

SULFUR-SPECIFIC METABOLISM OF ORGANIC COMPOUNDS

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All fossil fuels (except most natural gas) contain sulfur. When these fuels are combusted, the sulfur present is released into the atmosphere contributing to air pollution in the form of "acid rain." Current air quality standards have already placed strict limitations on the amounts of sulfurous air emissions allowed, and these regulations may well become even more stringent in the future. There is then a need and an incentive to limit sulfurous emissions resulting from the combustion of fossil fuels. The current technology focuses on sulfur removal achieved during or after combustion. At this time there is no cost effective technology that can desulfurize fuel prior to combustion.

The cost of desulfurization equipment currently used to remove sulfur during and/or after combustion, the problems associated with maintaining that equipment, and disposing of the copious quantities of sulfurous wastes generated by these processes are expenses and problems that industry and the utilities would prefer to avoid. If clean low-sulfur fuel (other than natural gas) could be made available, then fuel users could avoid these concerns. Although there are naturally occurring sources of low-sulfur oil and coal, these high-quality fuels are not abundant enough to serve all of the world's energy needs. Biological processes occur under very mild reaction conditions compared to chemical reactions, so it is hoped that if a suitable means of desulfurizing fuels using biological systems can be found, then economically favorable pre-combustion fuel desulfurization processes will result.

It has been known for many years that acidic drainage associated with coal mines or coal storage piles is the result of microbial oxidation of sulfur in coal, which produces sulfuric acid. This microbial desulfurization of coal is almost completely limited to the inorganic forms of sulfur (pyrite) found in coal and is accomplished by the action of well characterized bacteria: Thiobacillus ferrooxidans, Thiobacillus thiooxidans, and Sulfobolus acidocaldarius. The microbiological removal of sulfur from coal has been shown to be capable of removing 90% or more of the inorganic sulfur, while the microbiological removal of organic sulfur from coal is certainly less complete and is less well-documented. More than 90% of the total sulfur must be removed for any pre-combustion coal desulfurization process to obviate the need for subsequent desulfurization. Therefore, the focus of current research concerning the microbiological removal of sulfur from coal centers on the removal of organic sulfur.

Inorganic sulfur generally exists in coal in the form of discrete particles or crystals of pyrite; whereas organic sulfur occurs as an integral part of the molecular coal matrix and is not readily accessible for microbial attack. The goal, then, of developing a microbiological process for the removal of organic sulfur from coal — while retaining the fuel value of that coal — is a most difficult goal indeed. What is needed are microorganisms that have an affinity for cleaving carbon-sulfur bonds, that is, microorganisms capable of sulfur-specific metabolism of organic compounds.

Enrichment culture techniques are typically used to isolate microorganisms with desired traits, and the search for microorganisms capable of organic sulfur removal from coal is no exception. The chemical dibenzothiophene (DBT) is generally regarded as a good model compound representative of organic sulfur found in coal; it is used as the substrate of choice in most enrichment culture experiments. There are two fundamentally different pathways for the microbial degradation of DBT that have been proposed. Both pathways are outlined in Figure 1. Pathway A (the sulfoxide/sulfone/sulfonate/sulfate "4S" pathway) is the sulfur-specific metabolic pathway that results in the removal of sulfur from DBT, while leaving the carbon intact. Pathway B is a carbon-destructive metabolic pathway which results in the overall degradation of DBT. Numerous investigators¹⁻⁶ have attempted to isolate or develop microbial cultures that can metabolize or co-metabolize DBT. A summary of the published results of these attempts to isolate DBT-utilizing cultures is presented in Table 1. The nearly universal experience has been the isolation of microorganisms that metabolize DBT by the carbon-destructive pathway. The implication is that the methods of screening bacterial cultures and of performing enrichment culture experiments may be inappropriate and in need of revision. The published reports concerning CBI,⁶ the only bacterial strain claimed to metabolize DBT via Pathway A exclusively, do not give any indication of a novel culture enrichment procedure or offer an example of how to purposefully isolate microorganisms with sulfur-specific metabolic capabilities.

There are several major research needs that can clearly be identified at this time. Since the rate and extent of organic sulfur removal from coal by characterized microorganisms is insufficient to warrant process development at this time, there is a need to identify new microorganisms that have sulfur-specific degradation abilities toward the full array of sulfur-containing organic molecules. Toward the isolation of new bacterial strains with appropriate desulfurization activities, there is a need for a convenient and powerful strain selection technique rather than relying on laborious culture screening techniques. There also exists a need to compare the cultures and the results obtained from different laboratories concerning microbial desulfurization. We need a way to normalize the results obtained by different researchers, using different microorganisms, different substrates, and different growth/reaction conditions. Existing strains of bacteria with documented desulfurization abilities are in need of strain improvement and techniques to conveniently monitor the progress of strain improvement efforts. Lastly, there is a need for better analytical techniques to assess the removal of organic sulfur from coal, as the existing methods of physical/chemical analysis of sulfur by type in coal are costly, time consuming, and not particularly accurate — especially regarding the organic sulfur in coal. All of these research needs can be served by taking advantage of the fact that all living organisms require sulfur for growth.

Enrichment cultures can be established to isolate microorganisms that possess an array of characteristics reflective of the incredible diversity present in the microbial kingdom. The challenge is to manipulate the culture conditions such that a very powerful selective pressure exists favoring only those microorganisms that possess the desired trait; then all that remains is to provide a suitable inoculum and allow the selective pressure and the "survival of the fittest" to interact and allow only those microbes that possess the desired characteristic to predominate in the enrichment culture. Enrichment cultures are startlingly simple in theory. The compound to be degraded is supplied as the growth-limiting source of an essential nutrient,

while all other growth requirements are supplied in abundance. Growth under such conditions favors those microorganisms that possess degradative activities toward the target compound — whereby the "appropriate" microorganisms should outgrow all "inappropriate" competitors and rapidly come to dominate the culture. A growth limitation can be achieved by removing, reducing, substituting, or altering any essential nutrient; however, in practice it is usually only the carbon source that is manipulated in enrichment culture experiments. That also seems to be the case for enrichment culture experiments reported for the isolation of microorganisms with the ability to desulfurize DBT or the organic sulfur in coal. It would be much more to the point to establish enrichment cultures utilizing DBT as the growth-limiting source of sulfur.

Enrichment culture experiments were carried out at the Institute of Gas Technology (IGT) in two ways: traditional shake flask enrichment cultures and sulfur-limited chemostats. A sulfur-limited chemostat is a continuous culture bacterial growth experiment in which all of the nutrients, except sulfur, that are needed for growth are supplied in abundance, and sulfur is supplied in the form of coal or organic chemicals. The use of traditional shake flask enrichment culture techniques resulted in the isolation of a *Pseudomonas* species, designated TG-232, which is capable of co-metabolizing DBT while growing at the expense of naphthalene. The sulfur-limited coal chemostat resulted in the isolation of a mixed bacterial culture, designated IGT-S7, capable of co-metabolizing DBT while growing at the expense of glucose, succinate, or benzoic acid. The bacterial metabolites of DBT produced by TG-232 and IGT-S7 were identified by gas chromatography and mass spectrometry (GC/MS). The results from these analyses are presented in Table 2. It can readily be seen that TG-232 metabolizes DBT by the carbon-destructive Pathway B, while IGT-S7 is capable of sulfur-specific metabolism, which removes sulfur from DBT and leaves the carbon intact. Sulfur-limited chemostats appear to be a useful tool for the purposeful isolation of microorganisms with sulfur-specific metabolic capabilities.

Growth of cultures in a sulfur-limited medium is not only useful for the selection of microorganisms but also the sulfur requirement of all microorganisms for growth can be used as the basis of a quantitative sulfur-specific bioassay by correlating bacterial growth to substrate metabolism. A bioassay can detect sulfur utilization from organic substrates more easily and with greater sensitivity than physical/chemical analyses. Moreover, a bioassay can be used to compare the results of different bacterial strains acting on different substrates. A bioassay relating sulfur metabolism of organic substrates to the rate and extent of growth observed can be a universally applicable method useful in normalizing the data obtained from every researcher in the field of microbial desulfurization of coal.

Because all life requires some amount of sulfur for growth, a situation can be created such that by quantifying bacterial growth one can quantify the utilization of any organic or inorganic compound as a source of sulfur. In work funded by the U.S. Department of Energy, IGT developed a sulfur bioassay to do just that. An inorganic sulfate is usually the form in which sulfur is supplied in a bacterial growth medium. Similarly, various organic sulfur compounds can be tested for their ability to serve as sources of sulfur for bacterial growth. Growth curves of IGT-S7 with glucose serving as a carbon source and various sulfur sources (nothing, sulfate, DBT, and trithiane) are presented in Figure 2. These curves, which constitute sulfur bioavailability assays, illustrate several points. The no-sulfate control shows some growth

because trace amounts of sulfur are present in this medium, but this growth is comparatively slow and essentially complete after 24 hours. The experiment that contains sulfate shows extremely rapid growth that is largely complete within 8 hours. When the same bacterial culture is grown with either DBT or trithiane as sulfur sources, about 78 hours are required to attain maximal growth. Inspection of these growth curves reveals that the bacterial cultures that contain DBT or trithiane grow at approximately the same rate as the no-sulfate control for the first 24 hours. This suggests that DBT and trithiane are not utilized appreciably as sulfur sources until the trace amounts of sulfur compounds present in the medium have been exhausted. Thereafter, a slow rate of growth continues at the expense of the organic sulfur compounds. Moreover, there is no pronounced lag in the growth curves of the DBT or the trithiane cultures after 24 hours coinciding with the exhaustion of the trace sulfur in the medium. This implies that no lengthy induction/adaptation period is required for IGT-S7 to utilize organic sulfur sources.

IGT-S7 is capable of utilizing a variety of organic compounds as sulfur sources including, but not limited to, dibenzothiophene, dibenzothiophene sulfoxide, dibenzothiophene sulfone, thianthrene, thioxanthene, and trithiane. While all of these compounds can be used as sulfur sources by IGT-S7, the time required to achieve maximal cell growth is longer than when sulfate is present. However, when bacterial cultures capable of metabolizing DBT by Pathway B are tested in the sulfur bioavailability assay, the vast majority of these cultures show no growth whatsoever in excess of that observed in the "no-sulfur controls." TG-232 is capable of utilizing DBT as a sulfur source, but it never grows to more than 40% of the maximum growth observed in the sulfate-containing controls, even upon lengthy incubation and multiple sequential subculturing. The sulfur bioavailability assay, therefore, is a convenient inexpensive method to compare quantitatively the abilities of bacterial cultures to utilize organic substrates as sulfur sources.

When IGT-S7 is used to treat coal, as much as 25% removal of organic sulfur has been observed. These results are encouraging, but organic sulfur removal efficiencies of about 90% are required if a microbial coal desulfurization process is to be developed that is competitive with current post-combustion desulfurization technologies. Significant strain improvement research is needed to enhance the desulfurization activity in bacterial cultures, and physical problems associated with the pre-combustion desulfurization of coal must be addressed if a viable technology for the microbial removal of organic sulfur from coal is to be realized.

Sulfur-limited continuous culture chemostats can be used to isolate/develop bacterial cultures capable of sulfur-specific metabolism of organic compounds. Similarly, the sulfur bioavailability assay can be used to evaluate quantitatively the desulfurization abilities of bacterial cultures and can aid in culture evaluation and strain improvement experiments. If the goal of developing a microbiological process for the removal of sulfur from coal is to be achieved, bacterial cultures with expanded and improved abilities to desulfurize organic substrates are needed. The techniques described in this paper are useful tools that can help to achieve that goal.

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Table 1. SUMMARY OF THE IDENTIFICATION OF DBT METABOLITES PRODUCED BY MICROBIAL DEGRADATION

Research Group	Pathway A	Pathway B	Most Complete Degradation Products Observed*
	Degradation	Degradation	
	%		
Kodama et al. ⁵	8	92	A1, B3
Laborde & Gibson ³	Minor	Major	A1, B3
Monticello et al. ⁴	0	100	B3
Kilbane: TG-232	32	68	A1, B6, B10
IGT-S7	100	0	A4
Hou & Laskin ²	0	100	B2
Malik & Claus ¹	0	100	B2
Isbister & Kobylnski ⁶	100	0	A4

* The designations of A1, B1, etc. refer to structures included in the pathways presented in Figure 1.

Table 2. GC/MS ANALYSIS OF BACTERIAL-DERIVED METABOLITES OF DBT

The most abundant metabolites of DBT degradation were analyzed by GC/MS. The concentration of DBT was arbitrarily set at 1.0, and the concentrations of metabolites were reported relative to the concentration of DBT. The numbers in parenthesis (A1, B1, etc.) refer to structures included in the pathways presented in Figure 1.

Compound	Mol. wt	TG-232	IGT-S7
Dibenzothiophene	184	1	1
(A1) Dibenzothiophene-5-oxide	200	0.30	1.8
plus Phenoxathiin	200	--	--
(A4)* Dihydroxybiphenyl	186	BDL**	0.033
(A4)* Hydroxybiphenyl	170	BDL	59
(B3) 3-hydroxy-2-formyl-benzothiophene	178	0.21	BDL
Biphenyl	154	BDL	0.001
(B6) Benzothiophene	134	0.016	BDL
Naphthalene	128	0.001	BDL
(B4) Three isomers of C(8)H(6)OS: (hydroxybenzothiophene)			
No. 1	150	0.01	BDL
No. 2	150	0.02	BDL
No. 3	150	0.048	BDL
(B9) C(9)H(8)OS	164	0.12	BDL
(B7) C(9)H(8)O(2)S	180	0.067	BDL
(B5) C(9)H(6)OS	162	0.022	BDL
C(10)H(10)OS or C(9)H(6)O(2)S	178	0.035	BDL
(B10) C(8)H(8)O(2)S isomers	a) 168	0.033	BDL
	b) 168	0.025	BDL
Formula (?)	220	0.036	BDL
Total Excluding DBT		0.94	60.8

* Hydroxybiphenyl is the terminal metabolite in a modified A/"4S" pathway.
 ** BDL = below detection limit.

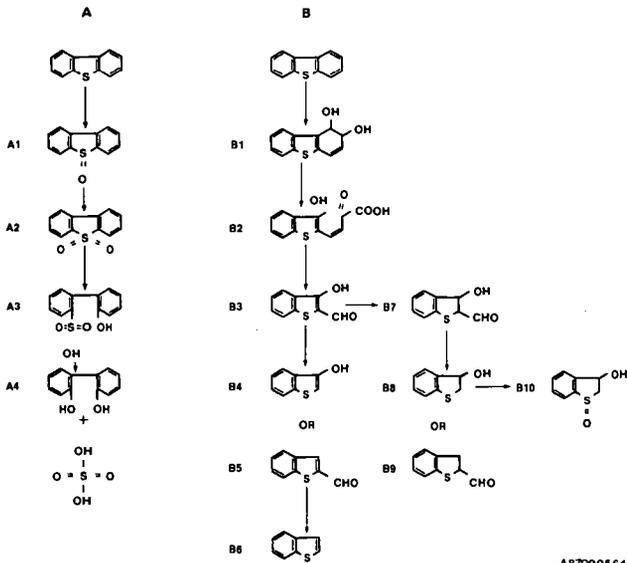


Figure 1. PROPOSED PATHWAY OF DBT DEGRADATION

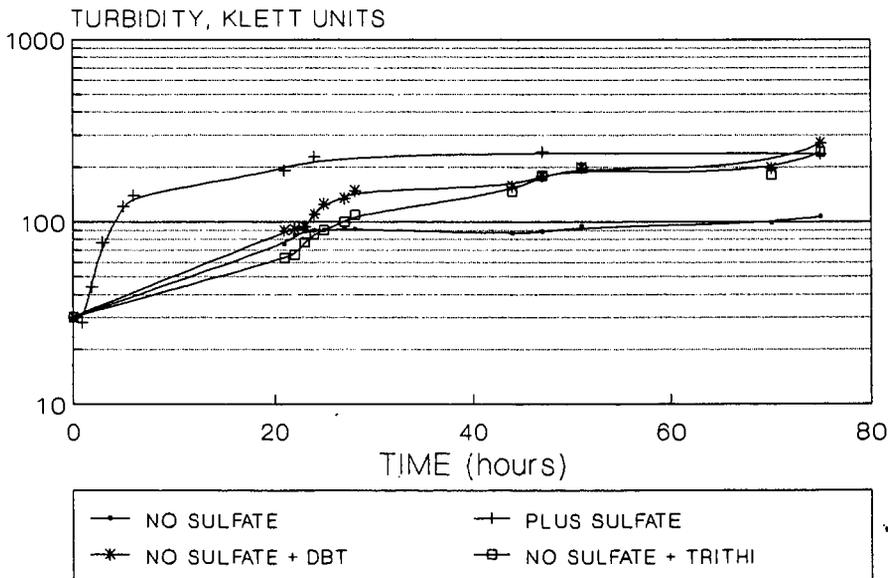


Figure 2. GROWTH CURVES OF IGT-S7 USING ORGANIC AND INORGANIC SULFUR SOURCES