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RESEARCH TITLE: Biocatalytic Removal of Organic Sulfur from Coal

PRINCIPAL INVESTIGATOR: Dale A. Webster[1]

CO-PRINCIPAL INVESTIGATOR: John J. Kilbane II[2]

STUDENTS AND THE DEGREE FOR WHICH THEY ARE REGISTERED: Sandip B. Patel, M.S. in Biology; Chae-Ok Yun, Ph.D. in Biology

INSTITUTION / ORGANIZATION: Illinois Institute of Technology[1]
and Institute of Gas Technology[2]
Chicago, IL 60616
(312) 567-3491

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FINAL REPORT
Note: The Final Quarterly Report is Incorporated Here

OBJECTIVE: To characterize more completely the biochemical ability of the bacterium, *Rhodococcus rhodochrous* IGTS8, to cleave carbon-sulfur bonds with emphasis on data that will allow the development of a practical coal biodesulfurization process.

WORK DONE AND CONCLUSIONS: There are no commercially useful chemical or physical procedures for the removal of organic sulfur from coal. An alternative would be to use a biological system. The purpose of the research reported here was to investigate this alternative. We used the microbe, *Rhodococcus rhodochrous* IGTS8, which can remove sulfur from various model organosulfur compounds and coal. Since substrates for the desulfurization enzymes are hydrophobic in nature, one of our goals was to develop relatively nonaqueous conditions for the biological desulfurization system. Dibenzothiophene (DBT) was used as the model organosulfur compound for most of our desulfurization studies.

Freeze-dried cells of *Rhodococcus rhodochrous* IGTS8 having 90% of the activity of wet cells can be obtained (although we frequently achieved less than this because our freeze-dryer is a small unit with inadequate cooling and vacuum). These freeze-dried cells can be stored in the freezer for at least four months with little or no loss of desulfurization activity (Table 1). Most of our data were quantitated using both the Gibb's assay, a colorimetric method to measure the product of the desulfurization, 2-hydroxybiphenyl (2-HBP), and HPLC which allows the simultaneous measurement of both 2-HBP formation and DBT removal. There was good correlation between the two methods although the Gibbs assay always showed yields of 2-HBP about 30% less than those obtained from HPLC (Table 2); we have no explanation for this phenomenon.

One of the first variables studied was the how much water was necessary in oil/water mixtures for good desulfurization to occur. We used hexadecane as our oil. The results indicated that 50% oil/50% water gave the best activity (Fig. 1), and we used this ratio for most of our subsequent studies. Later, however, it was discovered that it was not the oil/water ratio that determined the maximum amount of oil that could be used, but rather there is a minimum water requirement. This was 1.25 ml per gram of freeze-dried cells, and when this hydration requirement is met, activity was maximal at 80% oil and high activity even in 90% oil was oberved (Fig 2).

As reported briefly in previous Quarterly Reports, the addition of surfactants stimulated activity; this will be discussed in detail here. The effect of two surfactants at different oil/water ratios is shown in Fig. 3. The optimum concentration for stimulation of desulfurization activity was examined for three detergents. Oleic diethanolamine was optimal at 5% (Fig. 4), Triton N101 at 0.2% (Fig. 5), but glycerol monooleate (GMO) plus EM600 (1/1) had little effect on activity at concentrations between 0.5% and 10% (Fig. 6). The order of addition of the four components was also important, the best results being obtained when the oil, water (basal salts medium, BSM), and surfactant were first emulsified together and then the lyophilized cells added (Table 3, order of addition # 5). This has the additional advantage of easier mixing and fortunately would be the most convenient for a practical desulfurization system.

The kinetics of desulfurization of DBT by *Rhodococcus rhodochrous* were also investigated. In an aqueous system, initial rates were about the same during the first three hours for freeze-dried and wet cells and both reached a plateau after four hours, the latter cells having reached a slightly higher level of desulfurization (Fig. 7). In a 50% oil/water emulsion, freeze-dried cells reached a slightly higher level of desulfurization than wet cells even though their apparent initial rate was less (Fig. 8). However, when a correction is made for the loss of activity that occurred during freeze-drying, the freeze-dried cells had nearly twice the desulfurization activity. The leveling-off of activity observed in both Figs. 7 and 8 may be due to product inhibition, but this needs further study.

The ability of the desulfurization enzymes to cleave carbon-sulfur bonds raised the possibility that they may also be capable of cleaving carbon-selenium bonds since selenium is an analog of sulfur. Since *Rhodococcus rhodochrous* IGTS8 can remove sulfur from cystine, Se-cystine was incubated with the cells and the results analyzed using HPLC. This experiment included minus substrate and minus cells controls, and also cystine and cysteine as positive controls. Cysteine, cystine, and Se-cystine all decreased in the biotreated samples (Table 4) indicating that the desulfurization enzymes can also cleave carbon-selenium. Two potential products of these reactions, alanine and serine, were included in the HPLC analysis but were not detected in the assay mixtures. This does not exclude them being products as they may have been rapidly taken

up and utilized by the cells as soon as they were produced.

The above research was the work of student Sandip Patel who completed the requirements for the M.S. degree in October, 1994. Another approach for increasing the desulfurization activity of the IGTS8 cultures is to produce strains genetically that have higher activity. This approach is being pursued by student Chae-Ok Yun, and it is anticipated that this work, the subject of her dissertation, will be completed in the summer of 1995. The progress achieved to date and the directions that it will take are reported here.

The goal of this research is to achieve strain improvement by introducing a stronger promoter using genetic engineering techniques. The promoter regulates the transcription of the genes for the desulfurization enzymes, and a stronger promoter would up-regulate the expression of these genes, resulting in cells with higher desulfurization activity. Promoter probe vectors are used to identify and isolate promoters from a DNA library of the experimental organism. Promoter-probe vector pEBC26 was used for this research because it functions in both *R. rhodochrous* and *E. coli*; the latter organism is the one most commonly used for genetic engineering because it has been so well studied. *Vector pEBC26* contains the β-galactosidase gene without its native promoter. Thus, the β-galactosidase gene is expressed when a DNA fragment containing a promoter from *R. rhodochrous* is cloned in this plasmid, and the level of expression can be tested with a simple colorimetric assay for β-galactosidase. The levels of this enzyme in candidates for strong promoters in *Rhodococcus rhodochrous* IGTS8 is shown in Table 5. Further studies are required before we can test their ability to express the desulfurization enzymes at a higher level. Thus, the most promising candidates were selected from colonies exhibiting high β-galactosidase, analyzed by agarose electrophoresis, and then subcloned into pUC19, an *E. coli* plasmid, for nucleotide sequencing of the inserted DNA fragments. We have currently finished the sequence of eight of the putative promoters from *R. rhodochrous*. One, in the plasmid designated pYUCE, is shown here.

GGGCT CGCGA GTGTC GGTGT CGCGT CGGCA ACCTC CTGCA TACTC GGGAG
TCCAC TCGGC AGTCA CACCG GCCGA AGATG ACGCC GTGCC ACGAT AGCCG
CCGTG GTCTG GACTA CTCGA CTGAT CGAGC ACCAC CTGTT CCCGA TCCCC

Other promoters are in the process of being sequenced, and once the sequences are complete we will determine the transcriptional start sites using primer extension analysis and RNAse protection assays. The purpose of these techniques is to determine exactly where in the sequence the promoter region ends and the sequence for the mRNA begins, thereby pinpointing the location of the promoter sequences. Our progress to date on these different analyses are summarized in Table 6. We have also completed restriction site analyses on some of these sequences; An example is shown in Table 7. The information provided by the restriction site maps is needed to replace the native promoter of the *R. rhodochrous* desulfurization

genes with other, stronger promoters to produce cells with higher levels of desulfurization enzymes. Relatively little is known of *R. rhodochrous* genetics, including what constitutes a strong promoter in this organism. Comparisons of the nucleotide sequences of promoters isolated from *R. rhodochrous* with those of well characterized organisms, such as *E. coli* and *Bacillus subtilis*, will provide this basic information.

SIGNIFICANCE TO THE FOSSIL ENERGY PROGRAM: Most of the coal from the coal-rich midwest states has a high organic sulfur content. To meet current and future clean air standards this sulfur must be removed. The precombustion removal of organic sulfur by a biocatalytic system is an attractive alternative to other methods of coal cleaning. The research funded by this grant has been directed toward making this process practical. We have shown that *R. rhodochrous* IGTS8 can desulfurize model organic compounds in non-aqueous systems without the loss of caloric value in the coal. Further, freeze-dried cells had higher desulfurization activity than wet cells, which has a practical consequence: cells could be grown, processed, and stored at one location and shipped to another location when needed. Genetic experiments are underway to obtain strains of *R. rhodochrous* with increased desulfurization activity. The work accomplished during this grant and the work in progress will contribute to making the biocatalytic removal of organic sulfur from coal commercially feasible.

PLANS FOR THE UPCOMING YEAR: Although this grant terminated on September 30, 1994, with a no-cost extension to December 31, 1994, some research will continue. This will include finishing the sequences of the promoters isolated from *R. rhodochrous* IGTS8, analyzing them to identify consensus sequences of strong promoters in this organism, using site-directed mutagenesis to attempt to make the promoters even stronger, and finally inserting the strongest candidates upstream of the genes for the desulfurization enzymes to achieve higher levels of expression of these enzymes. We also plan to test our active nonaqueous model system for the actual removal of organic sulfur from coal and coal derived liquids. Some of the above plans can only be accomplished with additional funding.

II. HIGHLIGHT ACCOMPLISHMENTS

The major accomplishments have been to obtain high biodesulfurization activity in nonaqueous media, especially using freeze-dried cells, and to have isolated strong promoters from *R. rhodochrous* IGTS8 which will be used to engineer the organism to produce strains with higher biocatalytic activity.

III. ARTICLES AND THESES

1. S. Patel (1994). "Investigations of biodesulfurization of dibenzothiophene in non-aqueous media by *Rhodococcus rhodochrous* IGTS8," M.S. thesis, Illinois Institute of Technology, Chicago, IL 60616.

2. S. Patel, J. Kilbane, D. Webster (1994). "Desulfurization activity of *Rhodococcus rhodochrous* in nonaqueous media," manuscript in preparation.

3. C.-O. Yun (1995, anticipated). "Characterization of promoters from *Rhodococcus rhodochrous*," Ph.D. dissertation, Illinois Institute of Technology, Chicago, IL 60616.

4. C.-O. Yun, J. Kilbane, D. Webster (1995). "Isolation of promoters from *Rhodococcus rhodochrous* and analyses of their sequences," in preparation.

Table 1. Effect of Freeze-Drying and Storage Conditions** on Desulfurization Activity.

Time when activity was checked	Cell identification	2HBP (ppm/1 hr)*
Activity right before harvesting the cells from **fermenter-1**	Fermenter-1	6.70
Activity after centrifugation i.e before freeze drying.	wet cells	4.60
Activity after freeze drying	F.D. cells	3.62
Activity after 1 week	F.D. cells	3.60
Activity after 4 weeks	F.D. cells	3.48
Activity after 9 weeks	F.D. cells	3.40
Activity after 10 weeks	F.D. cells	3.52
Activity after 5 days of incubation at room temperature	F.D. cells	1.10
Activity right before harvesting the cells from **fermenter-2**	Fermenter-2	4.56
Activity after centrifugation i.e. before freeze drying	wet cells	3.10
Activity after freeze drying	F.D. cells	2.00
Activity after 1 week	F.D. cells	1.95
Activity after 2 weeks	F.D. cells	1.80
Activity after 3 weeks	F.D. cells	1.84
Activity after 4 weeks	F.D. cells	2.00
Activity after 5 days of incubation at room temperature	F.D. cells	0.55
Activity after 1 week	wet cells	2.95
Activity after 2 weeks	wet cells	2.86
Activity after 3 weeks	wet cells	2.41
Activity after 4 weeks	wet cells	2.71
Activity after 5 days of incubation at room temperature	wet cells	0.65
Activity right before harvesting the cell from **fermenter-3**	fermenter -3	3.10
Activity after centrifugation i.e. before freeze drying	wet cells	2.61
Activity after F.D.	F.D. cells	1.69
Activity after 1 week	F.D. cells	1.68
Activity after 2 weeks	F.D. cells	1.62
Activity after 3 weeks	F.D. cells	1.66

Table 1. Continued.

Activity after 5 days of incubation at room temperature	F.D. cells	0.80
Activity after 1 week	wet cells	2.70
Activity after 2 weeks	wet cells	2.59
Activity after 3 weeks	wet cells	2.65
Activity after 5 days of incubation at room temperature	wet cells	No activity
Activity of **EBC cells** before F.D.	wet cells	2.30
Activity after F.D.	F.D. cells	1.55
Activity after 3 weeks	F.D. cells	1.53
Activity after 12 weeks	F.D. cells	1.41
Activity after 16 weeks	F.D. cells	1.38
Activity after 17 weeks	F.D. cells	1.40
Activity after 5 days of incubation at room temperature	F.D. cells	0.06
Activity of **EBC cells** before F.D.	wet cells	2.41
Activity after F.D.	F.D. cells	1.45
Activity after 3 weeks	F.D. cells	1.40
Activity after 16 weeks	F.D. cells	1.15
Activity after 17 weeks	F.D. cells	1.21
Activity after 5 days of incubation at room temperature	F.D. cells	No activity

* Desulfurization activity is expressed as ppm of 2HBP formed by cells at a density of 1.00 absorbance units at 600 nm during a 1 hour incubation with DBT.

** Freeze-dried and wet cell preparations were stored at -80°C until used unless indicated otherwise.

Table 2. Gibb's and HPLC Assays Comparison for 2HBP

Sample Experiment 1	2HBP in mg by Gibb's assay	2HBP in mg by HPLC
75% oil	0	0.0279
50% oil	0.093	0.1260
25% oil	0.087	0.1190
10% oil	0.084	0.1048
0% oil	0.078	0.0990
Experiment 2		
50% oil	0.096	0.1270
75% oil	0.099	0.1270
80% oil	0.108	0.1325
86% oil	0.076	0.1080

Table 3. Effect of Order of Addition of Oil/Water/Surfactant/Cell on Biodesulfurization.

Order of Addition #		2HBP** by Gibb's Assay
# 1 BSM (250 mg) → Freeze-dried cells (200 mg) ↘ Hexadecane (250 mg) ↗	Mixed	0.099 mg.
# 2 BSM (245 mg) → Freeze-dried cells (200 mg) ↘ Surfactant (5 µl) → Hexadecane (250 mg) ↗	Mixed	0.160 mg.
# 3 BSM (200 mg) → Freeze-dried cells (200 mg) ↘ BSM (50 mg) → Hexadecane (250 mg) ↗	Mixed	0.106 mg.
# 4 Freeze-dried cells (200 mg) ↘ BSM (250 mg) → Hexadecane (250 mg) ↗	Mixed	0.152 mg.
# 5 Freeze-dried cells (200 mg) ↘ Surfactant (5 µl) → BSM (245 mg) + Hexadecane ↗	Mixed	0.200 mg.*
# 6 BSM (50 µl) → Freeze-dried cells → Surfactant (2.5 µl) ↘ Surfactant (2.5 µl) → BSM (195 mg) + Hexadecane ↗	Mixed	0.205 mg.
# 7 BSM (195 µl) → Freeze-dried cells → Surfactant (5 µl) ↘ BSM (50 mg) + Hexadecane ↗	Mixed	0.148 mg.

* 2HBP is average of triplicate samples.
** 2HBP, other then # 5, are of single sample.

Table 4. Cleavage of C-Se Bonds by IGTS8.

Sample		Retention time in min.	Area of the peaks by HPLC
Cystine	(standard)	4.127	1565238
Cystine	(biotreated)	4.127	No peak
Cysteine	(standard)	7.497	5580450
Cysteine	(biotreated)	7.615	1643777
Se-cystine	(standard)	3.9880	1318446
		10.188	1377538
Se-cystine	(biotreated)	3.9880	No peak
		10.230	744511
Alanine*	(standard)	18.947	37447087
Serine*	(standard)	8.027	31371601

* These two amino acids were included in the study because they are potential desulfurization products of cystine, cysteine and Se-cystine.

Table 5. Beta-Galactosidase Assay on R. rhodochrous PYGAL Promoters A-M and 13-23

plasmid	A600	A420	incubation time (hour)	A562	protein conc, (ug/ml)	specific activity
pYGALA	0.477	0.249	3	0.04	33	838.3838
pYGALB	0.391	0.097	23	0.034	28.3	49.67481
pYGALC	0.678	0.123	15	0.063	52.5	52.06349
pYGALD	0.648	0.049	23	0.037	30.8	23.05665
pYGALE	0.445	0.118	23	0.042	35	48.86128
pYGALF	0.35	0.079	23	0.026	21.7	52.76164
pYGALG	0.431	0.077	23	0.07	58.3	19.14137
pYGALH	0.503	0.146	23	0.058	48.3	43.80832
pYGALI	0.424	0.166	23	0.026	21.7	110.8662
pYGALJ	0.173	0.068	23	0.009	7.5	131.401
pYGALK	0.371	0.078	15	0.02	16.7	103.7924
pYGALK2	0.964	0.249	15	0.093	77.5	71.39785
pYGALL	0.433	0.003	23	0.038	31.7	1.371554
pYGALM	0.704	0.064	15	0.055	45.8	31.05289
pYGAL13	0.734	0	15	0.132	110	0
pYGAL14	0.227	0.121	23	0.015	12.5	140.2899
pYGAL15	0.356	0.081	15	0.03	25	72
pYGAL16	0.177	0.05	23	0	0	ERR
pYGAL17	0.787	0.422	15	0.079	65.8	142.5194
pYGAL18	0.217	0.078	23	0.052	43.3	26.10704
pYGAL19	0.353	0.121	23	0.044	36.7	47.78265
pYGAL20	0.189	0.06	23	0.04	33.3	26.11307
pYGAL21	0.272	0.08	23	0.024	20	57.97101
pYGAL22	0.721	0.173	15	0.07	58.3	65.94244
pYGAL23	0.2	0.042	23	0.034	28.3	21.50868

Table 6. Progress on Sequencing and Transcription Site Analyses of pYGAL Plasmids

PLASMID	SIZE OF INSERT (Kb)	SEQUENCING	RNAse Protection assay	Primer Extention Experiment	pGEM3zf transf-ormants
pYGALA	1.2	in progress		in progress	
pYGALB	0.4				
pYGALC	0.8			in progress	pGEMC
pYGALD	3.0				
pYGALE	0.2	completed	completed	in progress	pGEME
pYGALF	1.1				
pYGALG	2.3				
pYGALH	1.1				
pYGALI	0.3	completed	completed	in progress	pGEMI
pYGALJ	0.6				
pYGALK	0.3	completed		in progress	pGEMK
pYGALK2	0.3	completed	completed		
pYGALL	0.15	completed			
pYGALM	0.15	completed		in progress	
pYGAL13	2.2				
pYGAL14	0.6	in progress		determined	
pYGAL15	0.8	completed			
pYGAL16	1.3				
pYGAL17	0.5	in progress			
pYGAL18	0.6	in progress			
pYGAL19	1.4				
pYGAL20	1.6				
pYGAL21	0.4	in progress			
pYGAL22	0.6	in progress		in progress	pGEM22
pYGAL23	0.4	in progress			

Table 7. Restriction Site Analysis of R. rhodochrous 16s RNA Promoter DNA (635 bp)

Analysis done on the complete sequence.

List of cuts by enzyme

AciI	:	65	341	344	442	456
AcyI	:	130				
ApoI	:	100				
AvaI	:	282				
BalI	:	306				
BbeI	:	133				
BcefI	:	160	333			
BclI	:	449				
BetI	:	289				
Bme142I	:	131				
BsiI	:	236				
BsiYI	:	340	462	611		
BspMI	:	227				
BsrI	:	133	270			
BsrBI	:	341				
BstNI	:	146	188	256	308	
Cac8I	:	606				
CauII	:	336				
CfrI	:	304	345			
Cfr10I	:	126	299	604		
Csp6I	:	245	431			
CviJI	:	306	321	347	604	608
DpnI	:	168	451			
DsaV	:	144	186	254	306	334
Eco56I	:	604				
EcoHI	:	338				
EcoRII	:	144	186	254	306	
EcoRV	:	624				
EheI	:	131				
FnuDII	:	89	444			
Fnu4HI	:	342	345			
FokI	:	425				
GdiII	:	345	345			
HaeI	:	306				
HaeII	:	133				
HaeIII	:	306	347	608		
HgiAI	:	140				
HgiCI	:	129				
HhaI	:	76	132	299		
HindII	:	16	124			
HinfI	:	211	579	630		
HinP1I	:	74	130	297		
HpaI	:	124				
HpaII	:	127	290	300	336	605
HphI	:	239	372	631		
MaeI	:	553	569	627		
MaeIII	:	176	231			
MboI	:	166	449			
MboII	:	161	270	511		
McrI	:	348				
MlyI	:	220	588	624		
MnlI	:	217	232	469		
MseI	:	123				
MwoI	:	73	303			
NaeI	:	606				
NarI	:	130				
NlaIV	:	39	49	131	288	
Nli387/7	:	286				
NruI	:	89				
PleI	:	205	573			
RsaI	:	246	432			
ScrFI	:	146	188	256	308	336
SduI	:	140				
SecI	:	334				
SelI	:	87	442			
SgrAI	:	299				
SspI	:	422				
StsI	:	424				
TaqI	:	104	401			
XbaI	:	626				
XcmI	:	424				
XmaIII	:	345				

Total number of cuts is : 139.

Sorted list of enzymes by number of cuts

DsaV	:	5	BsiYI	:	3	HaeI	:	1	SspI	:	1
CviJI	:	5	HindII	:	2	Nli387/7	:	1	AvaI	:	1
AciI	:	5	PleI	:	2	Eco56I	:	1	CauII	:	1
HpaII	:	5	GdiII	:	2	HgiAI	:	1	EheI	:	1
ScrFI	:	5	MboI	:	2	BalI	:	1	SduI	:	1
NlaIV	:	4	CfrI	:	2	MseI	:	1	Bme142I	:	1
BstNI	:	4	Csp6I	:	2	BclI	:	1	McrI	:	1
EcoRII	:	4	BsrI	:	2	HpaI	:	1	BbeI	:	1
HinP1I	:	3	SelI	:	2	Cac8I	:	1	ApoI	:	1
HinfI	:	3	MaeIII	:	2	FokI	:	1	BetI	:	1
MaeI	:	3	MwoI	:	2	SgrAI	:	1	BsiI	:	1
MlyI	:	3	BcefI	:	2	XmaIII	:	1	StsI	:	1
HaeIII	:	3	RsaI	:	2	BspMI	:	1	NaeI	:	1
Cfr10I	:	3	TaqI	:	2	HgiCI	:	1	NruI	:	1
MnlI	:	3	Fnu4HI	:	2	HaeII	:	1	SecI	:	1
HhaI	:	3	FnuDII	:	2	BsrBI	:	1	EcoHI	:	1
MboII	:	3	DpnI	:	2	AcyI	:	1	XcmI	:	1
HphI	:	3	EcoRV	:	1	NarI	:	1	XbaI	:	1

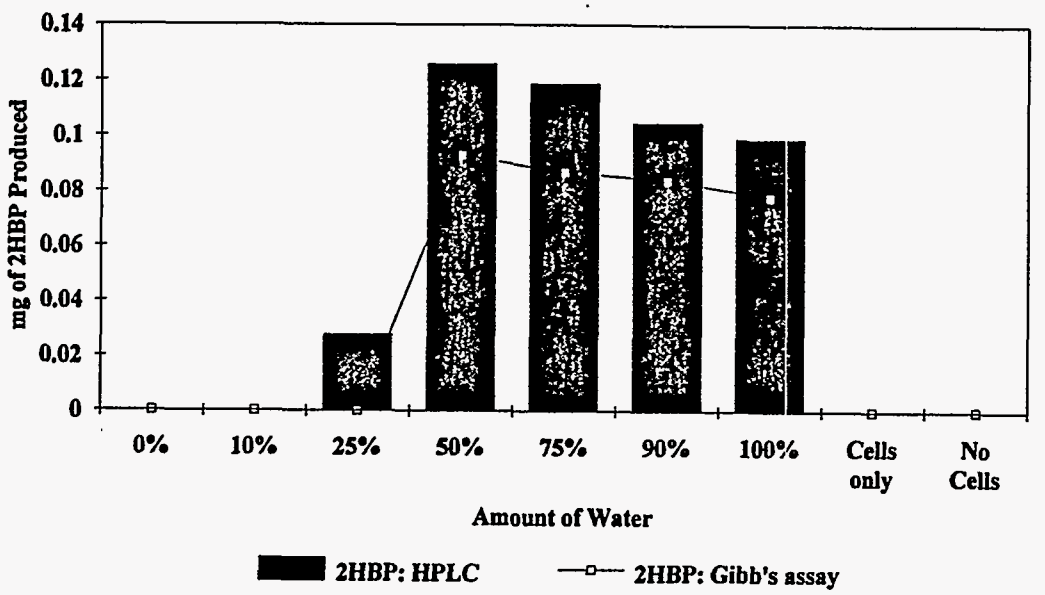


Figure 1. Effect of Water Concentration on Desulfurization Activity of Freeze-Dried Cells in Oil/Water Systems

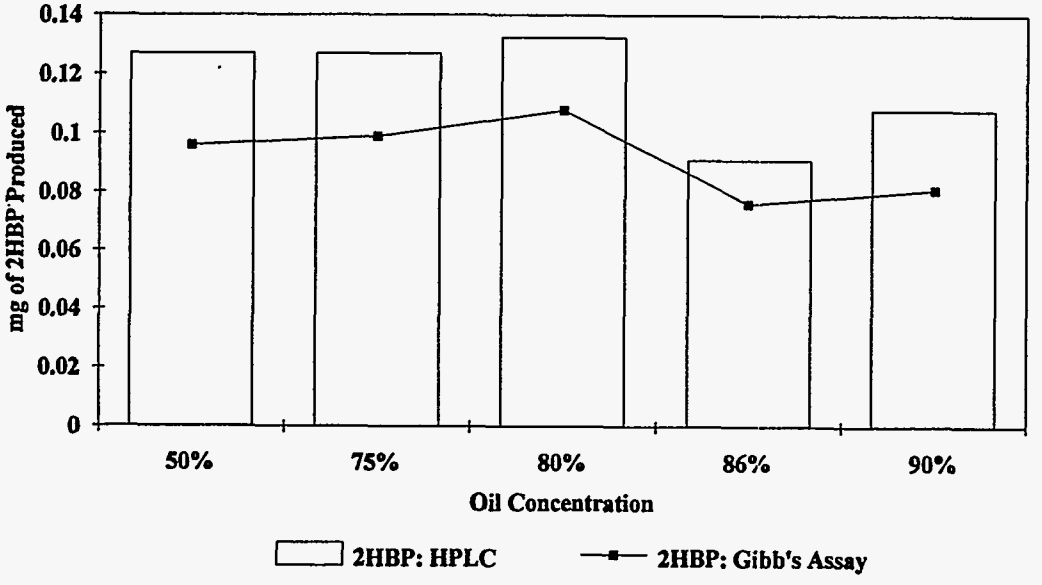


Figure 2. Effect of Oil/Cell Ratio on Desulfurization

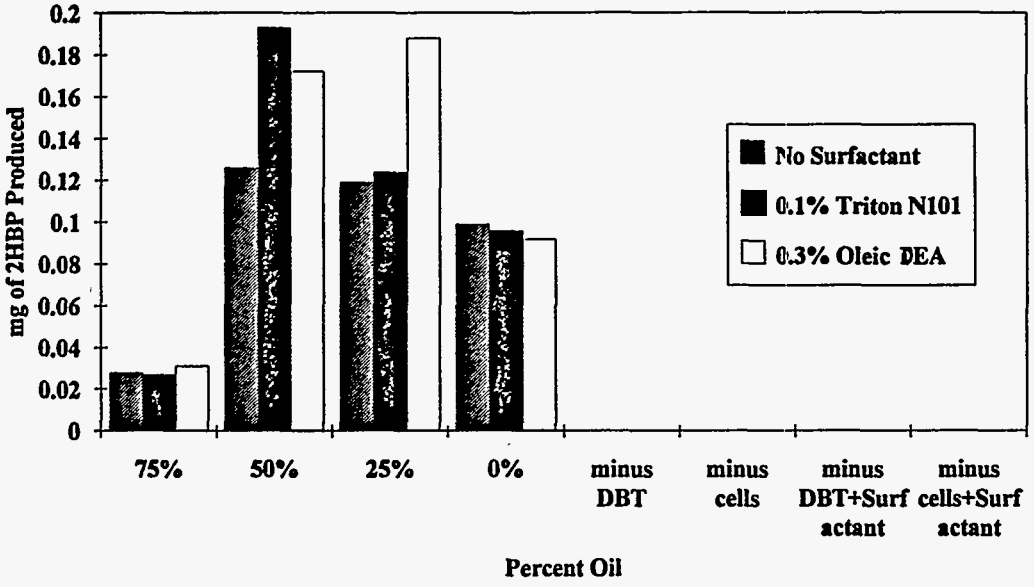


Figure 3. Effect of Surfactant at Different Oil/Water Ratios

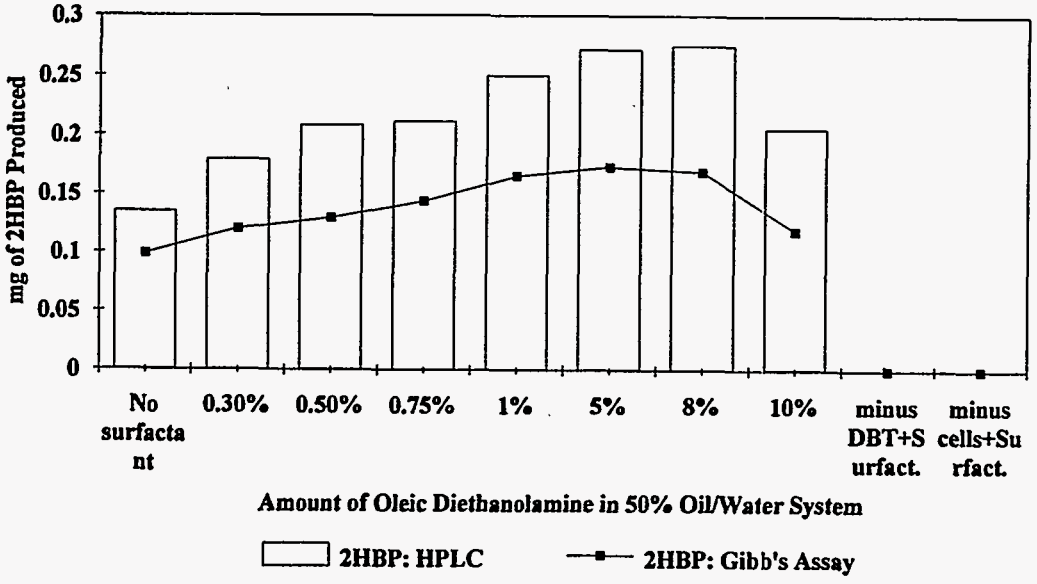


Figure 4. Effect of Different Concentrations of Oleic DEA on Desulfurization in 50% Oil/water System

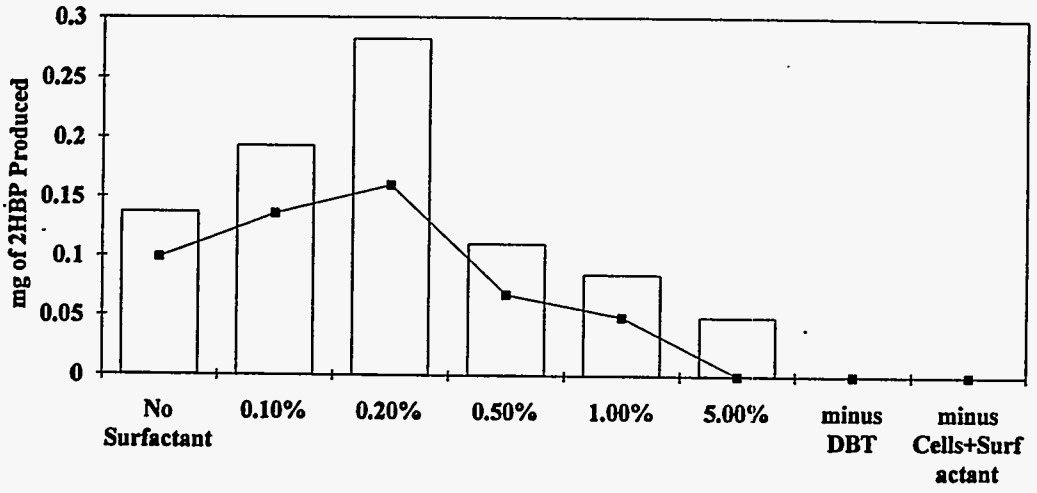


Figure 5. Effect of Different Concentrations of Triton N101 on Desulfurization in 50% oil/Water System

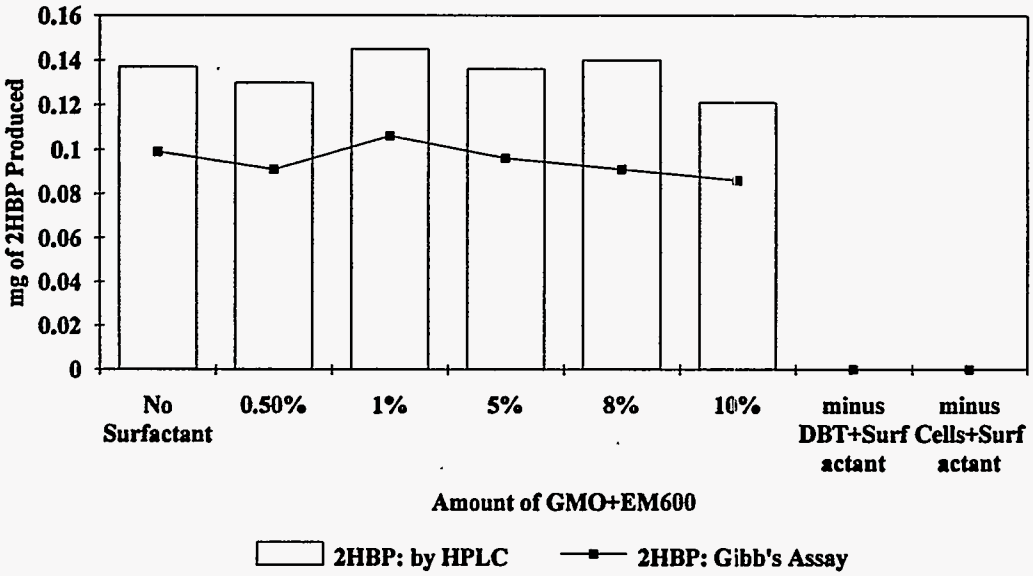


Figure 6. Effect of Different Concentrations of GMO plus EM600 on Desulfurization in 50% Oil/Water System

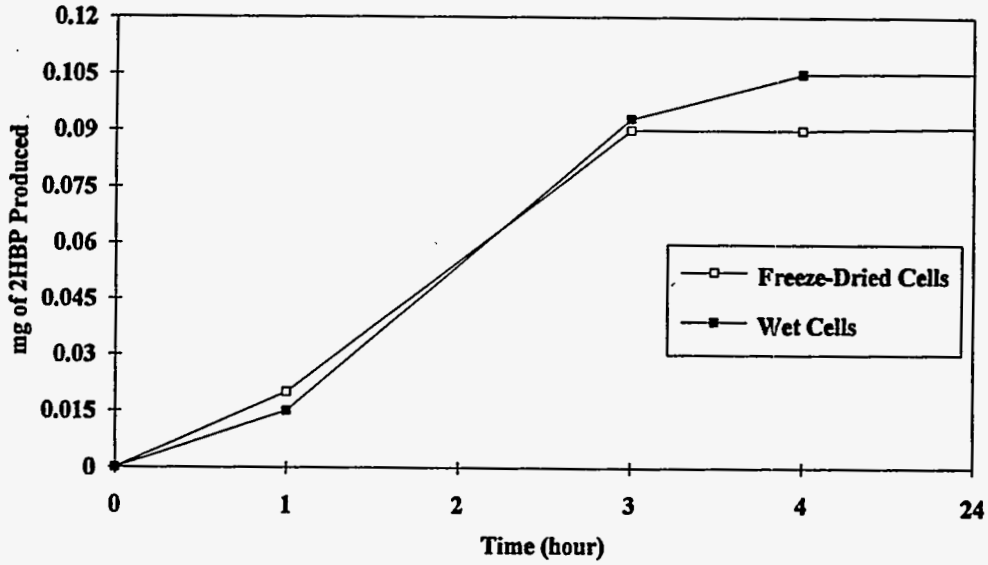
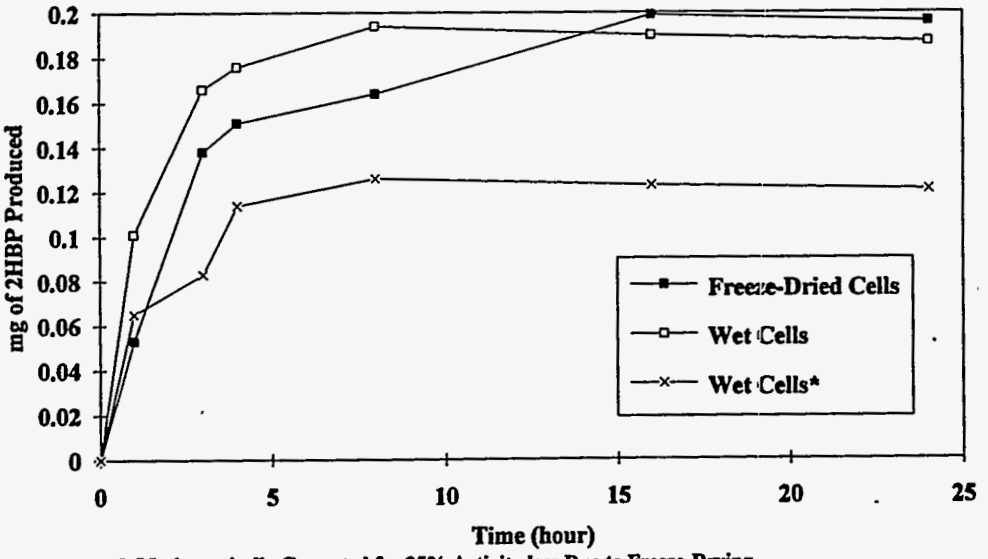


Figure 7. Kinetics of DBT Desulfurization in Aqueous System Using Freeze-Dried and Wet Cells



* Mathematically Corrected for 35% Activity loss Due to Freeze-Drying

Figure 8 Kinetics of DBT Desulfurization in Oil/Water Emulsion Using Freeze-Dried and Wet cells with Same Cell Density