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BIOPROCESSING OF LIGNITE COALS USING REDUCTIVE MICROORGANISMS

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Executive Summary

Huge world reserves and the low caloric value of lignite coal strongly suggests a need to develop alternative technology that can make better use of this fossil fuel. source. Its close structural similarity to lignin and high oxygen content are indicative of promising prospects for bioprocessing lignite coals. It is, however, certain that in order to convert lignite coals into liquid fuels, gases or chemical feedstock, the macromolecular structure of the coal must be broken down into low molecular weight fractions prior to further modification. Our research focused on this aspect of coal bioprocessing.

We isolated, characterized and studied the lignite coal-depolymerizing organisms *Streptomyces viridosporus* T7A, *Pseudomonas* sp. DLC-62, unidentified bacterial strain DLC-BB2 and Gram-positive *Bacillus megaterium* strain DLC-21. In this research we showed that these bacteria are able to solubilize and depolymerize lignite coals using a combination of biological mechanisms including the excretion of coal solublizing basic chemical metabolites and extracellular coal depolymerizing enzymes.

Project Summary

The initial hypothesis upon which this research was based was that microorganisms, particularly strictly anaerobic bacteria, would be able to metabolize and thereby biotransform lignite coals in ways that would completely or partially biotransform the coal into higher value liquid fuel products. By use of anaerobic bacteria it was thought that the biotransformations would be largely reductive, as opposed to oxidative. Thus, the liquid products generated would not be reduced in caloric fuel value as compared to the starting coal. During the course of research numerous bacteria were isolated and characterized for their abilities to biotransform coal and coal substructure model compounds. The isolates eventually chosen for detailed study turned out to be aerobic bacteria which nonoxidatively both solubilized and depolymerized the coal. Thus, the orginal objectives of the research wer met, but with aerobic instead of anaerobic bacteria. The mechanism by which selected of the bacteria solubilized lignite coals were determined. This mechanism involved the excretion of basic chemical metabolites which accumulated in the growth medium. These metabolites, in turn, acted chemically to solublize the coal. In addition, the mechanisms by which selected of the bacteria depolymerized solubilized lignite coal polymer were determined. This mechanisms involved the excretion of extracellular coal depolymerizing enzymes. Follow-up studies showed that the specific types of enzymes involved appeared to be extracellular peroxidases, esterases, and possibly etherases. The results show

that bacteria can be used to beneficially transform coal, by solublizing and depolymerizing the coal into lower molecular weight products that have not be extensively oxidized as a result of the microbial metabolism. Future work should focus research aimed at optimizing the biodepolymerization process, developing bioreactor configurations for scaling up the biodepolymerization process, and understanding the basic enzymatic biochemistry of the coal depolymerizing enzyme reactions.

Introduction

Coal constitutes the largest portion of the world's fossil fuel reserve. The current level of world coal production is estimated to be 5.2 billion short tons per annum, with the largest producers being China (1054 million short tons), U.S.A. (950 million short tons), Soviet Union (865 million short tons), and United Germany (556 million short tons).[1] Of these major producers, the United States, Soviet Union and China have by far the largest recoverable world coal reserves-29%, 26%, and 11% respectively.[2] Although coal represents a significant world fuel reserve, not all deposits are of high economic importance at present.

Because of its variance in chemical composition and corresponding degrees of economic importance, coal is classified by rank into four categories: Starting with the least degree of coalification, these are lignite, subbituminous, bituminous, and anthracite coals. Bituminous and anthracite are classified as high-rank coals because of their high caloric value; Lignite and subbituminous, on the other hand, are classified as low-rank, brown, or soft coals because of their low caloric value. High-rank coal deposits are estimated to be 6,900 billion tons- out of which 993 billion tons are recoverable under current mining techniques. Confirmed world reserves of low-rank coal are 6,500 billion tons- out of which 576 billion tons are recoverable.[1,3] Low-rank coals are characterized by low aromaticities, small aromatic ring clusters, and abundant aliphatic and heteroaromatic structures. The low calorific value (around 11,500 Btu/lb), due

to low carbon, and high oxygen and moisture contents, makes low-rank coals unsuitable for power production.[4] Low-rank coals also contain high levels of alkali and alkaline earth elements. In fact, these inorganic constituents are the cause of fouling, agglomeration, and slagging in conventional processes.[5] Such undesirable characteristics and lack of world supply strongly suggest that low-rank coals should be liquefied, gasified, or converted into chemical feedstocks. Biological processes may be useful for carrying out such conversions.[6-11]

As early as 1908 researchers were experimenting with the microbial utilization of coal,[12] but it was chiefly the work published by Cohen and Gabrielle in 1982 that stimulated present interest in coal bioprocessing.[19] They were the first to report the formation of water-soluble products by the action of the wood-rotting Basidiomycete fungi *Trametes versicolor* and *Poria monticola* on low-rank coals. Since then, there have been tremendous interest in and research efforts devoted to utilizing fungi,[19-40,50,52] actinomycetes[28,45-52] and bacteria,[12-18,29,52-60] for coal bioprocessing.

Investigative Approach and Research Results

Overall Research Results

In our efforts to utilize low-rank coals for energy and chemical feedstock production[48], we isolated several aerobic Gram-negative and Gram-positive bacterial cultures which depolymerize water-soluble lignite coals into low molecular weight products.[55-60] Production and involvement of

extracellular enzymes in lignite bio-depolymerization was proven by HPLC analysis.[57-60] We also characterized the extracellular enzymes in the culture supernatants of *Pseudomonas* strain DLC-62 and *Streptomyces viridosporus* T7A.[59] The enzymes in the case of *Pseudomonas* strain DLC-62 consisted primarily of peroxidases (two isoforms) and esterases (five isoforms). Etherases were probably also present, but their existence was not uneuqivocally shown. The two peroxidase isoforms were constitutively expressed. When partially purified, the esterases from periplasmic, cytoplasmic, and membrane fractions of the cells showed no coal depolymerizing activity. Unpurified crude enzyme preparations, on the other hand, containing both esterases and peroxidases did show coal-depolymerizing activity. The results with 3 day mineral salts-peptone + coal cell-free culture filtrates, particularly in case of *Pseudomonas* sp. DLC-62 and unidentified strain DLC-BB2, clearly-suggested free radical formation which could be due to the presence of peroxidase, laccase or multiple Cytochrome P-450 type of enzymes. It seems quite reasonable to expect these bacterial cultures to attack low-energy requiring bonds like ether and ester and bridged methylene groups rather than high energy carbon-carbon bonds. Resonance stabilization of phenolic radicals would certainly be responsible for lowering the activation energy required to cleave high- energy bonds inside the coal polymeric structure. Indirect oxygenation-either by direct action of oxygenases, or by oxidative or hydrolytic cleavage of ether and ester linkages must have been responsible for increasing the polarity of hydrophobic substrates like

lignite coal polymer. Peroxidases and ligninases have been implicated in the depolymerization of lignin and synthetic azo-dyes.[41]

Our research group has been involved in studies of the biodegradation of lignin for over fifteen years, and based upon our knowledge in that area, we noticed that the depolymerization mechanisms of lignin and lignite, and the nature of the enzymes involved, seemed to be similar.. It is fair to say that coal biotransformation and bioconversion will not be technically and economically feasible until and unless the coal macromolecular structure is broken down into low molecular weight fractions for further utilization or modification. Therefore, coal biodepolymerization seems to be the crucial step in efficient biogasification, bioliquefaction, biosolubilization and biological coal beneficiation. Our research showed that bacteria possess the ability to significantly depolymerize lignites. Our future goal should be to understand and develop precise bioreactor configurations, to further define the enzymatic mechanism responsible for the lignite depolymerization, and to increase the level of lower molecular weight products produced from lignite in order to provide economically and technically feasible processes for the production of liquid and/or gaseous fuels.

Coal biotransformations can be grouped into four categories: solubilization, depolymerization, gasification, and liquefaction. In solubilization, coal is microbially converted into water-soluble polymeric material; the product may or may not have been

significantly depolymerized when compared to the starting coal. In depolymerization, coal which has been solubilized microbially or chemically becomes significantly reduced in average molecular weight as a result of microbial action. This requires cleavage of macromolecular bonds in the coal. In gasification, the coal is microbially converted to methane and carbon dioxide gas. In this case, the starting coal may be solid, liquid, polymerized, depolymerized, or modified previously chemically or microbially. In liquefaction, coal is converted to a water-insoluble liquid-a conversion that probably requires both depolymerization and reductive transformation of the coal polymer.

Over the past nine years several research groups have reported the solubilization of low rank coals by the following fungi: *Acremonium*,sp.[22] *Aspergillus* sp.[20,23], *Candida* sp.[20,28,33-35,37] (Strain ML-13),[21,23,51] *Coriolous versicolor*[27,28,37,51] (also known as *Polyporous versicolor*[19,25,26] and *Trametes versicolor*[23,30,31,36]), *Cunninghamella* sp.[28,29,37,51], *Ganoderma applanatum*,[51] *Heterobasidion annosum*,[28] *Mucor* sp.[20], *Paecilomyces* sp.[20,23,32-34], *Penicillium* sp.[20,22,28,29,36,37,50] (*P. waksmanii* ML-20[21,23,51]), *Percniporia subacida*,[28] *Perenniporia tephropora*, (*Fomes lividus*),[51] *Phanerochaete chrysosporium*, [24,28,29, 37,39,40,51] *Pleurotus ostreatus*[51] *Poria monticola*[19,23] (also known as *Poria placenta*[28] and *Oligoporus placentus*), *Polyporous dryophilus* var. *vulpinus*,[28] *Pycnoporus cinnabarinus*,[51] *Rigidoporus ulmarius*,[51] *Sporothrix sp.*[23], and *Xylaria hypoxylon*.[51] Although certain of these reports have suggested

the possible involvement of oxidative extracellular enzymes in the coal solubilization process, others have been strongly indicative of non-enzymatic coal-solubilizing secondary metabolites.[11,30,31]

Fredrickson *et al.*[30] isolated a low molecular weight (500-1000 dalton) extracellular metabolite from cultures of the fungus *Trametes versicolor,* which appeared to be responsible for lignite coal-solubilizing activity. The compound was thought to be desferal mesylate,a hydroxamate siderophore-like compound. In contrast, Cohen *et al.*[31] have isolated a different coal-solubilizing metabolite from culture supernatants of *T. versicolor*(ATCC-12679). After purification and crystallization they characterized the solubilizing agent as ammonium oxalate. It seems rather plausible that after the uptake of iron and other transition metals by the siderophores and chelators, a disruption of charge-transfer interactions[42] between the coal molecule may be the real fungal coal solubilization factor.

Linehan[36] characterized the biosolubilization of a leonardite coal and a pre-oxidized Illinois #6 coal by two fungi, *Trametes versicolor* and *Penicillium* species. He found that biosolubilization was oxidative and that the biosolubilized product was slightly soluble in organic solvent and had a molecular weight below 20,000 daltons. Interestingly, as the coals were incubated with fungi, more coal became water-soluble over time, and the average molecular wt. of the soluble products progressively increased.

Stewart *et al.*[37] recently studied a *Penicillium sp.* strain RWL-5 which degraded an air-oxidized bituminous coal and caused erosion of the coal surface. The authors also believed that depolymerization of the coal occurred during biosolubilization. This was suggested by a comparison of the molecular weights of the biosolubilized product (1000 dalton) a the base-solubilized product (6000 dalton).

Fakoussa et al.[38] isolated and examined *Chaunopycnis alba* for the production of coal-solubilizing enzymes. They associated the ability of this fungus to solubilize lignite coal with the production of an extracellular peroxidase. They also concluded that solubilization was accompanied by increased hydrophilicity and depolymerization, although little data was supplied to support this conclusion.

Wondrack *et al.*[39,40] were the first to clearly demonstrate depolymerization of water-soluble lignite and subbituminous coals, which were depolymerized upon incubation with the lignin peroxidase of *Phanerochaete chrysosporium* (ATCC-34561). They based their conclusions on gel permeation, high performance liquid chromatography in which the average molecular weights of the soluble coal polymers incubated with peroxidase were reduced as compared to the control. While the accuracy of their gel-permeation procedure has recently been questioned (see chapter 3)[11] their data is neverthless fairly conclusive. Addition of veratryl alcohol to the reaction mixture stimulated depolymerization of the coal polymer, and veratryl alcohol oxidation by the peroxidase was inhibited by the coal polymer.

According to the authors, binding of coal polymer to the peroxidase might be responsible for inhibiting veratryl alcohol oxidation. However, very recent data indicate that veratryl alcohol might act as a third substrate (with H_2O_2 and a primary substrate such as coal) in the lignin peroxidase cycle during oxidation of the primary substrate.[41] The papers discused above are largely concerned with solubilization of coal, not depolymerization. However, it is clear from the limited data thus are published that solubilization of coals by fungi is sometimes accompanied by its depolymerization as well.

In 1964, Korburger[16] was the first to report the utilization of hydrogen peroxide-pretreated coal by bacterial cultures of *Escherichia freundii* and *Pseudomonas rathonis*. Fakousa and Trüper[18] later isolated a strain of *Pseudomonas fluorescens* from a mixed population collected in a burned forest region. The isolated organism could solubilize coal by the combined action of a bacterial surfactant and an extracellular enzyme produced by the organism.

In this research[43] we isolated several lignin-degrading *Streptomyces* species, including *S.viridosporous T7A, S. setonii 75Vi2, and S. badius 252*. According to Crawford and Crawford,[58] degradation of lignin by these *Streptomyces* involved oxidative depolymerization of the lignin polymer. The structural similarity between lignin and lignite prompted an investigation of the coal-solubilizing ability of these *Streptomyces* species. Strandberg and Lewis[45] reported that *S. setonii 75Vi2 and S. viridosporus T7A* solubilized several low-rank coals placed on the

surface of the agar cultures. They observed an extracellular coal-solubilizing component in culture supernatants of coal-containing submerged cultures of *S. setonii 75Vi2*, but not in cultures of *S.viridosporous* T7A.[46,47] The solubilizing component was heat stable, protease resistant, and had a molecular weight between 1000 and 10,000 daltons. They concluded that the activity was non-enzymatic and indicated the possible involvement of a basic polypeptide or polyamine. No attempt was made to determine if the soluble product had been depolymerized. We[48] also studied the coal-solubilizing ability of *S. viridosporous T7A, S. setonii 75Vi2, and S. badius 252*. After studying the chemical characteristics of the biosolubilized product and examining the mechanism of coal solubilization, we confirmed the involvement of a non-enzymatic mechanism mediated by a microbially-generated basic metabolite.

Initially we isolated the Gram negative bacterium *Pseudomonas cepacia* DLC-07 from Gasoyne soil (North Dakota) by enrichment in a mineral salts-coal polymer minimal medium over a period of several weeks.[55] This strain grew on water-soluble coal and depolymerized its macromolecular structure. Bacterial depolymerization was monitored by high performance liquid chromatograph (HPLC). *P. cepacia* DLC-07 was able to co-metabolize a number of coal model compounds and also utilized p-hydroxy substituted benzoic or cinnamic acids or aldehydes as a sole carbon and energy source.[55] *P. cepacia* DLC-07-mediated coal depolymerization was non-oxidative as shown by elemental analysis, ^{13}C-CP/MAS NMR, and IR data.[55] Later, tried to

increase the level of coal depolymerization by the addition of a rich carbon source like Sabouraud Dextrose Broth (SDB) or Peptone broth to the medium. We also sometimes supplemented the media with coal-substructure model compounds to act as potential inducers of coal-degrading enzymes. Interestingly, *P. cepacia* DLC-07 depolymerized coal best in peptone+coal broth.[56] The results clearly indicated a decrease in carbonyl content (ester and carboxylate groups), aromatic and conjugated carbon-carbon double bonds, methylene bridges, and etheric oxygen in the depolymerized coal product as compared to the starting coal.

In addition to *P. cepacia* DLC-07, we isolated four other aerobic bacterial strains from the water-soluble coal polymer minimal medium enrichments.[57,58] One of the bacteria in this group was a Gram positive spore-forming non-motile rod, while the other three were Gram negative rods. The Gram positive bacterium was identified as *Bacillus megaterium* DLC-21, on the basis of biochemical tests and a gas chromatographic analysis of its cellular fatty acids. The three Gram negative bacteria were strict aerobes ; DLC-62 and DLC-BB2 were both highly-motile rods, while DLC-63/9 was a very long and thin non-motile rod. The biochemical tests and cellular lipid analyses did not lead to their identification; it is certain, however, that DLC-62 is a *Pseudomonas*.

In order to increase the level of coal depolymerization by these bacteria, and to understand the enzymatic mechanisms involved , we grew the cultures in phosphate buffer-peptone broth containing water-soluble coal as a co-substrate.[57,58] Samples

were taken out at time 0,3 days, and thereafter at intervals of every 3 days for a total of 30 days. The 3-day old cultures showed excellent coal depolymerization. Coal depolymerization was clearly evident in all the bacterial cultures, including *P. cepacia* DLC-07, *Bacillus megaterium* DLC-21, *Pseudomonas sp.* DLC-62, and the unidentified strains DLC-63/9 and DLC-BB2.

Depolymerized coal was recovered from 3-day old control and inoculated cultures and then analyzed by FT-IR spectroscopy. The coal , although depolymerized, did not show increased oxidation relative to the control coal; this was clearly evident from I.R. difference spectra of all the samples. The difference spectra showed a decrease in carbonyl groups due to un-conjugated ketone and carboxyl groups and _,ß-unsaturated esters (1707-1719 cm^{-1}), and a decrease in carboxyl groups in conjugation with aromatic rings ,hydrogen bonded quinones or highly conjugated carbonyl groups (1666-1637 cm^{-1}). The other weak residual bands at 1525-1531, 1478,1431,1365,and 1225-1237 cm^{-1} could be due to carboxylates or C-O bonds in phenols or aryl-ethers.[58,60] Overall the results were indicative of a non-oxidative transformation of the coal polymer.

To investigate the long-term transformations of water-soluble coal polymer by these strains, Vermont lignite coal samples from a noninoculated control and from inoculated cultures incubated for five months in a minimal-mineral-salts+coal broth, were analyzed for their elemental composition, by FT-IR, and by ^{13}C (CP/MAS) NMR. The overall results showed that the long-term

coal biotransformations of coal by these cultures involved non-oxidative reaction mechanisms.

Having seen the depolymerizing activity by these five organisms, we wondered just how catabolically versatile they were. We therefore, investigated the co-metabolizability of a number of coal substructure model compounds by these organisms. The co-metabolizing ability of *Pseudomonas cepacia* DLC-07, (published earlier)[55] and the results from *Bacillus megaterium* DLC-21, *Pseudomonas* sp. DLC-62. strains DLC-63/9 and DLC-BB2 are presented in chapter-4.[60] *Bacillus megaterium* DLC-21 and *Pseudomonas* sp. DLC-62 could metabolize p-Hydroxycinnamic acid derivatives (p-coumaric acid, ferulic acid, caffeic acid and 3,4-dihydroxycinnamic acid) but none of the four cultures metabolized cinnamic acid. Strain DLC-62 was able to metabolize p-Hydroxybenzoic acid and p-Hydroxybenzaldehyde derivatives except syringic acid. All the four organisms (DLC-21,62,63/9 and DLC-BB2) metabolized vanillin and 3,4-Dihydroxybenzaldehyde, but syringaldehyde (a p-hydroxy derivative of benzaldehyde) was metabolized only by strains DLC-62 and DLC-63/9. *Bacillus megaterium* DLC-21 was the only organism to metabolize dibenzothiophene. Two of the organisms (DLC-62 and DLC-BB2) metabolized 8-Hydroxyquinoline. These results clearly demonstrate the catabolic versatility of these organisms.[60]

Since all five bacterial cultures depolymerized water-soluble coal, we investigated the effect of these cultures when incubated on coal together as a single mixed culture. The reason for doing such an experiment was to determine if these co-

cultures could depolymerize and modify water-soluble coal by functioning either in sequence or synergistically. After an incubation period of five-months in a minimal mineral-salts+coal broth, a coal sample from this consortium was analyzed and compared with the other previously characterized coal samples. The results were comparable to individual cultures.

To better understand the mechanism of depolymerization and the nature of the biocatalysts involved in coal depolymerization, we investigated the possibility of depoymerization reactions catalyzed by cell-free culture filtrates. Cell-free culture filterates, from 3-day old cultures grown in mineral salts-peptone broth supplemented with coal polymer, were used to study depolymerizing activity. Water-soluble coal was added to cell-free filtrate and incubated for 4 hr. The coal was then precipitated by acidification, dissolved in HPLC solvent, and subjected to size exclusion HPLC.[57] Heat-inactivated filtrates were used as controls; the rest of the procedure was kept the same. The HPLC results with *Pseudomonas* sp. DLC-62 and DLC-BB2 indicated that an extracellular biocatalyst was responsible for the depolymerizing activity.[60] The HPLC profiles of the control (heat inactivated) and active samples of *Pseudomonas* sp.DLC-62, clearly show a major peak at a retention time 10.32 corresponding to MW 162,200 D. and a shoulder at RT 12.45 (MW 18,200 D) in 4 hr old control sample. Active filtrates on the other hand show two peaks at RT 10.29 and 11.49 corresponding to molecular weights 165,900 D and 47,800 D. Sodium borohydride reduction of a 4 hr active cell free filtrate treated-coal fraction showed a major

peak at RT 11.55 (MW 45,700 D) and a shoulder at RT 10.30 (MW 165,900 D) instead of two major peaks. This kind of behavior strongly suggested that free-radicals were formed during depolymerization of the coal polymer. In the case of strain DLC-BB2, active filtrate plus coal incubations after a period of 3 hr, showed two peaks at RT 10.04 (MW 213,800 D) and 11.83 (MW 35,000 D). The heat inactivated control sample, on the other hand showed a sharp peak at RT 9.98 (MW 229,000 D) and a shoulder at RT 11.80 (MW 35,500 D). The sodium borohydride reduction of the 3 hr fraction leads to a major peak at RT 11.83 (MW 35,000 D) and a shoulder at RT 10.04 (MW 213,800 D). The results from these two experiments indicated cleavage of macromolecular bonds which in turn resulted in its depolymerization. The sodium borohydride reduction also indicated the formation of free-radicals, whose existence must be verified by ESR spectroscopy

Future Research Needs

Additional research is needed to characterize the depolymerization of lignite by these bacteria, particularly with regard to gaining a further understanding of the enzymology involved, and for scaling up the depolymerization process to examine its practical feasibility. Below is a list of the specific research objectives we feel are appropriate for future research.

1. Bench scale (1 to 2 liter capacity) three phase sparged slurry bioreactor in batch mode should be used to study the kinetics of bio-depolymerization of lignite by the organisms (*Streptomyces viridosporus* T7A, *Pseudomonas* sp. DLC-62, DLC-BB2, and *Bacillus megaterium* DLC-21).

2. Low-molecular weight compounds produced in the bioreactors should be separated continuously, fractionated and analyzed by GC/MS and by HPLC/MS. The bio-depolymerized lignite should be characterized by using size-exclusion HPLC, elemental analysis, FT-IR, proton and ^{13}C (CP/MAS) NMR spectroscopy.

3. The bio-depolymerized lignite should be fractionated by solvent extraction into oils (hexane or pentane soluble), asphaltenes (benzene soluble), pre-asphaltenes (pyridine soluble), and pyridine insoluble residues. These fractions should then be analyzed for their yields, bulk properties to estimate the relative saturated, monoaromatic, diaromatic, polyaromatic and hetrocyclic compounds, using chromatographic (GC & HPLC) methods coupled to a mass spectrometer.

4. Using classical enzyme purification techniques, the lignite depolymerses should be isolated and purified from *Streptomyces viridosporus* T7A, Gram negative bacterial strains *Pseudomonas* sp. DLC-62, DLC-BB2, and Gram positive *Bacillus megaterium* DLC-21.

5. Purified coal depolymerases should be characterized for their etherase, esterase, peroxidase and laccase activities.

6. Optimization of the enzymatic process for lignite depolymerization should be done to ensure optimal substrate concentration, oxygen concentration, temperature, pH, and enzyme to enzyme ratios (if multiple enzymes are involved).

7. From the data obtained above, the correct kinetic parameters and the necessary bioreactor configurations for a pilot scale depolymerization study should be determined.

Materials and Methods Used in This Research

Coal Substrates.[55,56] The coal used as a substrate for enrichments was weathered Cuba Alabama lignite which was supplied by Prof. H. Bailey Ward (University of Mississippi). This coal was treated with 20% HNO_3, for 6 h. The treated coal was washed, dried, powdered and dissolved in 0.1N NaOH, and then the solution was neutralized with 0.1 N HCl to pH 7.1 before use. A second polymeric coal substrate, soluble in water at pH 5.5, was prepared from 200 g of Vermont lignite coal. Powdered lignite was soaked in water for 6 h and then treated with 20% HNO_3 for 6

h. This coal was washed, dried, powdered and dissolved in 1 L 1N NaOH solution. The solution was centrifuged to remove any undissolved particles, and the supernatant was acidified to pH 7.0 with dilute HCl. The precipitated coal was centrifuged, washed, dried, and powdered (yield: 100 g). The supernatant from the above precipitation was acidified to pH 5.5. Precipitated coal was recovered by centrifugation, washed (pH 5.5), dried, and powdered (yield: 20 g). The remaining coal in solution was precipitated at pH 1.5. The coal was recovered by centrifugation, washed (pH 1.5), dried, and powdered (yield: 20 g). The later lignite coal polymer dissolved in solutions of pH 5.5 or higher and was used as a substrate for coal depolymerization studies.

Nitric acid un-treated Vermont lignite : The procedure was same as above except there was no HNO_3 treatment of the coal.

Isolation of Aerobic Bacteria.[55] The bacteria were isolated from soil associated with coal seams and from other soils rich in decomposing plant residues. The enrichment medium contained a mineral salts solution of the following composition g/l: $(NH_4)_2SO_4$, 2.0; KH_2PO_4, 2.0; $MgSO_4$, 0.5; and $CaCl_2$, 0.1. The medium also contained 0.1 g/L yeast extract (Difco, Detroit. MI) and 2.0 g/L of soluble Alabama lignite coal. The pH was adjusted to 5.5 before autoclaving. Soil (1 g) was added to 100 ml of the medium in a 250 ml flask. Shaking incubation (250 rpm) was for 2 wks. at 30°C. Then, 100 μL of the enrichment was transferred to 100 ml of fresh medium, and incubation was continued. After two more such biweekly transfers (a loopful of the inoculum was used in

the final transfer), pure cultures were isolated by streaking onto Sabouraud Maltose Agar (SMA) plates. Pure colonies were streaked onto agar plates of soluble Vermont lignite coal (2 g/L)-mineral salts medium (pH 5.5), and only organisms that showed growth were selected for evaluation. *P. cepacia* DLC-07 was isolated from Gascoyne soil (North Dakota), *Bacillus megaterium* DLC-21 from William Loam soil (North Dakota), *Pseudomonas* sp. DLC-62 & unidentified gram negative long rod DLC-63/9 from Woodland floor soil (Mississippi), and another gram negative strain DLC-BB2 was isolated from organic rich peat soil (Florida). Stock cultures were maintained on Sabouraud Dextrose Agar (SDA) and on SDA containing 200ppm soluble coal solution. Cultures are also maintained in minimal mineral salts-soluble coal medium shaking at 30°C.

Growth of Organisms in Minimal Medium Containing Coal.[55,57] The growth medium contained mineral salts solution as above) and soluble Vermont lignite coal (2.0 g/L dry wt.). In earlier studies[55] with *Pseudomonas cepacia* DLC-07, the coal used was HNO_3 treated Vermont lignite, while in our latter studies the coal used was HNO_3 un-treated Vermont lignite solution. Six 1 liter flasks containing 500 ml of the mineral salts solution (as in isolation of aerobic bacteria above) and soluble Vermont lignite (2.0 g/l) were sterilized by autoclaving. One served as an uninoculated control, while others were inoculated separately with a loopful of cells of DLC-07, DLC-21, DLC-62, DLC-63/9, DLC-BB2 and a loopful of all of these individual cultures, in the case of mixed cultures. All flasks were incubated with shaking

(250 rpm) at 30°C. After a period of five months, a serial dilution plate count was performed on SDA. After five month period the viable cell counts for DLC-07 ,DLC-21, DLC-62, DLC-63/9, DLC-BB2,and DLC-Mix were 1.1X 10^7, 1.22 X 10^3, 1.08 X 10^6, 6.8 X 10^3, 6.0 X 10^5, and 4.3 X 10^5 cells per ml.

Coal Depolymerization in Peptone Broth.[57] Cultures (DLC-07, DLC-21, DLC-62, DLC-63/9, DLC-BB2, and DLC-Mix.) were grown in 250 ml of mineral salts-peptone broth (Na_2HPO_4 , 4.26 g/l; KH_2PO_4 , 2.65 g/l; $MgSO_4$ 200 mg/l; $CaCl_2$, 20 mg/l; and peptone, 5.0 g/l; pH 5.5) supplemented with 100 mg of water soluble coal polymer supplied in 5.0 ml of aqueous solution. The sterile coal solution was added to the autoclaved medium afterwards to avoid coal precipitation and coagulation. For assay of coal transformations, 5 ml of samples were periodically (3d, 6d to 30 days at 3d intervals) withdrawn, centrifuged, and acidified to pH 2.0. The precipitated coal from 3 day old samples were collected, washed, and dried for FT-IR analysis. For HPLC analysis, 1 ml of a periodically withdrawn sample from inoculated and un-inoculated flasks was acid precipitated (pH 2.0), washed (with water, added very gently from the sides of micro-fuge tubes to avoid any spurting). The precipitated coal was then redissolved in 1 ml of HPLC solvent and subjected to HPLC analysis. The pH values for 3 day old centrifuged sample for Control, DLC-07, DLC-21, DLC-62, DLC-63/9 DLC-BB2 and DLC-Mix. were 5.55, 6.24, 6,44, 5.98, 5.59, 6.04, and 6.48, respectively.

HPLC Analysis for Molecular Weights Determinations.[55-59] Coal samples from mineral salts-peptone experiments and from cell free extract experiments were analyzed on a Hewlett Packard 1090A high performance liquid chromatograph (HPLC) equipped with an HP-1040A diode array detector and a Synchropack GPC-300 column (Synchrom, Inc., Lafayette, IN). The mobile phase consisted of buffer (0.02M KH_2PO_4 containing 0.5% Tween-80, pH 7.1) set at a flow rate of 0.25 ml/min. Elution of the coal polymer was monitored at 254nm. High and low molecular weight protein standards from Pharmacia were used for plotting a standard curve.

Coal Depolymerization in Cell-Free Extract.[57] Culture strains of *Pseudomonas* sp. DLC-62 and DLC-BB2 were grown for 3 days (250 rpm, and at 30°C) in 250 ml mineral salts-peptone broth, supplemented with 2 mg of water soluble coal polymer as an inducer. Cultures were harvested, and the cells were removed by centrifugation. The supernatant was used as the source of the extracellular coal-depolymerizing enzymes. For the assay, 100 μL of soluble coal polymer solution was added to 10 ml of the crude cell-free extract. After 0 and 4 hr incubation (25°C) the reaction was stopped by acidifying the solution (1.0 ml) to pH 2.0. The precipitated coal was centrifuged, washed (gently with water), redissolved in 1.0 ml of HPLC solvent, and subjected to HPLC. Controls consisted of reaction mixtures treated similarly, but heat-inactivated (15 min.) supernatant was used as the enzyme source.

The 4 hr old active cell free extract-coal solution from strain DLC-62 was reduced by adding 2 drops of 1 M sodium borohydride solution (To make up, dissolve 0.4 g NaOH in 75 ml of water, add 3.86 g of $NaBH_4$ and stir to dissolve. Dilute the solution to 100 ml with water and filter.), allowed to stand for 5 min., acidified to pH 2.0, and worked up as above for HPLC analysis. In case of strain DLC-BB2 , 3 h old active cell-free extract-coal fraction was reduced with sodium borohydride solution. Coal depolymerization was monitored over time by monitoring of the HPLC elution profiles.

Cometabolism of Substructure Model Compounds.[55] A loopful of cells from the SDA stock slants of *Bacillus megaterium* DLC-21, DLC-62, DLC-63/9, and DLC-BB2 were used to inoculate 5 ml of Sabouraud dextrose broth in big size test tubes. After an incubation of 18-20 h shaking (250 rpm) at 30° C, 0.5 mg of model compound dissolved in 25 μL of dimethylformamide (DMF), was added to the test-tubes and to a corresponding set of uninoculated control. After a period of 7 days cells were removed and the UV-visible spectra of the supernatants were measured. The recordings were made on a Hewlett Packard 8452A Diode Array UV/VIS spectrophotometer.

Elemental Analysis. Elemental analysis of 1-3 mg of control and depolymerized coal samples were performed by Desert Analytics, Inc. (Tucson, AZ), using standard procedures.

Infrared Spectroscopy. FT-IR spectra of coal samples were recorded using Perkin-Elmer 1600 spectrometer. KBr pellets, which had been well-dried and stored under vacuum, were used for all analyses. Spectra were obtained using 2 mg of coal polymer and 150 mg of KBr. Scans were signal averaged to obtain a primary spectrum in wave numbers 4400 to 450 cm^{-1}.

NMR Spectroscopy. The solid state ^{13}C cross-polarization magic angle spinning (CP/MAS) NMR was measured at 75.4 MHz on an IBM NR-300 NMR spectrometer with a 0.35 cm^3 vol. doty probe (Doty Scientific, Inc., Columbia, SC). The pulse width of 90^{o} was 6 μs. A repetition time of 1 s, 5 kHz spinning rate, and 36,000 scan time were selected.

Enzyme Assays. Extracellular enzymes (esterases, and peroxidases) will be assayed using methods used in our group over the years.[59,61-64] Naturally occurring products like eugenol methyl ether (4-allylveratrole), asarone (2,4,5-trimethoxy-1-propenylbenzene), and safrole (4-allyl-1,2-methylenedioxybenzene) will be used to monitor etherase activity.

Enzyme Purification. Combination of techniques including ultrafiltration, ammonium sulfate precipitation, gel permeation chromatography, ion exchange chromatography, HPLC, electrophoresis and affinity column chromatography will be used to purify extracellular depolymerizing enzymes.[59, 64-67]

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