known to encode promoters. The ability of existing computer programs to identify promoters in DNA sequences derived from less well studied microbial species, such as *Rhodococcus rhodochrous*, is almost non-existent. The problem stems from the fact that existing computer programs examine DNA sequences to find regions that match precisely, or nearly precisely, to a given DNA sequence specified in the search program. Unfortunately the DNA sequences of known promoters in *E. coli* and other species display a great deal of diversity

so that while promoter sequences all have much in common on a functional level (i.e. the recognition and binding of RNA polymerase) they do not have nearly as much in common at the level of DNA sequence comparison. A way to address this problem has been developed in the computer science department at the Illinois Institute of Technology through the creation of a computer program, designated as the W-curve, that can convert DNA sequence data into structural data. Thus by using the novel W-curve computer program it may be possible to detect structural similarities between rather different DNA sequences. We have begun to use the W-curve program to examine DNA sequences known to encode promoters derived from *Rhodococcus rhodochrous*, *E. coli*, and other species. The results of preliminary tests are highly encouraging.

OBJECTIVES

The objective of this research is to develop improved derivatives of *Rhodococcus rhodochrous* IGTS8 with greatly enhanced levels of desulfurization activity and to evaluate the ability of improved cultures to remove organic sulfur from coal. Specifically, recombinant DNA techniques will be used to identify DNA fragments that encode desulfurization genes which will be subsequently manipulated to achieve enhanced expression of these genes.

INTRODUCTION AND BACKGROUND

There are numerous physical, chemical, and microbiological techniques that can effectively remove inorganic sulfur from coal prior to combustion. Moreover, there are physical and chemical techniques for pyrite/ash removal that have been successfully commercialized and are routinely employed in the coal industry. While there are technologies capable of removing organic sulfur from coal prior to combustion, no commercially viable technology currently exists. The removal of organically bound sulfur from coal by physical/chemical techniques requires harsh conditions as compared with microbiological techniques; therefore a microbiological approach may result in more favorable economics than currently available technologies.

Unfortunately, with nearly all Illinois coals, physical cleaning alone will not produce a coal that complies (if burned without additional sulfur removal from the combustion gases) with New Source Performance Standards (NSPS). To remove the additional organic sulfur, another method such as microbiological treatment that breaks the chemical bonds binding the sulfur to the carbon must be developed. A coal cleaning strategy employing only physical or chemical cleaning methods to remove as much of the pyrite and other mineral matter as economically possible will be unlikely to produce a product capable of meeting emission standards. A separate step such as microbial cleaning will likely be needed that focuses on the removal of the organic sulfur. A microbial culture, *Rhodococcus rhodochrous* IGTS8, has been identified that is capable of selectively cleaving carbon-sulfur bonds in model compounds and in coal; however, the level of expression of desulfurization enzymes by IGTS8 is low. Improved bacterial cultures are needed if a practical coal biodesulfurization process is to be developed. The goal of this project is to use recombinant DNA techniques to develop microbial cultures with enhanced desulfurization abilities.

EXPERIMENTAL PROCEDURES

Restriction digests were performed at 37°C for 1 hour at a concentration of at least 1 enzyme unit per microgram of DNA (Gibco BRL, CA). Dephosphorylation of vectors was performed with HK phosphatase (Epicenter Technologies, WI) as instructed. Ligation was accomplished using T4 DNA ligase (Gibco BRL, CA). Southern hybridization and colony hybridization were performed by the published methods in Perbal, A Practical Guide to Cloning, pp. 424-428 and 422-423. DNA probes were labelled using ^{32}P dCTP and a nick translation kit (Amersham, IL). Polymerase Chain Reaction (PCR) was accomplished using published methods and apparatus of Perkin Elmer, CT. Sequencing reactions were carried out using the Sanger chain termination method with Sequenase 2.0 (USB, OH). Sequencing gels were run on a 7M urea, 6% Long Ranger gel (AT Biochem, PA) in 1XTBE buffer for 2 hours at 1500 volts with the Sequigen (Bio-Rad Laboratories, CA) sequencing apparatus. DNA sequences were computer analyzed with the PC Gene program (Intelligentics, Inc., CA).

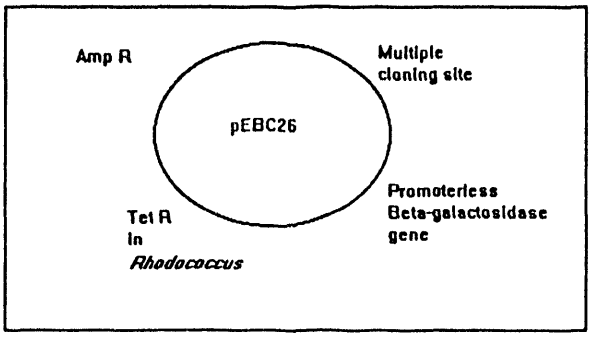
RNA isolation and characterization protocols were as follows:

Formaldehyde denaturing gels were made with 4.2g Agarose in 304.5 ml H_2O. Heat to dissolve agarose. Cool to ~55°C. Add 35 ml 10x MOPS buffer and 10.5 ml 37% formaldehyde. Premix 5ul 10x MOPS buffer, 8.75ul 37% formaldehyde, and 25ul formamide. [38.75ul] Add RNA to 50ul. Heat for 15 mins at 55°C. (10x MOPS = 800 ml H_2O; 41.8g MOPS; Adjust pH to 7.0; 16.6 ml of 3M Na Acetate; 20 ml .5M EDTA(pH 8.0); DEPC treatment.)
RNA Isolation protocol:
Grow cells to desired cell density. Harvest cells in centrifuge. Resuspend cell pellet in 100mM Tris-100mM EDTA. DEPC treated. pH = 8.0. Add VRCs to final concentration of 10mM. Freeze overnight at 70°C. Thaw cells. Add equal volume phenol\chloroform\isoamyl alcohol, 25:24:1. Add 1% SDS w/vol. Add 20mM DTT. Disrupt culture with glass beads for several minutes. Centrifuge for 15-30 mins. Remove aqueous layer. Phenol\chloroform 2x's. Chloroform\Isoamyl alcohol 1x. Isopropanol precipitation. 70% Ethanol wash. DNAse I treatment if necessary.
Radiolabelling Total RNA for subtractive hybridization:2.5 to 10 μg total RNA. 7.5 μl 10x T4 kinase buffer.66 pmol [γ-32P]ATP (200 μCi).25 to 50 U T4 polynucleotide kinase
H_2O to 75 μl. Incubate 37°C for 30 minutes. Prehybridization\Hybridization solution 50% formamide, 5% SSPE, 2x Denhardt's reagent, 0.1% SDS.

Vanadyl ribonucleoside complexes were purchased from Gibco BRL, MD and Trisolv was purchased from Biolecx Laboratories, Inc., TX. Primers for primer extension and RNA for subtractive hybridization were labelled with gamma ^{32}P dATP (Amersham, IL) and T4 polynucleotide kinase (Promega, WI). Primer extension experiments were performed using the methods of Ausubel et al., Current Protocols in Molecular Biology, pp. 4.8.1-4.8.3; Sambrook et al., Molecular Cloning A Laboratory Manual, pp. 7.79-7.83; and Forsman, M. et al. in Molecular Microbiology (1989), Vol. 3(10), pp. 1425-1432. Avian-reverse transcriptase was purchased from Promega, WI. Subtractive hybridization experiments were carried out according to the method of Apte, S. K. et al. in Plant Molecular Biology (1990), Vol. 15, pp. 723-733.

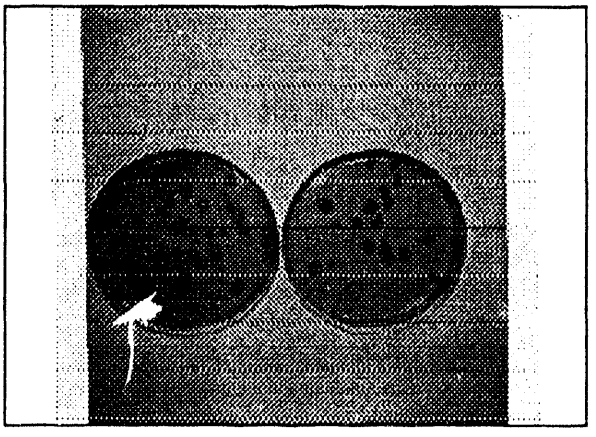
RESULTS AND DISCUSSION

Figure 1 shows the promoter probe vector pEBC26. It was constructed using the β-galactosidase gene from the *E. coli* plasmid pSV-gal and a tetracycline resistant derivative of pRF29. pEBC26 has a multiple polylinker site upstream from the promoterless β-galactosidase gene.



β-galactosidase (Lac Z) is used as a reporter gene in many bacterial and eukaryotic systems. An easy assay to gauge β-galactosidase activity is available and simply uses the production of varying intensities of blue colored colonies to monitor promoter strength. The β-galactosidase enzyme catalyses the degradation of the colorless substrate 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) to yield a blue colored product. Weak promoters yield a light blue color whereas stronger promoters yield dark blue colonies. On the right side of the photograph below (Figure 2) *Rhodococcus* colonies growing in the presence of X-Gal show expression of the β-galactosidase gene regulated by the *Rhodococcus* promoter isolated in our laboratory designated pYGAL-A. The left side of the photograph shows normal *Rhodococcus* colonies.

Control



pEBC26 with *Rhodococcus* promoter

Characterization of *Rhodococcus rhodochrous* IGTS8 promoter fragments in pEBC26 was initiated. *Rhodococcus* colonies that exhibited the deepest blue color on the substrate X-gal were picked and the sizes of the promoter fragments was determined. The promoter fragments range in size from about 100 bp to over 2000 bp. The smallest of the fragments were chosen for the first round of DNA sequencing. The larger fragments will be addressed after removing unnecessary DNA leaving only the smallest fragment that retains promoter function. Four of these small promoter fragments were sequenced. They have been designated YUC18, YUC21, YUC22, and YGALK. The DNA sequence is shown in Figure 3. Confirmation of these sequences and computer analysis will be completed next quarter.

YUC18
CGCCTGCAGG CATGCAAGCT TTTTGCAAAA GCCTAGGAGG CCGCGGCCGC
TCTAGAACTA GTGGATCGCC ACCCAGTAGA AGAGATAGCC GCTGAGGATG
AAGTGCAGGT TCATCAGCAG GTGAGCGCTG TGCAGTCGTA CCGTCGCTAC
CGTAGATTCC GCCGAGTCAT ACAGCGCGTA GAAA

YUC21
AGCTTTTTGC AAAAGCCTAG GAGGCCGCGG CCGCTCTAGA ACTAGTGGAT
CCGTCTCATC GCCACCGGTG GGATGGGTCA GGTGTGGGAA GCCACCGACT
CCCGGCTCAA CCGTCGCGTC GCGTCACATG GTACTCAAGT CCGAGTTCTC
TTCCGA

YUC22
ATATCGAATT CCTGCAGCCC GGGGGCAATC GATGACGTGC TGATAGCGCT
CGGCTGCGTC CTCGGTGTAG AAGAGGTCGT CCTCGCATTC GTACCGGCGG
GAGAGTGCAC CGTTCAACAG TTCGGTGATC CGAGTTCTCG C

YGALK
TCGAGGTCGA CGGTATCGAT AAGCTTGATA TCGAATTCCT GCAGCCCGGG
GGATCTTGTT CGCGATGCCG GCTACTGGCG TTCGCTCGTG GATGACGATC
GACGGAGATG GCGTCCGCAG TGGGCACGTC GCCGGATATC CAGTTCACCG
CGTAACTAGC TTCGGACAGC TGTA

Figure 3

Mapping of the start site of Rhodococcus promoters through primer extension is continuing. Four Rhodococcus promoters have been chosen for these studies at this time, other promoters of interest will be analyzed using this method when the system is optimized. Difficulties with these experiments has been traced to problems of RNA purity and integrity so that currently transcription start site determination studies are proceeding well. Preliminary results suggest that the transcriptional start site of pYUCE (whose DNA sequence was reported previously) is located at position 47 of that sequence. This result will be confirmed during the next quarter.

Ribonuclease protection is another means of determining RNA trasnscript sizes. This method utilizes sequence specific hybridization probes that are prepared by cloning the probe sequences downstream of bacteriophage promoters T7 or SP6. These promoters are found in the plasmid vector pGEM-3Z and pGEM-5Z (Promega, WI). The plasmid is cleaved and the DNA transcribed with bacteriophage RNA polymerase which transcribes the cloned sequence into an RNA that extends from the initiation site of the promoter to the end of the DNA fragment. This labelled RNA is hybridized to the RNA of interest. All unhybridized material is digested with RNAase. The labelled RNA:RNA hybrid is then detected and quantified on a sequencing gel. The pGEM vectors have been purchased and DNA promoter fragments are being subcloned into these vectors.

Determination of the rules for a consensus sequence of *Rhodococcus* promoters is very important. To begin defining the rules a large database of *Rhodococcus* sequences containing known promoters (defined by expression but not exact promoter location) is being actively compiled. The sequences of six *Rhodococcus* promoter areas were added to the collection. Also another 14 sequences from a closely related organism, *Corynebacterium* were added as well. Computer program manipulation is essential for this identification; however, computer identification is limited by the fact that the program only searches for *E. coli* promoter consensus sequences. Even within known E. coli promoter sequences a large degree of variability exists such that the computer program cannot identify all of these promoters since the program can only search for homologies.

A way to address this problem has been developed in the computer science department of the Illinois Institute of Technology by the creation of a computer program, designated as the W-curve, that can convert DNA sequence data into structural data. This program plots each base so that similarity patterns in large DNA sequences can be identified. Figure 4 is an example of a typical plot using this program. Patterns that emerged are highlighted. We are beginning to use this program to analyze smaller fragments of DNA containing promoters. This program has not been used with small DNA sequences; however, preliminary results are encouraging. Figure 5 is the plot of 50 base pairs of the *E. coli* promoter fragment for the vitamin B12 receptor gene. Interestingly there is a valley corresponding to the -35 region of the promoter and a plateau corresponding to the -10 region which are known to be important regions to functional *E. coli* promoters. Many promoter fragments must be carefully examined to establish true W-curve patterns for promoters in *E. coli*, *Rhodococcus* and other species.

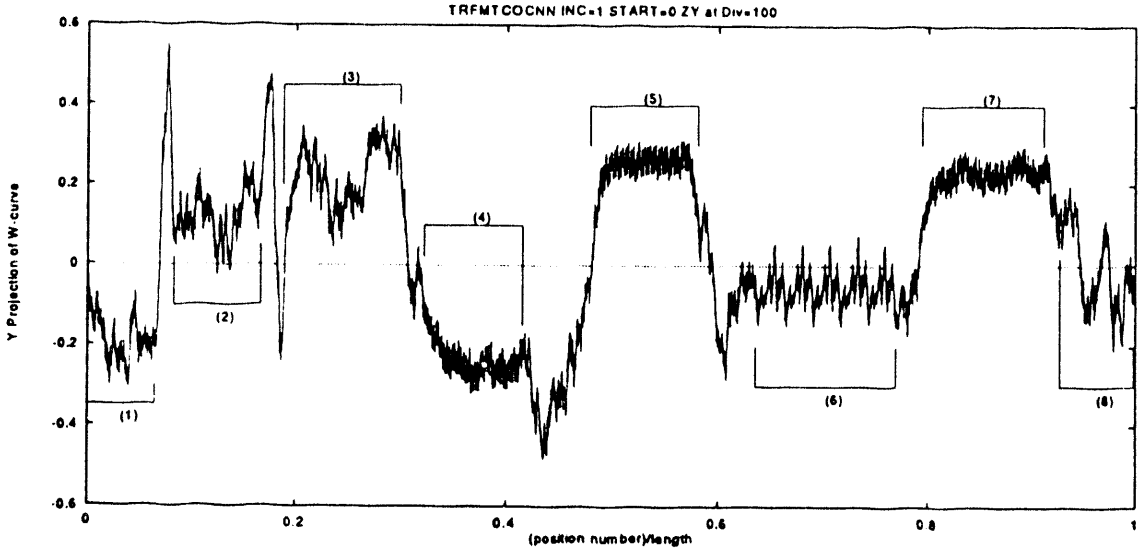


Figure 4

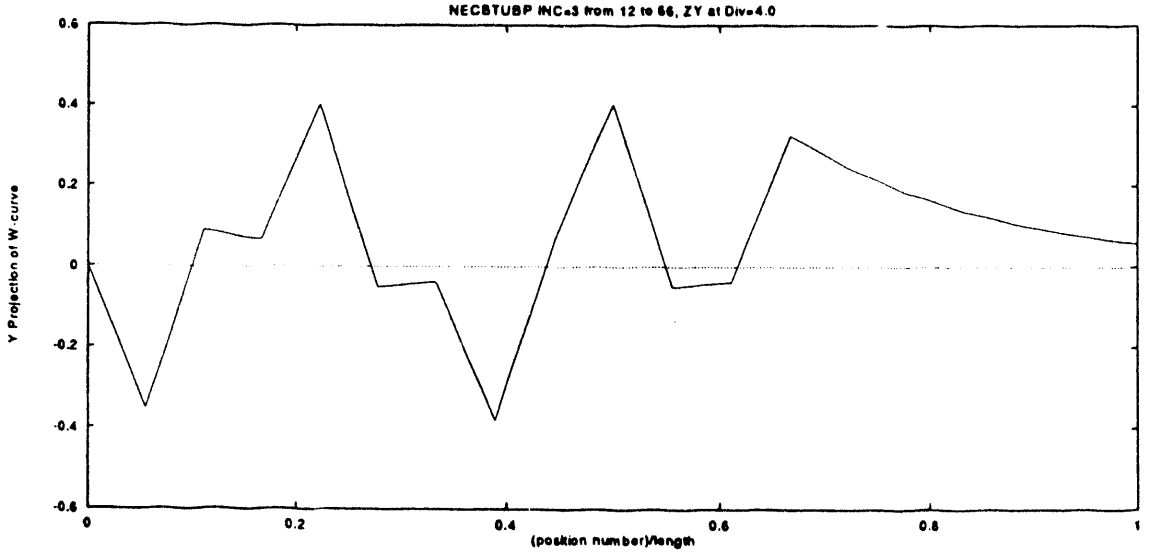


Figure 5

Figure 6 is an autoradiograph from a denatured RNA gel that was blotted to a nylon filter and probed with a radiolabelled portion of the desulfurization genes. Lanes 1 through 6 contain total RNA isolated from IGTS85 cells grown on DBT. The most remarkable thing to comment on is the extent of the smearing of the probed bands in contrast to the quality of the RNA gel in Figure 7. The ribosomal bands in the photograph are very distinct suggesting good quality RNA, yet the probed desulfurization mRNA is smeared. This suggest that the desulfurization mRNA is subject to rapid degradation.

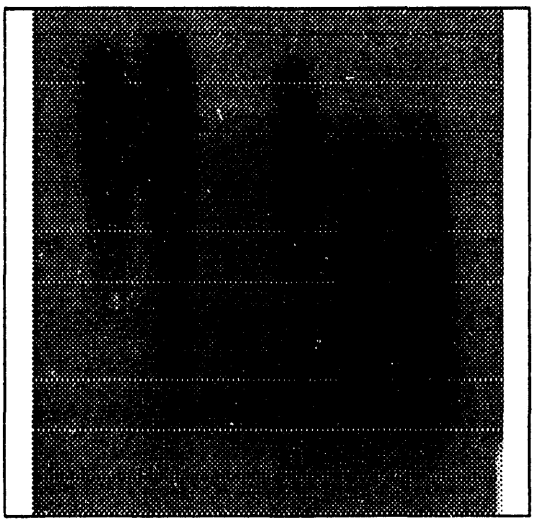


Figure 6

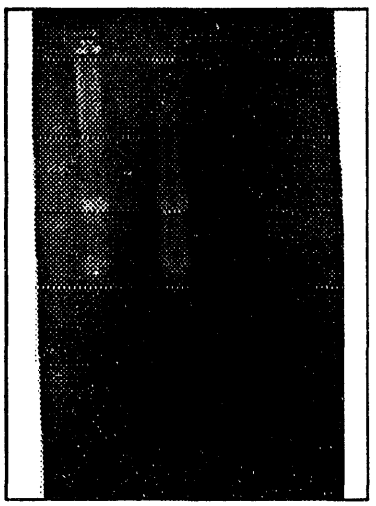


Figure 7

Figure 8 is from an autoradiograph of a dot blot performed on nylon filters. The RNA samples were treated with DNAseI, to insure purity, and loaded onto the filter. They were probed with a portion of the desulfurization gene. Signal intensity (How dark the region is) should correspond to desulfurization mRNA quantities in the sample. Sample number 1 and 2 are RNA harvested from IGTS85 cells grown to log phase in BSM containing dibenzothiophene(DBT). Samples A and B are pregrown IGTS85 cells switched to DBT for 1 hour. This technique allows us to quantify desulfurization mRNA transcripts in IGTS85 cells grown using varying conditions and to ultimately use this data to optimize desulfurization.

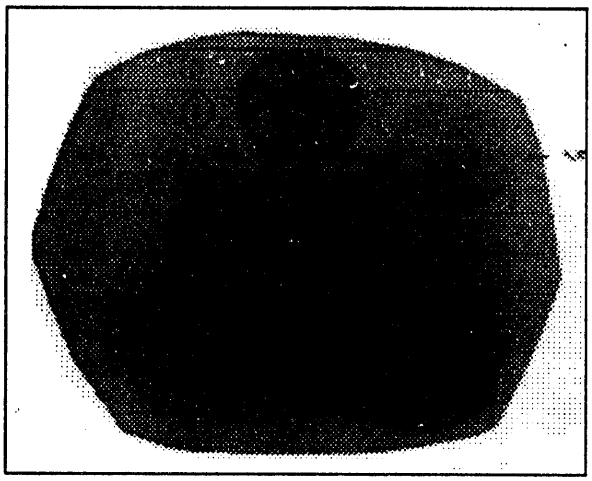


Figure 8

Figure 9 is an autoradiograph showing colonies with higher level of transcription than the actual protein being made (as measured by the amount of β-galactosidase activity). Fifty colonies were grided on to a nitrocellulose filter. The colonies were chromosomal promoter fragments cloned upsteam of the β-galactosidase gene in the vector pEBC26. The colonies ranged from light blue to dark blue (Levels of β-galactosidase protein being made). The image in Figure 9 is a RNA colony hybridization technique where mRNA quantities are determined. The colonies 5,6,16 and 19 did not show a dramatic blue color, they were light to medium blue. Colony numbers 7,8, and 14 were darker blue. We would suspect the darker colonies to yield higher levels of mRNA transcripts and thus darker signal intensity on the filter than that of the lighter colonies. Although a slight color change was noted between all the colonies, the transcript levels (mRNA) differs considerably. The other colonies on the plate showed little to no mRNA transcripts in this experiment.

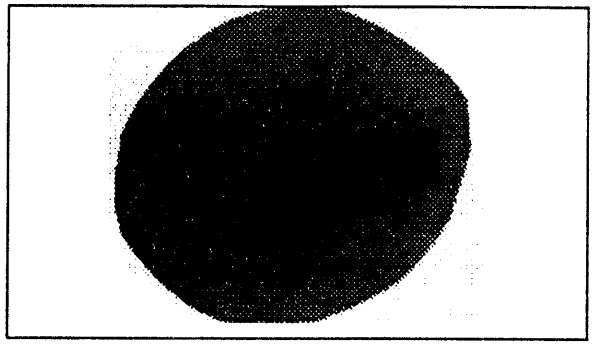


Figure 9

Gel motility shift assays search for DNA binding proteins. When a DNA binding protein binds radioactive labelled DNA its motility through an acrylamide gel is altered because DNA-protein interactions migrate slower than just DNA. Many conditions must be met for a proper DNA-protein association to form such as: salt concentration, protein concentration, DNA concentration and many other critical parameters. Figure 10 is a autoradiograph of a gel motility assay experiment that titrated increasing levels of crude protein concentration from IGTS85 versus otherwise standardized conditions. The native promoter from the desulfurization genes was examined for any interactions with DNA binding proteins. Lane 1 contains control DNA (no protein). Lane 2 contains 2μg IGTS8 crude protein extract. Lane 3 contains 5μg IGTS8 crude protein extract. Lane 4 contains 7.5μg IGTS8 crude protein extract. Lane 5 contains 10μg IGTS8 crude protein extract. Lane 6 contains 20μg IGTS8 crude protein extract.

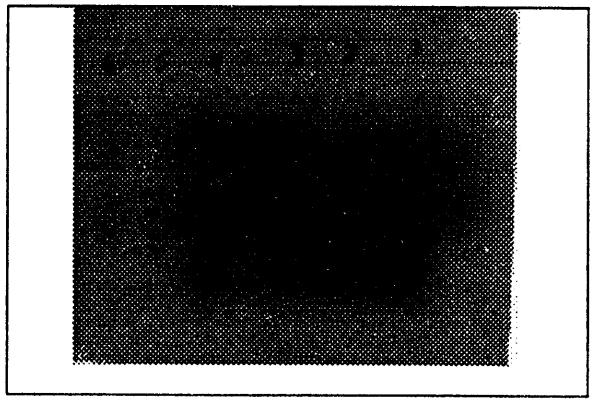


Figure 10

Conclusions and Recommendations

Several possible strong promoters have been isolated and are in the process of being analyzed. When these promoters have been characterized for inducibility, strength, transcriptional start sites and other physical properties, they will be placed in front of the desulfurization genes and expression will be monitored. Improved promoter probe vectors have been constructed, allowing a conclusive screen of all putative *Rhodococcus* promoters. With the improved methodologies in the handling of *Rhodococcus* RNA, we have begun to gauge promoter expression using Northern blots and this research should continue. Analysis of DNA binding proteins using gel motility shift assays has yielded some results in preliminary experiments and should provide detailed understanding of the mechanisms that control the desulfurization genes expression. Our research is continually providing insightful information about genetic expression in Rhodococcus, we feel confident that all of our endeavors will soon provide enhanced genetic expression of the desulfurization genes.

PROJECT MANAGEMENT REPORT
December 1, 1993 Through February 28, 1994

Project Title:	**MOLECULAR BIOLOGICAL ENHANCEMENT OF COAL BIODESULFURIZATION**
DOE Grant No.:	DE-FC22-92PC92521 (Year 2)
ICCI Project No.:	93-1/5.4A-1P
Principal Investigator:	Dr. John J. Kilbane II, Institute of Gas Technology
Project Manager:	Dr. Ken Ho, ICCI

COMMENTS

The project is proceeding as scheduled. The computer analysis using the W curve for the examination of DNA sequences that encode Rhodococcus promoters will be performed by a graduate student at IIT as a component of his thesis and at no cost to this project.

EXPENDITURES - EXHIBIT B

CUMULATIVE PROJECTED AND ESTIMATED EXPENDITURES BY QUARTER

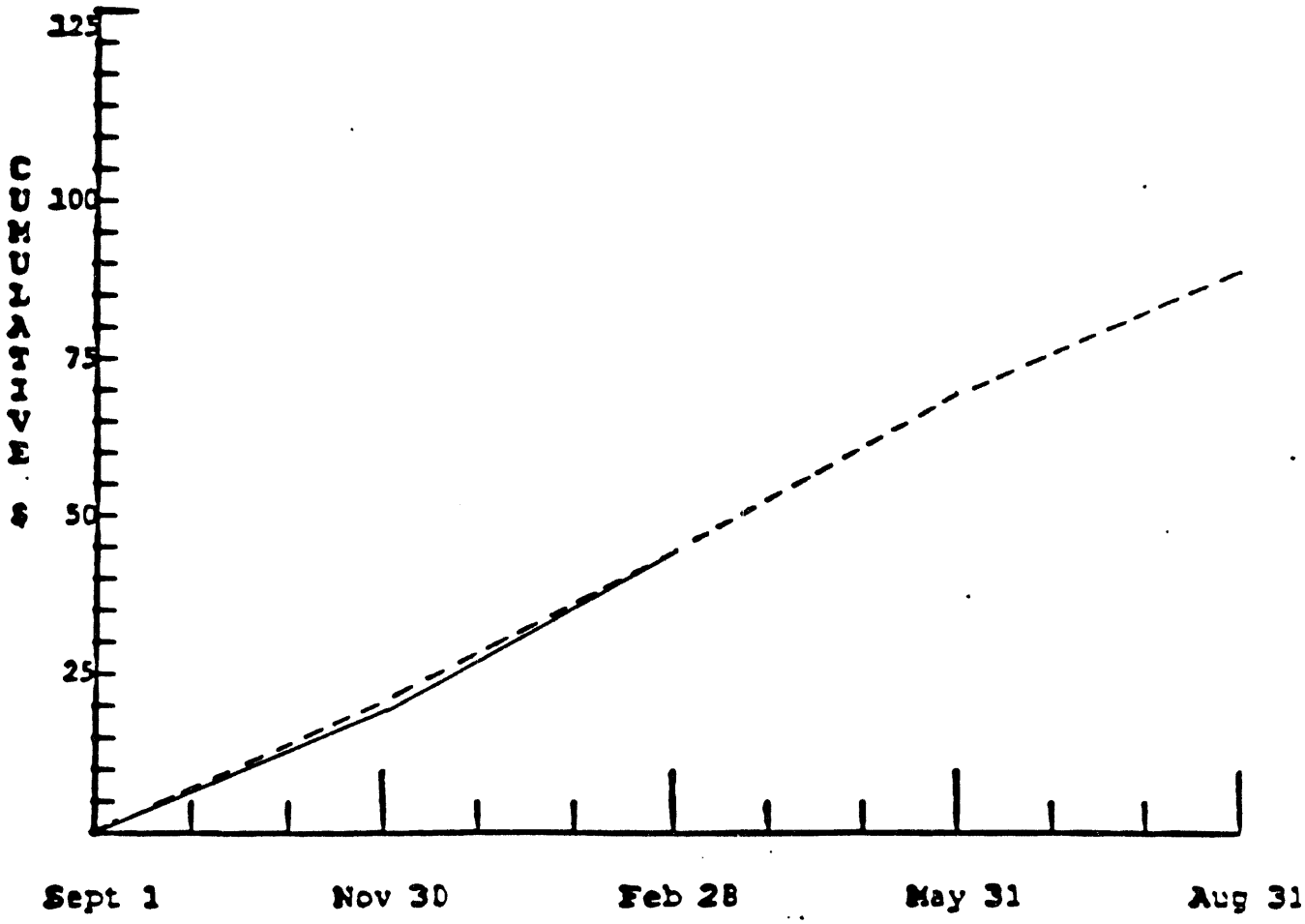
Quarter*	Types of Cost	Direct Labor	Fringe Benefits	Materials & Supplies	Travel	Major Equipment	Other Direct Costs	Indirect Costs	Total
Sept. 1, 1993 to Nov. 30, 1993	Projected	6,270		1,500				11,347	19,117
	Estimated	6,000		1,500				10,873	18,373
Sept. 1, 1993 to Feb. 28, 1994	Projected	12,540		8,500				23,905	44,945
	Estimated	13,984		5,200				25,716	44,900
Sept. 1, 1993 to May 31, 1994	Projected	18,810		12,500	1,630			36,162	69,102
	Estimated								
Sept. 1, 1993 to Aug. 31, 1994	Projected	25,080		15,000	2,040			47,818	89,938*
	Estimated								

*Cumulative by Quarter

*Requested from ICCI

CUMULATIVE COSTS BY QUARTER - EXHIBIT C

Molecular Biological Enhancement of Coal Biodesulfurization
(IN THOUSANDS)

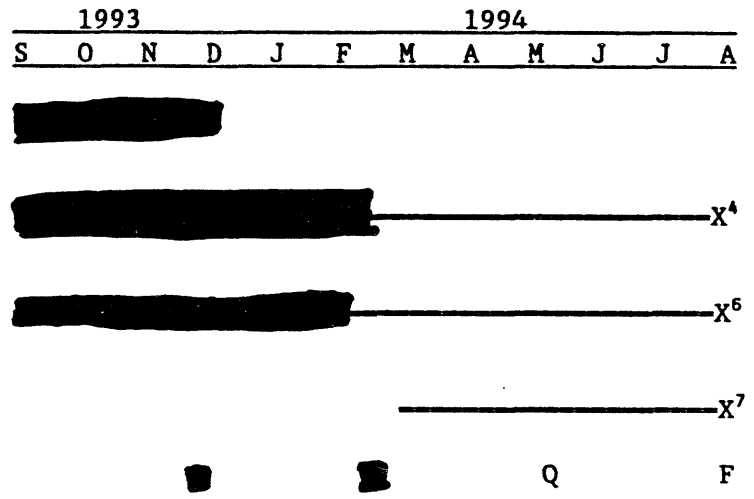


Months and Quarters

O = Projected Expenditures ----------

Δ = Actual Expenditures ——————

Total ICCI Award $_____



X = Milestone
Q = Quarterly Report
F = Final Report

[1] The role of each dsz gene is determined.

[2] The expression of dsz genes with at least one alternative promoter is accomplished.

[3] The 16S-RNA promoter is cloned and sequenced.

[4] Genetic manipulation experiments are completed.

[5] Preliminary results are available regarding the isolation of improved cultures by non-recombinant DNA techniques.

[6] Strain improvement experiments using non-recombinant DNA techniques are completed.

[7] Coal/coal product biodesulfurization data is obtained and analyzed.

DATE

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7/6/94

END