

**CHARACTERIZATION OF EXTRACELLULAR BACTERIAL ENZYMES WHICH
DEPOLYMERIZE A SOLUBLE LIGNITE COAL POLYMER**

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ABSTRACT

Several Gram negative and positive soil bacteria, isolated by enrichment technique in a liquid minimal medium containing water soluble lignite coal polymer as a sole source of carbon and energy, were screened for their abilities to depolymerize coal when growing in peptone broth containing soluble coal. One Gram positive and 3 Gram negative strains significantly depolymerized the coal within 3-6 days. These strains were screened for the production of extracellular coal depolymerizing enzymes. Each bacterium, including Gram negative strains DLC-BB2, DLC-62 and DLC-63/9, and Gram positive strain DLC-21, produced lignite depolymerizing enzymes. Extracellular filtrates from 3-day cultures grown in peptone medium supplemented with an inducing level of soluble coal polymer, contained an enzymatic activity which caused significant depolymerization of the coal polymer after 4 hr of incubation of enzyme with coal (30°C), as shown by High Performance Liquid Chromatography. Each bacterium produced similarly acting enzymes which progressively converted the principal broad coal polymer peak of about 174,000 MW into a much sharper peak of about 113,000 MW. In some cases, even lower molecular weight products appeared upon prolonged incubation of the reaction mixtures. The activities were inactivated by boiling of culture supernatants. While chemical analyses of enzymatically depolymerized coal products indicated that the depolymerization was non-oxidative, the specific type(s) of enzymes involved in the depolymerization remain to be identified. The results show that nonoxidative, enzymatic depolymerization of coal is possible.

INTRODUCTION

Selected biotransformations of coal may aid in its conversion to liquid and/or gaseous fuels. Such transformations include depolymerizations (1), reductions (2), and/or solubilization (3-6). Both fungi and bacteria have been shown to solubilize coal. Some appear to excrete

enzymes that oxidize coal into water soluble polymeric products (7-9). However, coal solubilization more typically involves the excretion of basic, low molecular weight coal-solubilizing metabolites by the microbes, and coal solubilization is nonenzymatic (3-5, 10-12). Few papers have reported the microbiological depolymerization of coal. One paper (13) reported that lignin peroxidase from the white-rot fungus Phanerochaete chrysosporium oxidized water soluble coal polymers, converting them into smaller molecular weight polymers. Pseudomonas cepacia Strain DLC-07, when growing in liquid media at pH 5.5 depolymerized a water soluble Vermont lignite coal polymer (1). Depolymerization was found to be optimal when the bacterium was grown on coal in a mineral salts-peptone-soluble coal polymer medium (14). Here, we report on characterizations of 3 Gram negative and 1 Gram positive aerobic bacterial strains which metabolize lignite coals. These strains significantly depolymerize base-solubilized lignite when growing on the coal in liquid media and excrete enzymes which catalyze lignite depolymerization via a non-oxidative mechanism. This is the first report of such enzymes from both Gram negative and positive bacteria. Our results are indicative of the positive potential for using bacteria to biotransform coal into useful liquid fuels.

MATERIALS AND METHODS

Isolation of Aerobic Bacteria. The bacteria were isolated from soil associated with coal seams (1) and from other soils rich in decomposing plant residues. The enrichment medium (pH 5.5) was a mineral salts solution supplemented with 0.01% (w/v) of yeast extract (Difco, Detroit, MI) and 0.2% (w/v) of soluble, nitric acid pre-treated Alabama lignite coal polymer (1). The enrichment-isolation procedure was carried out at 30°C as described previously (1). Stock cultures of each bacterium were maintained on Sabouraud Dextrose Agar (SDA) slants at 4°C. Strains DLC-62, DLC-63/9, and DLC-BB2 are as yet unidentified aerobic Gram negative rods, while strain DLC-21 is a spore-forming, Gram positive Bacillus species.

Assay for Extracellular Coal Depolymerizing Enzyme Activity. Each bacterium was grown in shake flasks for three days (30°C) in 250 ml of mineral salts-peptone broth (14) also containing 2mg of water soluble coal polymer. Cultures were then harvested, and the cells removed by centrifugation. The supernatant was used as the source of enzyme. If desired, the supernatants can be concentrated 10-fold by ultrafiltration prior to use. For the assay, 100 ul of soluble coal polymer solution was added to 10 ml of crude or concentrated supernatant. After 0 and 4 hr incubation (30°C; pH 5.5) the reaction was stopped by acidifying 1.0 ml of the solution to pH 2.0. The precipitated coal was collected by centrifugation, washed, redissolved in 1.0 ml

of HPLC solvent, and analyzed by HPLC. Controls contained reaction mixtures treated similarly, but heat-inactivated supernatant was substituted for active enzyme. Coal depolymerization was monitored by observing the appearance of lower molecular weight HPLC peaks.

Coal Substrate Used and Growth of Bacteria on Coal. The coals used was a weathered Vermont lignite (1,2). A coal substrate water soluble at pH 5.5 or higher was prepared from the Vermont lignite as described by Gupta *et al.* (1). One hundred grams of powdered coal were soaked in water for 8 hr and then dissolved in 1 liter of 1N NaOH. The solution was centrifuged to remove undissolved coal, and the pH of the supernatant was adjusted to pH 7.0 with HCl. Coal that precipitated was collected, washed, dried, and powdered (yield: 40 g). The supernatant from the precipitation was acidified to pH 5.5, and the resulting precipitate was collected, washed (pH 5.5 water), dried, and powdered (yield: 15 g). The remaining coal in solution was precipitated at pH 1.5, collected, washed (pH 1.5), and powdered (yield: 10 g). This final coal polymer precipitate, which was soluble at pH's of 5.5 or higher, was used as the substrate in both growth and enzyme studies.

Molecular Weight Distribution of the Coal Polymers. Bacterial depolymerization of the soluble coal polymer was monitored by HPLC, as described previously (1), using a Hewlett Packard 1090A instrument equipped with an HP-1040 diode array detector and a Synchropak GPC-300 column (1000-500,000 MW separation capability) (Synchrom Inc., Lafayette, IN). The mobile phase consisted of phosphate buffer (0.02M KH_2PO_4) containing 0.5% (w/v) Tween 80, pH 7.1, set at a flow rate of 0.25 ml min^{-1} . The column was equilibrated with high and low molecular weight protein standards (1). Coal containing culture medium samples (0.5 ml) were centrifuged, the supernatants acidified to pH 2, and the precipitated coal recovered by centrifugation. The wet precipitate was redissolved in 0.5 ml of HPLC solvent and injected into the HPLC. Elution of the coal polymer was then monitored at 254 nm.

Elemental Analysis of the Coal Polymers. Elemental analysis of 1-3 mg samples of control and depolymerized coal polymers were performed by Desert Analytics, Inc. (Tucson, AZ) according to the procedure described by Gupta *et al.* (5).

RESULTS

Coal Depolymerization During Growth of the Bacteria. From among numerous bacteria isolated from the liquid enrichments, 4 were selected for their ability to depolymerize the soluble Vermont lignite coal in liquid mineral salts-peptone broth. Micrographs of each organism, including Gram negative strains DLC-62, DLC-63/9, and DLC-

BB2 and Gram positive strain DLC-21 are shown in Figure 1. DLC-21, the only Gram positive bacterium in the group, is a nonmotile, spore-forming rod (Fig 1,A). We have classified it as a Bacillus species. All of the Gram negative bacteria are strictly aerobic. DLC-62 and DLC-BB2 are both highly motile, short rods (Fig 1,B and D), while DLC-63/9 is a very long and thin nonmotile rod (Fig 1,C).

As shown in Figures 2 and 3, each bacterium significantly depolymerized soluble Vermont lignite coal polymer within 3 days when growing in mineral salts-peptone-coal polymer broth. Over the same period, the molecular weight of the coal polymer (130,000) remained unchanged in incubated, uninoculated, controls (Figs. 2A, 2B). In contrast, depolymerization resulting from bacterial metabolism (Figs. 3A-D) was significant, and greater than that previously seen with Pseudomonas cepacia DLC-07 after growth for 2 weeks on coal in the same medium (1).

Detection of Extracellular Coal Depolymerizing Enzymes.

Preliminary assays for the presence of extracellular enzymes catalyzing coal depolymerization were carried out by incubating cell free culture filtrates from 3-day cultures grown in mineral salts-peptone broth supplemented with an inducing level of coal polymer (2mg/250ml). Each bacterium produced lignite depolymerases. Cell free culture filtrates from each of the cultures enzymatically transformed the coal polymer within a period of 1-4 hr. (data not shown). HPLC elution profiles shifted to lower molecular weight peaks in a pattern like that observed when the bacteria were grown on the coal polymer in peptone broth. In contrast, elution profiles of reaction mixtures incubated with heat-inactivated (boiled; 5 min) culture filtrates replacing untreated filtrate (=inactivated controls) did not change over the incubation period. In active filtrates HPLC elution profiles of the coal polymer shifted from a single major peak with a retention time corresponding 167,000-174,000 MW to a sharper peak averaging about 113,000 MW, and having a higher molecular weight shoulder of 144,000-170,000 MW. With several of the reaction mixtures, a shoulder peak corresponding to about 87,000 MW also appeared after extended incubation. All of the bacteria produced similarly acting enzymes. The time of harvesting of the filtrates was critical to their activity. If harvested too early or too late, activities were low.

Elemental Composition of the Coal Polymers.

Elemental analyses of the depolymerized coal polymers showed no major changes in C, H, O, N, or S content relative to the starting coal (Table 1). The ash contents increased slightly. These data show that the depolymerizations were non-oxidative, although additional data will be needed to refine this conclusion.

DISCUSSION

This is the first report of coal depolymerizing enzymes in Gram positive and negative bacteria. Previously, Wondrack et al. (13) reported that the lignin peroxidase of the fungus Phanerochaete chrysosporium would depolymerize soluble base-solubilized coal polymers. The enzyme was oxidative, utilizing H_2O_2 to oxidize the coal. The enzymes produced by our bacterial isolates, in contrast, act non-oxidatively, perhaps hydrolytically.

We have found that careful timing of culture harvest is critical to obtaining optimally active enzyme preparations. Thus, we need to carry out a study that relates coal depolymerizing enzyme production to the growth curves of each of these bacteria. Then, we can establish the optimal incubation times for production of the enzymes by each strain. The rapid rate of depolymerization we observe (1-4 hr), and the appearance of a sharp product peak in HPLC chromatograms lead to some intriguing hypotheses concerning the nature of these enzymes. The enzymes may act to cleave structurally important internal linkages such as ether or ester bonds within the lignite macromolecule, thereby significantly depolymerizing the polymer. The remaining undepolymerized polymer, accumulating as the 113,000 MW peak in HPLC chromatograms, may represent a macromolecular structure more resistant to the enzymes and/or not containing susceptible linkages. Additional research will hopefully determine if this is the case. Regardless, the overall data show clearly that bacterial enzymes may be useful in breaking down the macromolecular structure of coal to produce lower molecular weight products potentially useful in the microbiological production of liquid or gaseous fuels.

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REFERENCES

- 1) Gupta, R.K., L.A. Deobald, and D.L. Crawford. 1990. Depolymerization and Chemical Modification of Lignite Coal by Pseudomonas cepacia Strain DLC-07. Appl. Biochem. Biotechnol., 24/25: In press.
- 2) Deobald, L.A. and D.L. Crawford. "Isolation of Microorganisms Able to Reductively Transform Aromatic Compounds and Their Relevance to Coal Liquefaction. In Proceedings of the Second International, Institute of Gas Technology Symposium on Gas, Oil, and Coal Biotechnology. December 11-13, 1989. New Orleans, LA.
- 3) Scott, C.D., G.W. Standberg, and S.N. Lewis. "Microbial Solubilization of Coal," Biotech. Progress, 2: 131-139 (1986).
- 4) Quigley, D.R., B. Ward, D.L. Crawford, H.J. Hatcher, and P.R. Dugan. "Evidence that Microbially Produced Alkaline Materials are Involved in Coal Biosolubilization." Appl. Biochem. Biotechnol., 20/21: 753-763 (1989).
- 5) Gupta, R.K., J.K. Spiker, and D.L. Crawford. "Bio-transformation of Coal by Ligninolytic Streptomyces," Can. J. Microbiol., 34: 667-674 (1988).
- 6) Cohen, M.S. and P.D. Gabriele. "Degradation of Coal by the fungi Polyporus versicolor and Poria monticola," Appl. Environ. Microbiol., 44: 23-27 (1982).
- 7) Cohen, M.S., W.C. Bowers, H. Aronson, and E.T. Gray, Jr. "Cell-free Solubilization of Coal by Polyporus versicolor." Appl. Environ. Microbiol., 53: 2840-2843 (1987)/
- 8) Pyne, J.W., D.L. Stewert, J. Fredrickson, and B.W. Wilson. "Solubilization of Leonardite by an Extracellular fraction from Coriolus versicolor," Appl. Environ. Microbiol., 53: 2844-2848 (1987).
- 9) Moolik, R.T., J.C. Linden, and M.N. Karim. "Bio-solubilization of Lignite," Appl. Biochem. Biotechnol., 20/21: 731-742 (1989).
- 10) Maka, A., V.J. Srivastava, J.J. Kilbane II, and C. Akin. "Biogeochemical Solubilization of Untreated North Dakota Lignite by a Mixed Bacterial and a Mixed Bacterial-Fungal Culture," Appl. Biochem. Biotechnol., 20/21: 715-729 (1989).

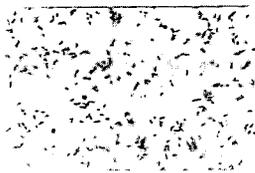
- 11) Strandberg, G.W. and S.N. Lewis. "The Solubilization of Coal by an Extracellular Product of Streptomyces setonii," J. Ind. Microbiol., 1: 371-375 (1987).
- 12) Faison, B.D. and S.N. Lewis. "Production of Coal-Solubilizing Activity by Paecilomyces sp. During Submerged Growth in Defined Liquid Media," Appl. Biochem. Biotechnol., 20/21: 743-752 (1989).
- 13) Wondrack, L., M. Szanto, and W.A. Wood. "Depolymerization of Water Soluble Coal Polymer from Subbituminous Coal and Lignite by Lignin Peroxidase," Appl. Biochem. Biotechnol., 20/21: 765-780 (1989).
- 14) Crawford, D.L. and R.K. Gupta. "Influence of Cultural Parameters on the Depolymerization of a Soluble Lignite Coal Polymer by Pseudomonas cepacia DLC-07," Resources, Conservation, and Recycling, In Press.

Table 1. Elemental Analyses of Control and Bacterially Depolymerized Coal Polymer Samples after Incubation in the Peptone-Coal Polymer Broth for ? days.

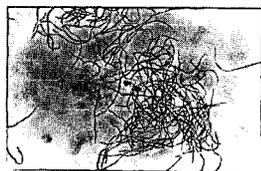
SAMPLE	%C	%H	%N	%O	%S	ASH
Control	49.1	4.1	3.3	35.6	1.0	3.8
DLC-21	49.9	4.0	3.1	33.8	1.5	4.8
DLC-62	50.7	4.0	3.1	35.0	1.2	4.2
DLC-63/9	51.0	4.0	3.1	35.4	1.3	5.1
DLC-BB2	50.6	4.0	3.2	35.5	1.2	5.1



A



B



C



D

Figure 1. Light micrographs of aerobic bacterial strains a) DLC-21, b) DLC-62, c) DLC-63/9, and d) DLC-BB2. All micrographs are from wet mounts photographed at 1000X magnification.

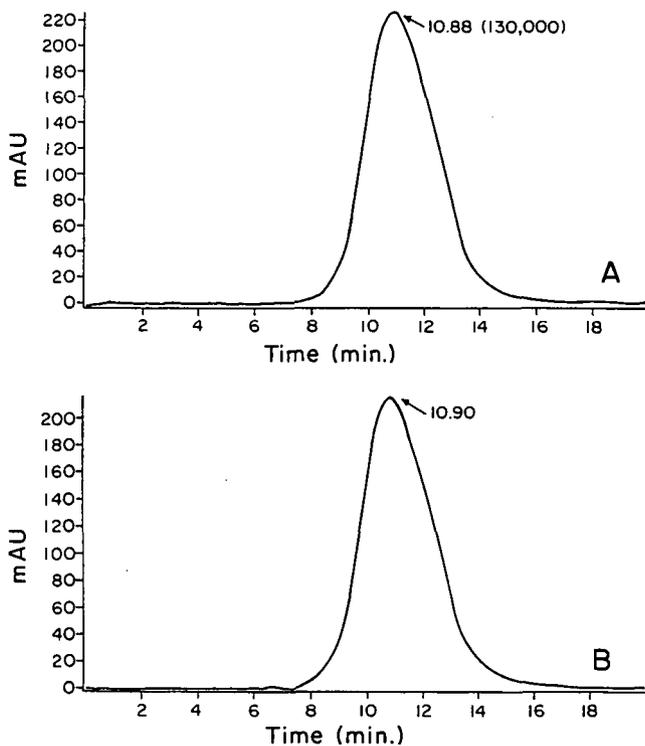


Figure 2. HPLC chromatograms showing the molecular weight distributions of the soluble lignite coal polymer in uninoculated controls at time 0 and after 3 days incubation in the mineral salts-peptone-coal broth at 30°C. A) Uninoculated control, time 0; B) Uninoculated control, 3 day. The retention time for the principal peak (10.9 min) represents an averager molecular weight of 130,000 daltons.

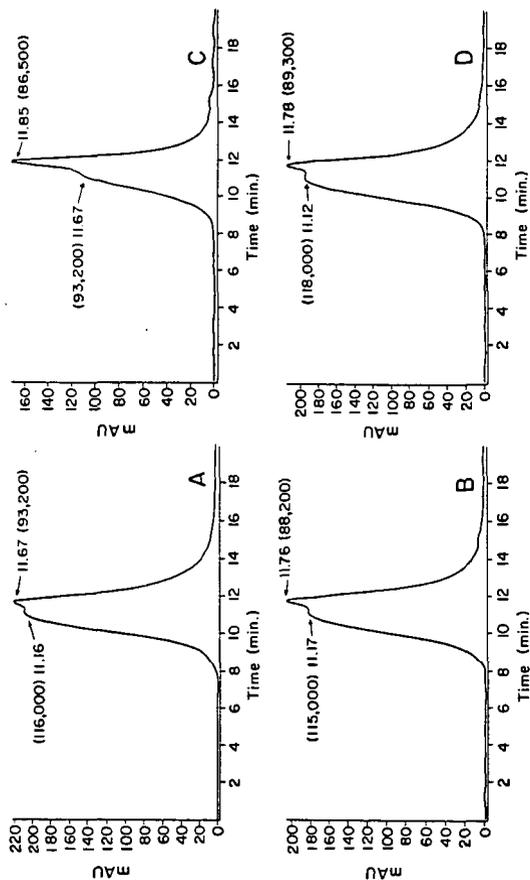


Figure 3. HPLC chromatograms showing the molecular weight distributions of the soluble lignite coal polymer in bacterially inoculated cultures after 3 days incubation in the mineral salts-peptone-coal polymer broth at 30°C. A) DLC-21; B) DLC-62; C) DLC-63/9; D) DLC-BB2. The retention times for the principal peaks are given in minutes, and the average molecular weights for each are presented beside the retention times in parentheses.