

MICROBIAL DESULFURIZATION OF COALS
BY ORGANISMS OF THE GENUS *PSEUDOMONAS*

CHARANJIT RAI AND JON P. REYNIERS

TEXAS A&I UNIVERSITY, KINGSVILLE, TEXAS 78363

INTRODUCTION

Organisms of the genus *Pseudomonas* are widely distributed in the environment and are found in high-sulfur content coals. These organisms are strictly aerobic chemolithotrophs which require minimal organics for growth.

Preliminary studies indicated that *P. aeruginosa* and *P. putida* may function in desulfurization of bituminous coals and lignite (1,2). However, *P. aeruginosa* was much less effective than *P. putida*. Incubation of these organisms at temperatures less than 37°C could favor growth of *P. putida* over *P. aeruginosa* since optimum growth of *P. putida* occurs at 26°C (3). Further, incubation of these organisms in the presence of semi-purified sources of inorganic sulfurs, such as pyrite (PYR), marcasite (MAR), and melanterite (MEL), or organic sulfur such as dibenzothiophene (DBT) could result in restricted growth of the organisms. Finally, differences in the enzymatic profile of the organisms could suggest possible mechanisms of sulfur processing.

The purpose of the present study was to characterize *P. aeruginosa* and *P. putida* in their ability to desulfurize coals and to evaluate their performance with specific natural sources of inorganic and organic sulfurs known to be constituents of coals. The results show that *P. aeruginosa* uniformly grows better than *P. putida* in the presence of the sulfur sources and coals and suggest that the mechanism of desulfurization is not similar to that of the genus *Thiobacillus*.

EXPERIMENTAL

Organisms

Pseudomonas aeruginosa (ATCC 27853) and *Pseudomonas putida* (ATCC 12633) were obtained in pure culture from the American Type Culture Collection, Rockville, MD.

Media

P. aeruginosa and *P. putida* were maintained at 5°C on minimal agar slants containing the following in g/l, final volume: solution A [potassium dihydrogen phosphate (3.0), disodium hydrogen phosphate (6.0), ammonium chloride (2.0), sodium chloride (5.0), agar (15.0) in 800 ml with deionized, distilled H₂O]; solution B [glucose (6.0), magnesium sulfate (0.1) in 200 ml with deionized, distilled H₂O]. Solutions A and B were autoclaved at 15 psi for 15 minutes and then aseptically combined after cooling. The final pH was 7.0. All reagents were obtained from MCB, except as noted. For experiments, HPLC-grade H₂O (Burdick & Jackson Laboratories, Inc.) was used, and agar was not incorporated. Where other materials were used as sulfur sources, magnesium sulfate was replaced by magnesium chloride. In shaker-flask experiments, the organisms were grown in a nutrient broth (DIFCO) containing beef extract (3.0 g/l) and peptone (5 g/l). Tryptic soy broth (DIFCO) served as a complete medium and contained the following in g/l: tryptone (17.0), soytone (3.0), dextrose (2.5), sodium chloride (5.0), dipotassium phosphate (2.5 g) in 1.0 l with deionized, distilled H₂O prior to autoclaving.

Coal and Sulfur Sources

Samples of Illinois #6 bituminous coal and lignite were obtained from Amax Coal Company. The coal samples were ground in a ball-mill and sieved to 147-1651 μm particle size for shaker-flask experiments or to a fine powder for other experiments.

Samples of pyrite (FeS_2), marcasite (FeS_2), and melanterite ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were obtained from the Department of Geology, Texas A&I University. These samples were ground to a fine powder equivalent in particle size to the coal samples. High-purity (>99.9%) pyrite was obtained from the Aesar Group, Seabrook, NH. The forgoing samples served as experimental sources of inorganic sulfur.

Dibenzothiophene (DBT) was obtained from Aldrich Chemical Company and served as a model organic sulfur source.

Organism and Enzyme Assays

Positive identification and the purity of cultures of *P. aeruginosa* and *P. putida* was made prior to each experiment, using 20 standardized biochemical and carbon assimilation tests. Systematic and rapid semi-quantitative microanalysis of 19 enzymatic assays were obtained from DMS Laboratories, Inc., Farmington, NJ, and were performed according to standard protocols provided by the manufacturer.

Analytical Procedures

Illinois #6 coal and lignite samples were analyzed for total sulfur by the Eschka method and pyritic and sulfate sulfur content was determined by chemical procedures (D-2494-79) described by the American Society of Testing Materials (3,4).

Filtered coal samples were washed with dilute hydrochloric acid followed by distilled water to remove trace adsorbed sulfate and iron. The filtrate was analyzed for sulfate and total iron. The Eschka method was used to estimate total sulfur content in coals. Extraction of 1 g coal samples by dilute hydrochloric acid was followed by turbidimetric determination of sulfate (4). Extraction of weighed coal samples with 2N nitric acid was followed by titrimetric determination of iron (5) as a measure of pyritic sulfur content.

Microbial Methods

Shaker-flask experiments were conducted to determine the effectiveness of *P. aeruginosa* and *P. putida* in removal of pyritic sulfur from coals. Weighed samples of coal were placed into 250 ml Erlenmeyer flasks and suspended in 75 ml nutrient broth. The flasks were charged with 5 ml of bacterial cells adjusted to 10^9 cells/ml. Incubation was allowed to proceed at 30°C for periods between 5-7 days in a shaker-bath. Following incubation, total and pyritic sulfur content of the coals were determined as previously described.

P. aeruginosa and *P. putida* were evaluated under various growth conditions for their response to the presence of saturating and dilute levels of pyrite, marcasite, melanterite, and dibenzothiophene. The organisms were also characterized with regard to their enzymatic patterns.

Preliminary growth experiments were performed in 13 x 100 mm screw-cap tubes containing 5.0 ml of complete medium and 0.1 g of pyrite, marcasite, or melanterite. The tubes containing the medium and sulfur sources were autoclaved (15 psi, 15 minutes), cooled, and inoculated with 0.1 ml of a suspension of an 18-hour mid-log phase culture of bacterial cells, adjusted to an optical density of 0.75 A_{540} (10^9 cells/ml) using a Baush and Lomb Spectronic 20 spectrophotometer. The tubes were incubated with occasional

shaking in water baths adjusted to temperatures between 20°C and 40°C. Growth of the organisms was monitored turbidimetrically by following absorbance at 540 nm. After 55 hours, the cells were removed by centrifugation (10,000 x g), and the pH of the medium was determined for each tube. In other experiments, growth of *P. aeruginosa* and *P. putida* was followed in tubes containing chemically-defined minimal medium and dilutions of the sulfur sources and coals. 0.1 g of pyrite, marcasite, melanterite, dibenzothiophene, bituminous coal, or lignite was added to 10 ml of HPLC-grade H₂O, autoclaved, and allowed to stand overnight. 0.5 ml of these suspensions was added to 4.0 ml of sterile solution A. The suspensions were clarified by centrifugation and the saturated supernate used to make 10-fold dilutions of the sulfur sources. 0.5 ml of these dilutions were added to 4.0 ml of sterile solution A. All tubes received 0.5 ml of solution B containing autoclaved glucose and magnesium chloride. Each tube received 0.1 ml of *P. aeruginosa* or *P. putida* grown to mid-log phase in 18 hours at 30°C in the chemically-defined minimal medium containing only magnesium sulfate as a sulfur source. Prior to inoculation, the organisms were washed with solution A without magnesium sulfate and the inoculous culture was adjusted to an optical density of 1.75 A₅₄₀. The tubes were incubated at 30°C and growth of the organism was followed spectrophotometrically for 45 hours. The cells were then removed by centrifugation and the pH of the medium in each tube was determined.

RESULTS AND DISCUSSION

1. Microbial Desulfurization of Coals by Organisms of the Genus *Pseudomonas*.

Preliminary investigations were conducted for pyrite desulfurization of Illinois #6 (Herrin Coal), Illinois #6 (Ziegler Coal) and Atascosa-McMullen lignite. The initial (As Received) pyritic sulfur content of Illinois #6 (Herrin Coal) was 4.9%; Illinois #6 (Ziegler Coal), 4.2%; and Atascosa-McMullen lignite, 1.9%. In the laboratory experiments *Pseudomonas aeruginosa* was found to be barely effective (about 28%), whereas *Pseudomonas putida* reduced the pyritic sulfur content of Illinois #6 (Herrin Coal) 147 µm particles and that of Atascosa-McMullen lignite 147 µm particles by approximately 75%. The desulfurization rate for the Herrin Coal was about 659 mg/liter x day, for the Ziegler Coal, 557 mg/liter x day and that for Atascosa-McMullen lignite about 288 mg/liter x day for the initial 6 day period. The data on the effect of particle size distribution on the rate of pyrite desulfurization is presented in Table 1 with organisms of Genus *Pseudomonas*.

2. Characterization of Organisms of Genus *Pseudomonas*.

P. aeruginosa is differentiated from *P. putida* in its production or use of nitrate, urease, gelatin, n-acetylglucosamine, and adipate. All other biochemical and carbohydrate utilization tests were identical for both organisms.

Enzyme profiles were prepared on the organisms. No major differences were observed in the enzymatic profiles of the two organisms when grown in the absence of the sulfur or coal sources except those listed in Table 2. Incubation of the organisms in the presence of PYR, MAR, MEL or DBT as sulfur sources had no marked effect on any of the enzymes tested.

P. aeruginosa and *P. putida* were grown in complete medium in the presence of saturating levels of pyrite, marcasite, and melanterite at temperatures between 20 and 40°C. An objective of this experiment was to determine if these materials interfered with the growth of either organism and, if not, what temperatures would permit equivalent growth rates for both organisms.

The results of this experiment are summarized in Table 3.

TABLE 1

MICROBIAL DESULFURIZATION OF BITUMINOUS COALS
BY ORGANISMS OF GENUS PSEUDOMONAS

PSEUDOMONAS PUTIDA

COAL	PARTICLE SIZE	PYRITIC SULFUR, WT %		DAYS	PYRITIC SULFUR REDUCTION, (%)	RATE, (MGS/LITER x DAY)
		BEFORE	AFTER			
Illinois #6 Herrin Coal	+1397 μ m	4.10	1.25	5	69.15	586.75
Illinois #6 Herrin Coal	+1397 μ m	4.10	1.08	7	73.66	444.11
Illinois #6 Herrin Coal	- 147 μ m	5.13	1.47	5	71.34	753.52
Illinois #6 Herrin Coal	- 147 μ m	5.13	1.28	7	75.05	566.17
Illinois #6 Herrin Coal	- 147 μ m	4.40	1.02	6	76.82	579.89
Ziegler Coal Illinois #6	- 147 μ m	4.40	1.10	6	75.00	566.17
Ziegler Coal Illinois #6	- 147 μ m	2.22	0.54	6	75.67	288.23
Lignite Atascosa-McMullen	- 147 μ m	1.15	0.38	6	66.95	132.10
Lignite Atascosa-McMullen	- 147 μ m					

PSEUDOMONAS AERUGINOSA

Illinois #6 Ziegler Coal	- 147 μ m	4.40	2.97	6	32.50	245.34
Illinois #6 Ziegler Coal	- 147 μ m	4.40	3.25	6	26.14	197.30

These experiments were conducted at 70-85°F with continuous shaking.

TABLE 2. ENZYMATIC DIFFERENCES WITHOUT SULFUR AND COAL SOURCES.

ENZYME/SUBSTRATE	ACTIVITY ^a	
	<i>P. aeruginosa</i>	<i>P. putida</i>
Lipase esterase/2-naphthyl-caprylate	30	5
Lipase/2-naphthyl-myristate	10	0
Acid phosphatase/2-naphthyl-phosphate	5	30
All enzymes/no substrate	0	0

^a Nanomoles substrate hydrolyzed in 4 hours.

TABLE 3. INFLUENCE OF INORGANIC SULFUR SOURCES ON THE GROWTH OF *P. aeruginosa* and *P. putida*.

GROWTH TEMPERATURE, (°C)	GROWTH ¹	
	<i>P. aeruginosa</i>	<i>P. putida</i>
20	R (PYR, MAR, MEL)	N
25	R (PYR, MAR, MEL)	R (PYR, MAR, MEL)
30	R (PYR, MAR, MEL)	R (PYR, MAR, MEL)
37	R (MAR, MEL)	R (PYR, MAR, MEL)
40	R (PYR, MEL)	N

¹ Relative to controls, without PYR, MAR or MEL as sulfur sources, growth of organisms is repressed (R) or not repressed (N) by saturating levels of the sulfur sources in complete mediums.

At 20°C, growth of *P. aeruginosa* was $<0.4 A_{540}/45$ hours and the presence of pyrite, marcasite, and melanterite further restricted growth $\leq 0.2 A_{540}/45$ hours. At 25°C, the growth of *P. aeruginosa* improved. Maximum growth was reached with temperatures $\geq 30^\circ\text{C}$ typically $1.5 A_{540}/45$ hours. Stationary phase of growth was not reached until after 45 hours of incubation. The presence of the sulfur sources was generally restrictive at any incubation temperature. At or above 37°C , *P. aeruginosa* reached stationary phase between 15 and 20 hours of incubation.

P. putida grew slowly at 20°C , typically reaching $\leq 0.4 A_{540}/45$ hours. Above 20°C , the presence of pyrite, marcasite, and melanterite was not restrictive to growth. *P. putida* grew better at 25°C with or without the sulfur sources. Maximum growth of *P. putida* was achieved $\geq 30^\circ\text{C}$, typically being $\geq 1.4 A_{540}/45$ hours. However, the growth of *P. putida* at temperatures $\geq 30^\circ\text{C}$ in the presence of the sulfur sources was generally repressed, being typically $\leq 0.8 A_{540}/45$ hours. Elevation of incubation temperature above 37°C resulted in low-level growth of *P. putida* ($\leq 0.6 A_{540}/45$ hours) with or without the sulfur sources.

Following incubation of *P. aeruginosa* and *P. putida* with or without the sulfur sources, the pH of the medium was monitored. The results are shown in Table 4. Incubation of the organisms in the presence of the sulfur sources for 45 hours did not result in lowering of the pH of the medium relative to controls without the sulfur sources.

TABLE 4. EFFECT OF SULFUR SOURCES ON pH OF MEDIUM.

TREATMENT	<i>P. aeruginosa</i>	pH OF MEDIUM ^a	<i>P. putida</i>
None	7.2 ± 0.1		7.3 ± 0.1
Pyrite	7.3 ± 0.1		7.0 ± 0.3
Marcasite	6.9 ± 0.4		7.3 ± 0.1
Melanterite	7.2 ± 0.1		7.1 ± 0.1

^a Average of pH of all tubes ($n = 20$) of a given treatment for a given organism incubated between 20 and 40°C .

P. aeruginosa and *P. putida* were grown in a chemically-defined minimal medium containing various sulfur sources. The results are shown in Table 5. The presence of the sulfur sources or coals was, in general, repressive to the growth of both organisms and to *P. putida* more than *P. aeruginosa*. An attempt to limit availability of the sulfur sources by diluting from a point of solution saturation did not result in substantial effects to the growth of either organism. Growth of *P. putida* was better when the individual sulfur sources were provided rather than the coals. The pH of the medium after 55 hours of incubation remained near neutrality and was not significantly different from controls without the sulfur sources.

CONCLUSIONS

The results from the preliminary microbial desulfurization of Illinois #6 and Texas lignite by the organisms of the Genus *Pseudomonas* show that *P. putida* was much more effective than *P. aeruginosa*. The *P. putida* reduced the pyritic sulfur content of Illinois #6 as well as lignites by 69 to 76% in 5 to 7 days for coal particles from $147 \mu\text{m}$ to $1397 \mu\text{m}$. Whereas *P. aeruginosa* was hardly effective (26 to 32.5%) in reducing the pyritic sulfur content of Illinois #6.

TABLE 5. GROWTH OF ORGANISMS IN CHEMICALLY-DEFINED MINIMAL MEDIUM.

TREATMENT ^a	ORGANISM ^b	AVERAGE GROWTH ^c	
		SATURATED MEDIUM	DILUTED MEDIUM
PYR	P	0.40 ± 0.01	0.43 ± 0.01
	A	0.81 ± 0.01	0.58 ± 0.01
MAR	P	0.40 ± 0.02	0.48 ± 0.01
	A	0.68 ± 0.01	0.68 ± 0.02
MEL	P	0.50 ± 0.01	0.31 ± 0.02
	A	0.72 ± 0.02	0.60 ± 0.03
DBT	P	0.39 ± 0.01	0.53 ± 0.03
	A	0.59 ± 0.03	0.63 ± 0.02
BITU	P	0.34 ± 0.01	0.38 ± 0.01
	A	0.59 ± 0.02	0.64 ± 0.01
LIGN	P	0.39 ± 0.01	0.46 ± 0.03
	A	0.57 ± 0.01	0.64 ± 0.01

^a Sulfur sources: pyrite (PYR), marcasite (MAR), melanterite (MEL), dibenzothiophene (DBT), bituminous coal (BITU), lignite (LIGN).

^b Organisms: *P. aeruginosa* (A), *P. putida* (P).

^c All A_{540} values averaged along growth curve for 55 hours at 30°C. These averages then averaged for saturated medium (n=4) contain particulate sulfur sources or dilutions (n=12) of the sulfur sources.

P. aeruginosa and *P. putida* were characterized by standard enzymatic and carbon assimilation tests to assure the purity of the microorganisms. Preliminary screening of other enzymatic activities indicated semiquantitative differences between the two microorganisms which could not explain the observed differences between their activities related to pyritic desulfurization of coals.

It was further observed that the equivalent growth for both *P. aeruginosa* and *P. putida* was obtained at 30°C, and this temperature was used in further studies. The presence of pyrite, marcasite, and melanterite, although not toxic to the organisms, was generally repressive to their growth with *P. putida* being qualitatively more labile than *P. aeruginosa*. Incubation of both *P. aeruginosa* and *P. putida* for long periods with or without the sulfur sources did not significantly alter the pH of the medium, suggesting that these organisms process sulfur by different mechanism than the organisms of the genus *Thiobacillus*.

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