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Microbial Activity on Mississippi Lignites (Final)

Bailey Ward

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Microbial Activity on Mississippi Lignites

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Interim Report, 1982

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The Mississippi Mineral Resources Institute
University, Mississippi 38677

MICROBIAL ACTIVITY

on

MISSISSIPPI LIGNITES

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THE UNIVERSITY OF MISSISSIPPI

UNIVERSITY, MS 38677

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CONTENTS

PREFACE

In November, 1982, a presentation of this research was made at the Symposium on Biological and Chemical Removal of Sulfur and Trace Elements in Coal and Lignite, held at Louisiana Technological University, Ruston. In December, 1982, an interim report was submitted to the Mississippi Mineral Resources Institute. The reports adequately detail and summarize the research efforts through the second quarter of the project. A copy of the Symposium report serves as the introductory and first section of this final report. Beginning thereafter is a presentation of work accomplished subsequent to December, 1982, and up until the termination of the budget period for the project (June 30, 1983).

ACKNOWLEDGEMENTS

The second party of the "we" denoted in the text is Tammy M. Ensi in, graduate research assistant, who performed portions of this work and whose contributions are gratefully acknowledged. I wish also to acknowledge the contributions of Anuchit Sekthira, laboratory assistant, and Tommy Babin, undergraduate research assistant. Dr. M. B. Huneycutt provided his expertise in identification of hyphomycete fungi.

General Methodology and Special Techniques
Used for All Reported Research

1. Aseptic Technique, Sterilization, and Handling of Cultures,

All manipulations and transfers of cultures were carried out in a sterile chamber which received UV radiation when not in use. We were concerned about contamination of the laboratory, other cultures, and possibly humans.. The Candida, at least, represents a potential human pathogen, and the other fungi could be harmful plant pathogens. Sterilization of lignite, spent cultures, cultureware, nutrient agar, and mineral solutions was accomplished by autoclaving at 1.1 kg cm'\ 121°C for 15 minutes. Solutions of organics which might be altered by autoclaving were filter-sterilized and added to preparations aseptically.

2o Water and Aqueous Solutions.

Many hyphomycete fungi are opportunists with respect to organic nutrition and are capable of utilizing diverse kinds of organic carbon sources. Indeed, we have observed mycel ia growing in stock solutions of "pure" inorganic reagents prepared with all-glass-distilled water. We tested first our glass-distilled water alone in routinely-washed glassware (rinsed well with "house" distilled water)$_c$ Most of our isolates exhibited some growth in the "pure" water, presumably due to residual detergent which had adhered to the glassware—or to trace amounts of organics in the glass-distilled water. Next we washed the culture dishes in concentrated HC1 for 10 minutes, then rinsed with glass-distilled water; still some fungal growth occurred in glass-distilled

water only. We always were careful not to introduce any extraneous organic matter (cotton fibers, excess fungal hyphae with spores, etc.). Still perplexed, we purchased water of highest purity (HPLC grade, ChromarR 6795, Mallinckrodt) and repeated the acid-washed glassware tests; still a minimal amount of fungal growth occurred, especially in the case of the most active 1 ignite-degrading isolates L20 and L13, which we chose to use for most subsequent experiments. When the basal inorganic solution was prepared using the HPLC water, a greater amount of growth occurred relative to HPLC water only cultures. We have not been able to eliminate such growth from our control cultures and still do not know the source of organic carbon being used by the fungi. However, replicates of "control" cultures (minus added organic substrate) gave uniform and repeatable minimal growth. We designated this growth to be "background noise" and expressed enhancement of growth by lignite, extracts, or pure compounds by substracting control growth (means of replicates) from experimentals (means of replicates). That is, when growth is expressed as "++", this value was derived by correcting a mean of experimental replicates ("+++") for mean growth in replicates of control ("+"). Amount of growth was estimated by measurements of color, size or by visual estimates of total biomass.

3. Inocula.

We sought to inoculate each experimental culture with spores only; we were careful not to add exogenous organic material in the form of hyphae. We collected spores from-stock cultures by gently touching sporangia with a sterile probe. We did not attempt to enumerate spores of inocula.

4. Lignite.

Because lignite exposed to the elements, water, soil, plant material, etc., at the site might contain various and unknown non-lignite, organic materials, we performed our experiments using only lignite taken from the interior of previously unexposed samples. We were careful not to touch the samples with fingers or any other materials which might have introduced exogenous organic matter.

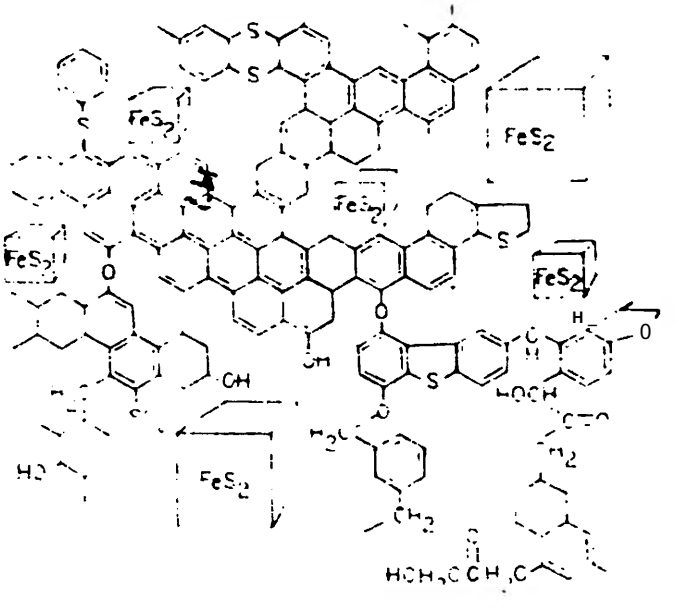
5. Identification of Fungal Isolates. Tammy Enslin and Dr. Bruce Huneycutt, an Ole Miss mycologist, labored for several months in attempts to identify to the level of species the most active lignite degraders. By using various culture media on which such hyphomycete fungi exhibit characteristic growth structures and habits, the following identifications were made: L20, Pénicillium waksmani; L27, Mucor lausannensis; L36, Asperigillus terreus. In spite of many tests and much microscopic study, we still cannot establish even the genus of the L30 isolate; perhaps this is a previously unknown fungus. Tests on other isolates still are in progress. For convenience of reference, the fungal isolates used for tests are designated in the text according to the isolate number for each (see also Table I, p. 11).

Section I

First and Second Quarter Research

PROCEEDINGS

SYMPOSIUM ON BIOLOGICAL AND CHEMICAL REMOVAL OF SULFUR AND TRACE ELEMENTS IN COAL AND LIGNITE



NOVEMBER 11-12, 1982

LOUISIANA TECH UNIVERSITY
RUSTON. LOUISIANA '1272

Coal Degrading Fungi Isolated from a Mississippi Lignite Outcrop

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Interest in the general microbiology of coals has increased in recent years because of the pressing need to exploit this abundant fossil material for fuel and chemical products. The mining, processing, and use of coal reserves introduce real and potential environmental problems in the forms of mineral elements and refuse released into the atmospheric, aquatic, and terrestrial environments. To date, the emphasis has been placed on microbiological demineralization of coals in efforts to develop inexpensive and efficient methods by which extraneous materials can be removed prior to combustion. Attention has been focused on the use of acidophilic chemoautotrophic bacteria such as Thiobacillus ferrooxidans (as described elsewhere in this Proceedings) and the thermophile Sulfolobus acidocaldarius (2) for removal of sulfur.

The present study was undertaken in an effort to identify other kinds of microorganisms which would act metabolically on lignite coals thus offering promise as being organisms useful for demineralization or bioconversion processes. Furthermore, we wanted to determine which, if

any, microorganisms would biodegrade lignite coal in the soil or water environment, in which capacity they would be important factors in determining the environmental fate of mining and processing refuses.

In recent years, there have been reports of biomass production using fungi grown on water-soluble components of oxidized coal (3) and on n-alkanes which might be derived from processed coal (4). For the first and only literature report describing growth of fungi directly on a lignite coal, Cohen and Gabriele (1) used two wood-decay basidiomycete fungi obtained from the American Type Culture Collection. Given the demonstrated ability of some fungi to grow on coal, we directed our efforts toward isolating coal-using fungal organisms from a natural lignite outcrop located in Northwest Mississippi. The outcrop is biologically interesting inasmuch as most of the requirements for successful microbiological exploitation of a natural organic substrate are present. The outcrop actually is a lignite seam exposed along the banks and bed of a small stream which (apparently) receives perpetual moisture via spring-water seepage from various areas along the banks. At least during the spring and summer, the dense overhead canopy of vegetation holds humidity high and buffers temperature fluctuations. Some of the exposed lignite appeared to be relatively unweathered and solid, whereas other sections blended with the soil and was obviously well-weathered in that the coal was brittle and appeared to be decomposing.

Isolation Methods

We used three methods for isolation of organisms. We pulverized weathered coal samples by grinding (using mortar and pestle) and "peppered" these onto the surface of Sabuaraud dextrose agar (SDA) plates. The plates were incubated at 23-25^{0}C until good growth occurred (2-5 days). From spores or hyphae of colonies growing on the coal particles, we obtained unifungal isolates.

At the outcrop site, we located areas under exposed roots, within crevices, etc., which were so protected that (with the use of hand lens) we were able to locate cotton-like tufts of fungi apparently growing on the exposed coal. Small pieces of these organisms were inoculated directly onto sterile SDA at the site and incubated at 23-25 °C after return to the laboratory. Successive transfers resulted in unifungal isolates.

A third technique involved loosely sealing chunks of exposed lignite in plastic bags. After incubation for 1-2 weeks at 23-25°C numerous surface colonies of fungi appeared from which we ultimately obtained unifungal isolates on SDA. All cultures were grown in the dark.

Assay for Growth on Lignite as Sole Source of Organic Carbon and Miner al s~~

Although we obtained approximately 40 isolates total (combined methods), any number of these could have been

utilizing organic carbon and minerals derived from sources other than the lignite coal. To demonstrate direct utilization of the lignite, we used the following technique. Large chunks of subsurface lignite were collected at the site and processed in the lab into small, irregularly-shaped pieces of 0.5-1.0 cm width. Care was taken to use only pieces of lignite which had not previously been exposed or otherwise available to contamination with exogenous materials. Only metal tools were used to avoid contamination with body oils, etc., which might occur with direct finger contact. The lignite pieces were sterilized by autoclaving at 1.1 kg cm^{-2}, 121°C, for 15 min., after which individual pieces were placed in pre-sterilized polystyrene tissue culture flasks. A small amount of glass-distilled or chemically pure water (HPLC grade, Mallinckrodt, Inc.) was added to the bottom of each flask to maintain high humidity. For inoculation, a few spores or small amounts of mycelium from each isolate were placed directly onto the surface of a lignite piece. All lignite cultures were maintained in the dark at 23-25°C. Growth was assessed visually using a stereoscopic dissecting microscope.

From the over 40 isolates, at least 12 exhibited growth at the expense of lignite as a sole source of organic carbon and minerals. Table 1 lists the coal-using isolates according to isolation method and relative rate of growth. This latter necessarily is quite subjective and "fast"

represents a casa in which the lignite piece was covered with fungal growth within 5-6 days. However, the considerable variations in natural growth habitats and reproductive structures among the several genera make direct growth rate comparisons tentative, at best. We have not yet been able to identify the LW-2, LW-5, and LW-30 isolates. Figures 1-3 represent typical growth patterns of three of the isolates.

We noted several features of the growth on lignite which might reflect the nature of biodegradation.

1) Most organisms grew steadily until the entire surface of the coal was covered with mycelium after which no additional (obvious) growth occurred. It appeared that surface nutrients were depleted and that the organisms were not able to utilize subsurface materials.

2) In the case of LW-20, a thin surface layer of the coal became changed in physical texture in that it became soft, "gummy," and could be scraped off using a metal probe. Control pieces treated as were experimentals remained unchanged in texture.

3) In the case of LW-13, but not for all samples, a viscous, liquid (non-cellular) material formed on the coal surface (Fig. 3). We interpret this phenomenon to indicate some degree of bioconversion of lignite organic compounds. Further work on this phenomenon is planned.

4) The unidentified LW-5 grew ¿is minute hyphal threads connecting isolated green clumps of spore-producing structures. Manipulation of the green structures revealed that the lignite immediately underneath the clumps had become degraded (pulverized) such that pits formed when the spore-producing structures were removed.

Studies on Mineral Metabolism

Inasmuch as the fungi were obtaining from the lignite all mineral elements required for growth, we performed experiments designed to provide some information about the possible value of these organisms for removal of sulfur and other elements.

Most microorganisms grown in the laboratory are provided with a mineral nutrient solution consisting of various salts known or believed to be required for growth. A rather typical basal mineral recipe was prepared at a strength routinely used for growth of microorganisms and at ten-fold dilutions. The composition of the full-strength basal medium is given in Table II. For all applications, glass-distilled water was used (the isolates would not grow on the water alone) and growth experiments were carried out in the dark. We chose four of the fastest growing isolates for initial tests. We ran a series of experiments designed

to establish pH and temporat ure optima Γor growth on coal of isolates LW-13, LW-20, LE-27, and LE-30. Best growth in all cases occurred at pH 5.5-6.0 and at 23-25^{0}C. Isolate LE-27 grew on lignite only at pH 5.0-5.5.

The experimental procedure for tests using basal mineral supplement was as described already except that the coal pieces were dried at 40°C for 24 hours after which they were soaked in basal medium, pH 5.5 for 20 hours. This method was used to enhance penetration of the test solutions into the coal. After treatment with basal medium, five lignite pieces per treatment variable were autoclaved in deep well "moist-chamber" culture dishes (Kimble Products No. 23087), and incubated at 23-25°C. Growth after 6 days' incubation was estimated visually by stereomicroscopic observation at 10-30X magnification. The results of these trials are presented in Table III. We observed consistent and uniform enhancement of growth at different dilutions of the basal medium for each microorganism.

We have attempted to identify basal mineral elements which produced increased growth on the lignite. We especially wanted to explore the potential for desulfurization or demineralization of lignite by our fungal isolates. Using the five-replicate, moist-chamber method already described, we substituted MgC^ for MgSO, in the diluted complete basal medium and monitored the growth of two isolates (LW-20 or LW-13) on soaked lignite. No differences in growth between MgSO, and MgC12 cultures were apparent. Inasmuch as sulfur is a macroelement with

respect to mineral nutrient requirements, we interpreted our observations to mean that each organism was obtaining all required sulfur from the coal. When the microelement mix was deleted from the diluted basal medium, we again observed no consistent or obvious differences in growth compared to that on complete medium plus trace elements. Apparently, most or all trace elements required for growth by the LW-13 and LW-20 isolates were obtained from the lignite, a conclusion supported by the observation that the organisms grew on sub-surface lignite which had not been exposed to any water or mineral supplement.

Because growth slowed or stopped after the surface of each lignite piece had been covered with fungal growth, we concluded that the organisms could utilize nutrients only from the surface of the coal. We have been able to enhance growth of LW-20 by growing the fungus on pulverized coal, otherwise treated as described for the soaked lignite pieces. Inoculation of the autoclaved slurry resulted in rapid and extensive growth of LW-20. Thus, surface area appears to be a factor in determining degree of biodegradation of lignite coal by at least one of our isolates. It is apparent that LW-20 is unable to penetrate and utilize sub-surface lignite nutrients.

We do not yet know if the fungi obtain required minerals from organic or inorganic components--or both. We have prepared ethanol extracts which support growth of the only

isolate tested to date, LW-20. The extracts were prepared by stirring equal volumes of absolute ethanol and pulverized subsurface lignite for 24 hours at 25°C, followed by centrifugation, after which the brown supernatant was decanted. The residue again was extracted for 24 hours with an equal volume of ethanol. The extracts were pooled and evaporated to dryness at 40°C using a Rinco vacuum evaporator. The "gummy" to brittle residue was dark brown in color and emitted an odor resembling that of "oily" aromatic organic compounds.# The extract was insoluble in water, soluble in acetone and benzene, partially soluble in petroleum ether and carbon disulfide, and only slightly soluble in hexane. For growth tests, the "dried" residue was floated on purified water in glass petri plates, autoclaved, and inoculated with spores of LW-20. Growth was slow but eventually extensive (2-3 weeks) and occurred only at the water/extract interfaces. Much additional research is needed before definitive statements can be made. We feel that the LW-20 isolate obtains at least part of its mineral--and sulfur--nutrition from organic components of the lignite. It is possible also that components of the ethanol extract (and the coal itself) are inhibitory to growth of some microorganisms and that fractionation and analysis of organic components will reveal growth-promoting and growth-inhibiting compounds. We should note that aqueous extracts, golden brown in color, would support growth, albeit slow, of some isolates. Inasmuch as the ethanol extracts

were hydrophobic, it is apparent that different compounds were utilized in aqueous versus ethanolic extracts.

In summary, we have isolated a dozen different fungi which are capable of growth on a Mississippi lignite coal which serves as sole source of organic carbon and mineral elements required for growth. Growth for some isolates can be enhanced by about 30% by supplementation with a dilute basal mineral solution. Deletion of sulfur from the mineral medium does not affect growth, an observation indicating a potential for use of the fungi for desulfurization as well as removal of other minerals. The preliminary data indicate also that one fungal isolate, LW-20, utilizes organic sulfur compounds.

This is the first and only report known to us describing biodegradation of lignite coal by these kinds of fungi and by any fungus isolated from a natural coal outcrop. These microorganisms will attack exposed lignite refuses such as those which are certain to enter the environment as a consequence of mining and processing activities. Many avenues are open for further research on these lignite-degrading fungi.

Table I

Lignite Degrading Fungal Isolates

Method	Organism	Relative Growth Rate
1) Weathered Particles	LW-2 (yeast)	Slow
	LW-5 (mycelial fungus)	Slow
	LW-13 Candida sp.	Fast
	LW-18 Pénicillium sp.	Fast
	LW-20 Pénicillium sp.	Fast
2) Site	LE-24 Mucor sp,.	Moderate
	LE-24 Mucor sp.	Slow
	LE-27 Mucor sp.	Moderate
3) Plastic Bag	LE-30 (mycelial fungus)	Fast
	LE-31 Pénicillium sp.	Fast
	LE-33 Paecilomyces sp.	Fast
	LE-34 Pénicillium sp.	Fast

^LW = Isolated by H.B. Ward

LE = Isolated by T.E. Enslin

Table II

Basal Mineral Medium

Mineral Components	Final Concentration (mg L~^)
NaCI	25
NH_4NO_3	250
$MgSO_4$ Or $MgCl_2$	75
C aC 12	25
K_2HPO_4	75
KH_2PO_4	175
$FeCl_3$	8
$ZnS0_4$*	8.8
$MnCl_2$*	1.4
$Mo0_3$*	0.7
(CusO,* 4 *	1.6
$Co(NO)_3$	0.5

*Added as "trace" elements.

Final solution adjusted to pH 5.5 prior to autoclaving.

Table III

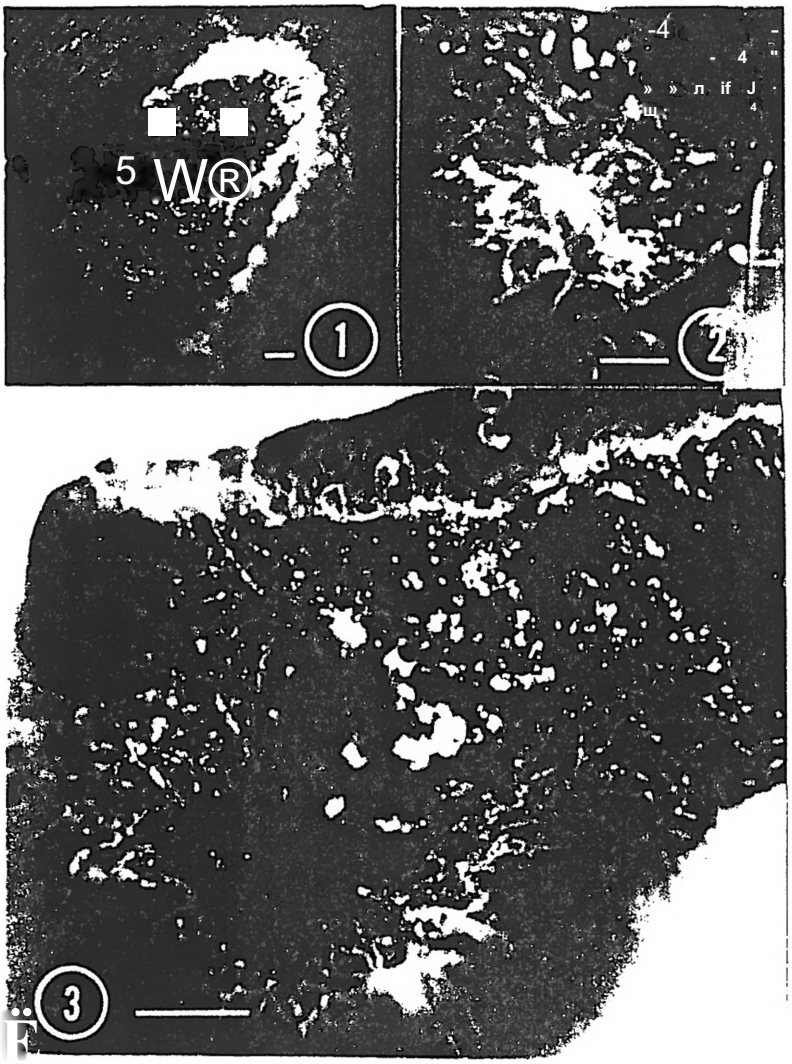
Growth on Lignite with Basal Mineral Supplement

Isolate	Basal Concentration 0[1]	0.0001	0,01	0.1	1.0
Pénicillium sp. (LW-20)	^2	44-	444-	++	44*
Candida sp. (LW-13)	+■	-1 ; j-	44-	44-	44-
Mucor sp. (LE-27)	4-	4-	44-	444-	44-
(Unidentified (LE-30) Fungus)	+	44-4-	44-	4~	4-

[1] **Soaked only in water.**

[2] **4- « relative degree of growth after one week.**

Fig. 1-3. Typical patterns of growth on untreated lignite of three isolates. 1. LW-20 (Pénicillium sp.) after approximately 7 days. 2. LE-30 (unidentified) after 5 days. 3. III-13 (Candida sp.) after 10 days, showing apparent formation of viscous nater ia 1 on top center surface of coal. Bar scale = 2 m



Section II

Research Efforts and Accomplishments for Third and Fourth Quarters

A. Studies on Apparent Bioliquefaction of Lignite

Two of the fungal isolates produce an apparent liquid product when cultured on chunks of lignite. The earlier report of Cohen and Gabriele (1) described liquefaction of a North Dakota lignite by mycel ia of basidiomycete wood-decay fungi which are quite unlike the fungal isolates described in the present reporto

Methods. To test our isolates, we set up culture preparations according to the methods described by Cohen and Gabriele (1). Into 100 ml wide-mouth, screw-cap jars, we dispensed approximately 20 ml of Sabouraud Dextrose Agar (Difco). The cooled agar was inoculated with spores from each of six fungal isolates and incubated until mycel ia were evident (5 days) after which a 50 mm diameter sterile glass fiber filter disc (Schleicher and Schuell No. 32) was placed on the agar surface. A piece of solid lignite taken from a previously unexposed sample was placed on the filter disc. The purpose of this technique was to isolate the lignite from direct contact with the nutrient agar but to allow the fungus to grow through or around the filter disc and onto the coal. Hypha in contact with the lignite thus could be induced to attack organic residues in the coal, yet receive a plentiful supply of nutrients from the agar medium. After about sixty days, depending on growth rate of the fungus being tested, mycelium usually covered the lignite.

Resul ts. Initially the mycelia of the isolates covered the lignite with a thick, cottony growth. After about two weeks in this phase for the L13 fungus, the lignite appeared to become oily over the exposed surface, and the fungus growth receded. The liquid product diffused onto/into the surrounding agar medium, turning it dark brown (Figure 4). In the case of the L20 fungus during two months' growth, there appeared droplets of a black liquid (Figure 5). The droplets appeared to form along fissures in the lignite piece. Within the two-month test period, only the L20 and L13 isolates exhibited apparent bioconversion of the lignite. Controls, consisting of all components minus a fungus, did not exhibit changes during the incubation period. Fungi minus lignite did not produce dark colored products.

We collected a sample of the black liquid produced by L20 and analyzed it by using infrared and ultraviolet spectroscopy. Drs. Norman Heimer and Jon Parcher of the Ole Miss Chemistry Department and Dr. Harold Drake of the Ole Miss Biology Department kindly provided instrumentation and assistance for the rough analyses. We learned that the liquid product was primarily water with no evidence of hydrocarbons. The UV spectrum revealed the presence of complex organics, but the material will have to be fractionated in order to identify components. At the.time of this report, no further analyses have been made. We did note, however, that the black liquid was produced continuously and that a droplet removed was slowly replaced within a week or two.

We also tested the two basidiomycete fungi (obtained from the American type Culture Collection) reported by Cohen and Gabriele (1) to liquify lignite. During a two-month period of cultivation, according to their reported methods, we observed no evidence of liquefaction of the Mississippi i igni te.

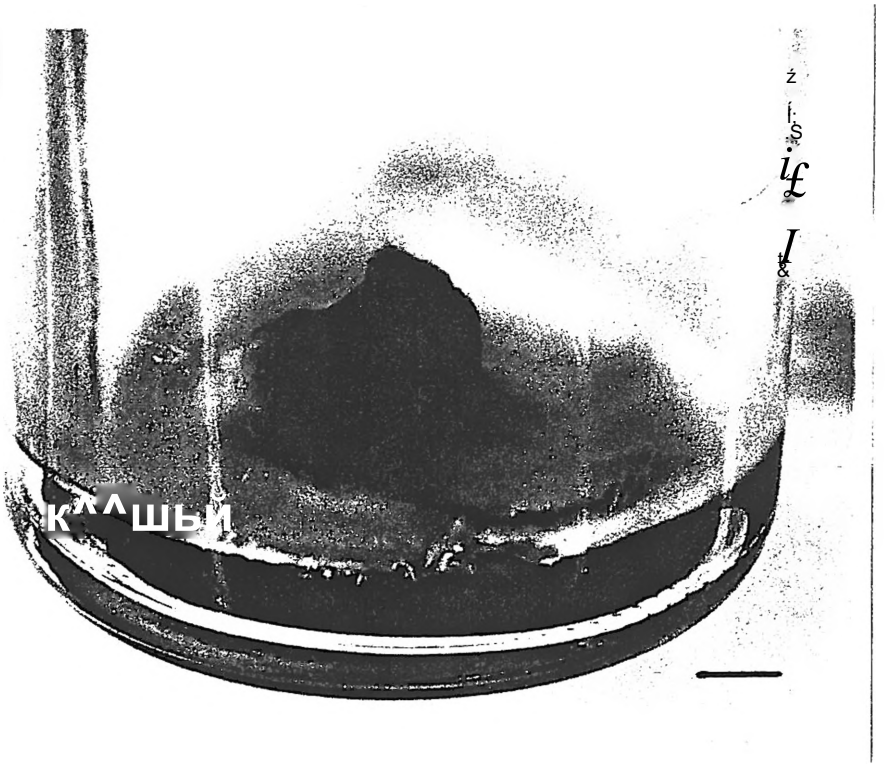


Fig. 4. Isolate L13 after about sixty days growth on nutrient agar and lignite chunk resting on glass fiber disc. The lignite in this case earlier was covered with the same type cottony mycelium which can be seen growing around the lignite. Note the gloss on the lignite and the dark product which has colored the originally very light amber nutrient agar. (See also Fig. 3, p. 14, for L13 growth directly on lignite.) Bar scale = 5 mm«

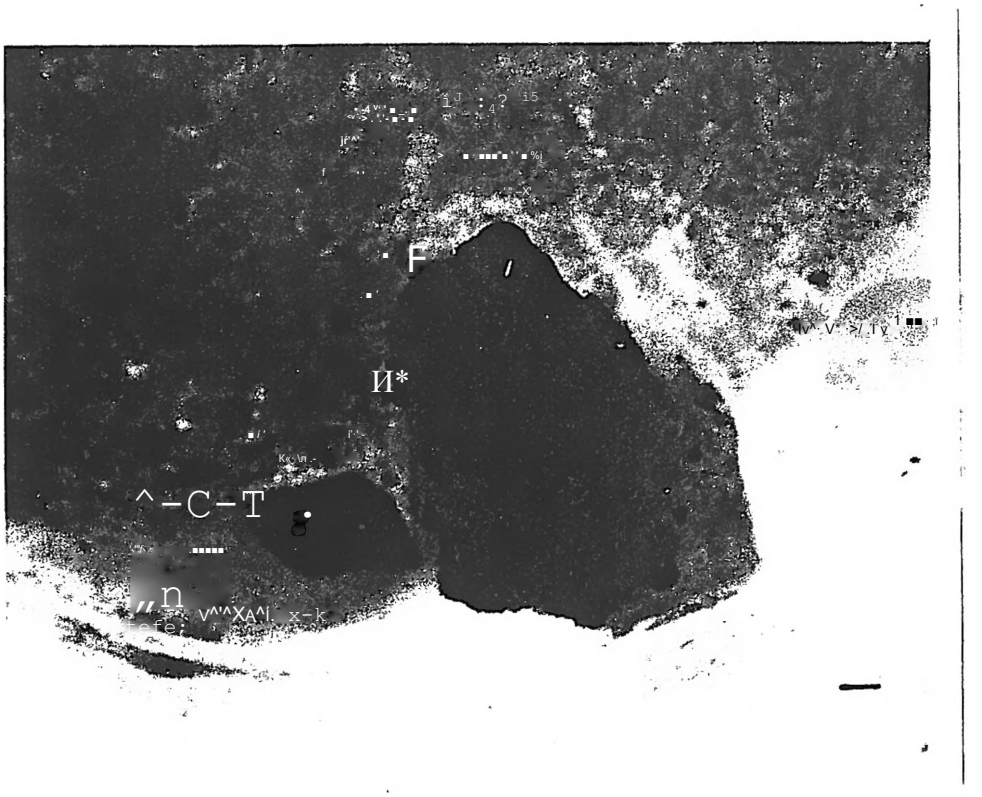


Fig. 5. Isolate L20 after about fifty days growth on lignite as described for Fig. 4. The almost black hydrophilic liquid product was produced continuously and appeared to be expressed from fissures in the chunk of lignite. Bar scale = 1 mm.

We can now state only that two of our hyphomycete isolates, L13 and L20, apparently can liquify some lignite coal, producing water-soluble organic products. The significance of this bioconversion phenomenon with respect to environmental effects or potential commercial value is unknown.

B. Growth of Fungi on Aqueous and Ethanolic Extracts of Lignite

We set out to attempt to learn more about the nature of the biodegradation phenomenon by testing aqueous or ethanolic extracts for their effects on fungal growths.

Aqueous Extracts. We extracted pulverized lignite with glass-distilled water with continuous stirring for twenty-four hours, after which the preparation was centrifuged. In other cases, we submerged small (ca. 0.5 cm wide) chunks of lignite in glass-distilled water and let them stand undisturbed for several days or weeks. When the extracts were to be used for growth tests, they were sterilized by autoclaving.

Results. Some aqueous preparations yielded an amber to brownish-colored, optically clear solution. Other samples, taken from other pieces of lignite, did not produce colored leachates. The reasons for these variations are not known. We do recognize, however, that the composition of lignite is quite variable and that we likely were testing samples (although taken from the same site) having different chemical properties.

The sterile colored aqueous extracts supported growth of L20 when cultured in stationary flasks. Non-steri ilzed extracts and the solutions

of standing chunk lignite (not sterile) exhibited fungal growth of several types similar to our L20, L13, etc., isolates—we presume these were the same organisms. Those preparations which did not yield a colored solution did not support fungal growth except that some fungi would grow on submerged lignite chunks in these cases.

Ethanolic Extracts. Pulverized lignite in absolute ethanol (equal volumes ethanol and lignite) was extracted at 25^0C for twenty-four hours. Suspensions were stirred continuously by use of magnetic stirrers. The first extraction supernatant was decanted after centrifugation. The lignite residue was reextracted with a second equal volume of ethanol as described above. The two ethanolic extracts, optically clear and amber-brown in color, were combined. The solutions were evaporated at 40°C to dryness with a Rinco rotary vacuum evaporator.

The residue was a dark brown to amber color (with variations of yellow in spots) haying an ”oily," somewhat pungent odor. To this writer, the odor was reminiscent of that which often pervades an engine-repair shop or heavy machinery facility. We tested the crude extract for its ability to support growth of the L20 isolate. Unmeasured amounts of the dried extract were added to a basal mineral medium. The colored components, at least, of the extract apparently were hydropholic in that no color leached out into the aqueous solution. The preparations were autoclaved at 120°C for fifteen minutes in 60 mm petri dishes. The extract became dispersed over or under the aqueous phase. After cooling, the preparations were inoculated with spores of L20. After about one week of incubation, mycelial growth was evident at the extract/aqueous interfaces. A control preparation consisted of basal mineral medium only plus spores. Growth in the control was minimal compared to that on extract.

Inasmuch as there might have been components in the crude extract which inhibit growth or otherwise be toxic, we attempted to fractionate the extract. Moreover, the crude extract would need to be fractionated for identification of components. Our fractionation procedure was "rough" in that we set out initially only to learn if we could separate from the crude extract organic solvent fractions showing activity versus others which either did not enhance growth or actually inhibited growth relative to controls (which always exhibited minimal growth).

Our procedure was as follows: First, we tested numerous organic solvents for immiscibil ity and extract solubility. The dried ethanolic extract exhibited varying degrees of solubility in methanol, heptane, and petroleum ether; that is, it appeared that color separation (our visual criterion for fractionation) occurred$_0$ We achieved fractionation by swirling (in test tubes) extract in combinations of two of the solvents, including ethanol, after which the solvents were allowed to separate. Solvent layers were collected by pipette, air-evaporated to dryness in wide-mouth test tubes, and redissolved in ethanol.

We applied the bioassay technique devised by Taniny Ensi in for the screening of hydrophobic compounds. Work from my lab (as well as that of others) has shown that a microorganism must maintain some degree of physical contact with a hydrophobic substrate in order to metabolize the compound. Furthermore, the organism must also be bathed in a water solution of required mineral elements. In the case of many mycelial fungi, a physical substrate to which the mycelium can become secured provides for enhanced growth; Ensi in's assay technique provides all three requirements. The assay consists of loading 5 cm diameter acid-washed glass fiber discs (Schleicher and Schuell No. 32 glass) with the test compound dissolved in an appropriate volatile solvent. After evaporation of the solvent, the loaded fiber discs were partially

immersed in basal mineral medium so as to provide a medium-to-organic substrate interface. The glass fiber discs provided a physical substrate for fungal attachment as well as direct contact with the hydrophobic organic substrate. Several inoculated discs per deep-well culture dish (page 7, Symposium Report, Section 1) provided replicates.

Results. Greatest growth occurred in methanol or heptane-parti tioned fractions. Growth in these cases equaled that of an ethanol-only (unpartitioned, crude extract) preparation. Petroleum ether fractions yielded growth 50-60% that of methanol or heptane fractions. These results indicate that the organic constituents of the Mississippi lignite tested readily can be separated from the crude extract by solvent partitioning. Following crude fractionation, further purification using thin-layer chromatography, for example, likely would be needed for identification analyses using gas chromatography.

COMMENTS

Fungi as a group attack and degrade diverse kinds of organic molecules and in this capacity are important agents in biogeochemical cycles. Mining and processing activities are certain to introduce raw lignites into the environment. When sufficient moisture is present for microbial growth, it is likely that several fungi, at least, will degrade some of the vaste i igni te.

The standing water of the creek at the collection site was of a color similar to that of the aqueous extracts. The creek waters contained an abundance of microbial life, which included mycelial fungi. We suggest herein that lignite leachates at the outcrop site cause inorganic and organic enrichment of the creek waters, and that these water-soluble compounds serve as mineral, carbon, and energy courses for various forms of microorganisms. Lignite mining and processing residues are likely to have a direct, and perhaps dramatic, effect on microbial populations in the environment.

C. Tests for Growth of Fungi on Pure Organic Substrates

The organic chemistry of lignite coals is complex and not yet completely elucidated. However, numerous literature reports are extant which describe organic analyses of lignite coal. (See Karr, 1978, 1979, Bibliography.) Due to a lack of analytical instrumentation for direct analysis of lignite extracts attacked by our fungi, we used an indirect method in an attempt to learn something about the nature of the organic constituents metabolized. For these studies, pure compounds which might exist in Mississippi lignite were tested for their ability to support growth of the fungi.

The pure compounds tested were the aromatics napthalene and benzene, a mixture of the n-al kanes tridecane, tetradecane, hexadecane, and octadecane, and the organic sulfur compounds dibenzothiophene (DBT) and thiophene carboxylic acid (TCA). The latter two compounds were tested because of the potential for using the fungi to remove organic sulfur from lignite prior to combustion. Each of the tested compounds is hydropholic and all but hexadecane and octadecane are soluble in ethanol, thus these could be the type of substrates in the lignite which are attacked by the fungi$_e$

Methods. Two methods were used to test the pure compounds. For DBT and TCA, the glass fiber technique described for ethanolic extracts was used. Concentrations of 0.001%, 0.01%, 0.1%, and 1.0% (v/v) DBT or TCA were prepared in absolute ethonol. Solutions were loaded onto glass fiber discs, air dried, placed in basal mineral solution in deep-well dishes, then inoculated with spores of each of six lignite-degrading isolates. Controls consisted of glass fiber discs treated only with ethanol, then air dried. Five disc replicates for each variable were incubated in basal

mineral medium at 25°C for eleven days. The relative degree of growth was estimated by assessing visually the averaged amount of mycelium on each set of five discs for each treatment.

Napthalene, benzene, or n-alkane mixtures were added directly to the basal mineral medium; napthalene remained a crystalline solid dispersed over the solution surface whereas benzene and the n-alkanes floated as liquids on the aqueous solution. Four replicates of each treatment were assayed. No concentration effects were studied; instead, "drops" of liquids or a "sprinkle" of napthalene were added to the mineral solution. Because these organic compounds are essentially insoluble in water, there should be no effect of different quantities other than that of increased surface area of organic substrate, and we added the same amount of each compound to each replicate.

Results. The results of growth tests for napthalene, benzene, and the n-alkane mixture are presented in Table IV. It is apparent that each fungus exhibited a quite different response to each organic substrate. Only isolate L20 exhibited much utilization of DBT (Table V), and in this case, a definite dose-response pattern was observed. Although L36 exhibited some enhanced growth at low DBT concentrations, growth apparently was inhibited at higher concentrations. Indeed, the lack of growth observed for other compounds by other fungi might have been due to inhibition whereas at lower concentrations (not tested) the compounds might have been metabolized. No growth enhancement occurred on TCA for any concentration tested for any fungus. Indeed, at concentrations of 0.1% and 1.0%, growth of isolates L36 and L19 was repressed compared to the minimal growth of control sets.

TABLE IV

Growth on Napthai ene, Benzene, or η- Al kanes

Isolate	L20	LI3	L30	L36	L27	L19
n-al kanes	++[1]	+	+	-	++	-
napthalene	-	-	-	-	-	-
benzene	+	++	-	+	+	-

[1] relative degree of growth compared to control after 15 days incubation

TABLE V

Growth of Fungi on Di benzothiophenel

Concentration (%) of dibenzothiophene	L20	Ll3	L30	L36	L27	L19
0.001	+	-	.	+	.	-
0.01	++	.	.	+	_	-
0.1	++	.	-	—	-	-
1.0	+++	-	-	-	-	-

iafter 11 days incubation

DISCUSSION AND CONCLUSIONS

We set out initially with the hope that we might be able to isolate one or several microorganisms which would degrade lignite coal. At the time the proposal was written and, indeed, after the search was begun, we knew of no reports in the literature describing direct utilization of lignite or coal by microorganisms. In August, 1983, a report (1) appeared describing for the first time growth of fungi on a North Dakota lignite. These fungi are of the "wood-rot" type and were obtained from the American Type Culture Collection. We immediately ordered a culture of each to test on the Mississippi lignite. By this time, we were getting evidence of biodegradation of Mississippi lignite by some of our own fungi isolated from the Tocowa outcrop.

Although we have not been able to duplicate the work of Cohen and Gabrielle (1) using their fungi on our lignite, we have isolated and identified several types of hyphomycete fungi which grow on the Mississippi lignite as sole source of organic carbon and mineral nutrients. Indeed, we were surprised to find the variety of fungus types which attacked the lignite and had to limit our research to a few of the most active lignite-degraders and to lignite collected only from the one site at Tocowa. On the other hand, we were disappointed in that we were not able to isolate other kinds of microorganisms which would degrade the lignite. It is likely, however, that some bacteria, other fungi, etc., can utilize some of the byproducts of the fungal biodegradation and that in nature, the lignite ultimately serves as the beginning of a complex food chain—after all, the lignite is derived from photosynthetic plants.

We have established firmly that several kinds of fungi existing as natural microbiota in the environment around at least one lignite deposit can and will attack, degrade, and apparently transform one or more organic components of the $coal_0$ Both hydrophilic and hydropholic components of the lignite are used as sources of energy and organic carbon by several of our isolates. From an environmental perspective, we can state that mining and processing activities associated with the exploitation of Mississippi lignites are certain to have an effect upon the biotic environment. We cannot yet predict the consequences of these environmental effects; much more research is needed. It is quite possible that some of the biodegradation products will represent environmental hazards.

There appears to be a real potential for using one or more of these fungi to produce economically valuable products from lignite. Further analysis, of course, will be needed to reveal the chemical nature of the bioconversion products and to provide an assessment of their potential applications.

The L20 isolate (Pénicillium waksmani) was the most active lignite-degrader. The growth of this organism also on pure DBT, one major form of organic sulfur in coals, indicates that DBT might serve as at least one organic carbon substrate attacked in the lignite. If L20 also consumes the sulfur from DBT, then the organism might have potential for pretreatment of coals to remove organic sulfur.

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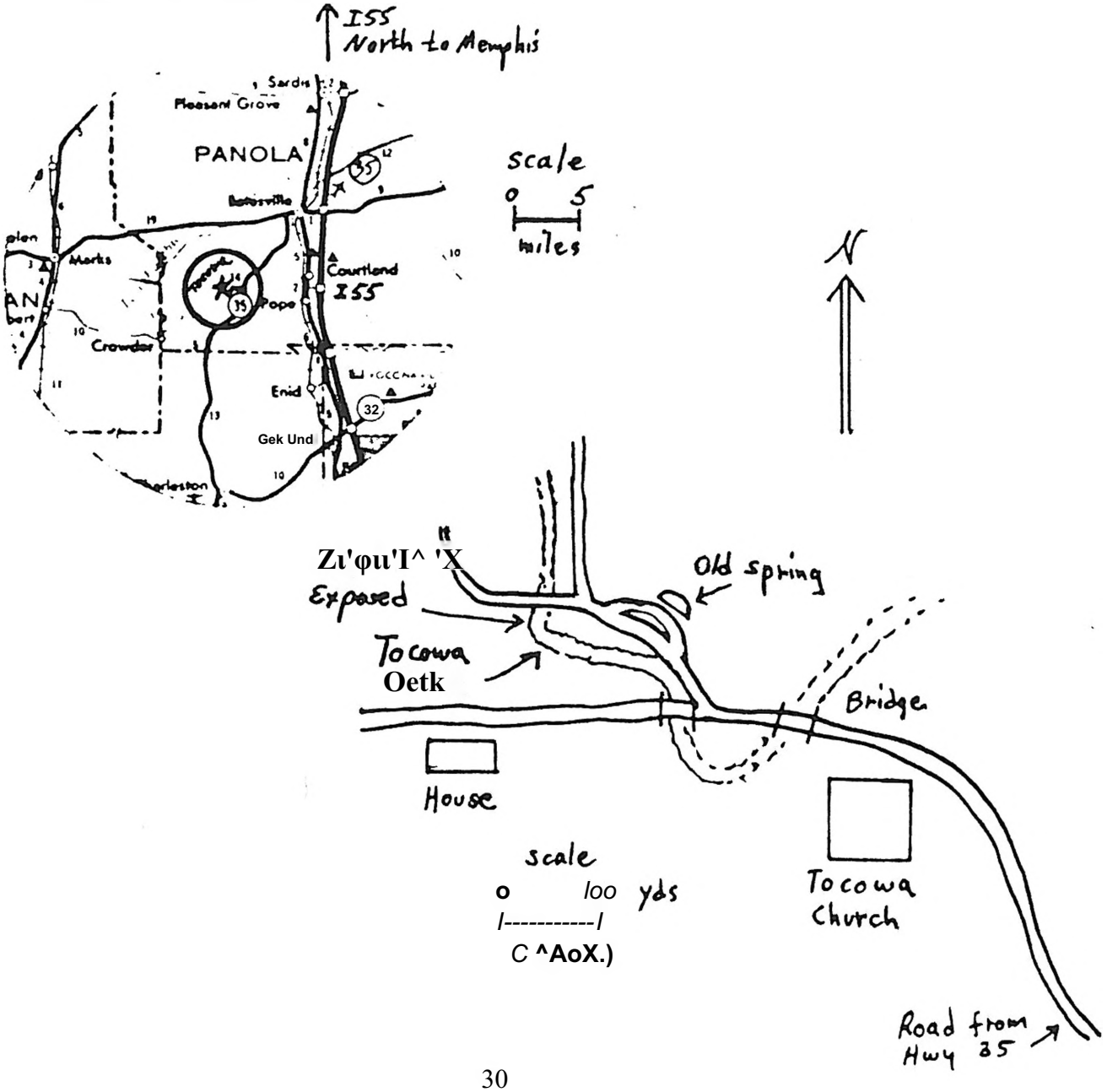
APPENDIX

Site of Exposed Lignite

The lignite outcrop referenced in this report is located near the Tocowa Baptist Church, east of Highway 35 South from Batesville, Mississippi. A turnoff from Highway 35 is indicated by a Tocowa Church sign approximately eight miles south of Batesville.



Microbial Activity on Mississippi Lignites

Interim Report

(December, 1982)

For

The Mississippi Mineral Resources Institute

by

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MMRI Contract No. 83-3S

Microbial Activity on Mississippi Lignites

Interest in the general microbiology of coals has increased in recent years because of the pressing need to exploit this abundant fossil material for fuel and chemical products. The mining, processing, and use of coal reserves introduce real and potential environmental problems in the forms of mineral elements and refuse released into the atmospheric, aquatic, and terrestrial environments. To date, the emphasis has been placed on microbiological demineralization of coals in efforts to develop inexpensive and efficient methods by which extraneous materials can be removed prior to combustion.

The present study was undertaken in an effort to identify new kinds of microorganisms which would act metabolically on Mississippi lignite coals thus offering promise as being organisms useful for bioconversion of lignite organic compounds to economically useful products. Furthermore, I wanted to determine which, if any, micro-organisms would biodegrade lignite coal in the soil or water environment, in which capacity they would act as important agents in determining the environmental fate of mining and processing refuses.

In recent literature reports, there have been accounts of biomass production using fungi grown on water-soluble components of oxidized coal and on n-alkanes which might be derived from processed coal (1,2). For the first and only literature report describing growth of fungi directly on a lignite coal, Cohen and Gabriele (3) used two wood-decay basidiomycete fungi obtained from the American Type Culture Collection. These latter workers reported a possible liquification of the coal as a consequence of growth.

Overall, the prospects of finding microorganisms which would biodegrade or biotransform Mississippi lignite initially appeared to be quite favorable.

With the help of Tammy Enslin, a graduate research assistant, the project was initiated with a search for a lignite outcrop which presented an environment favorable to growth of microorganisms. Such a natural outcrop occurs near the Tocawa Baptist Church, in Southwest Panola County. In that the exposed coal is constantly moistened by spring and creek water, the conditions are quite favorable for microbial activity. Samples were collected from various areas of exposed lignite representing different degrees of weathering or blending with the surrounding soil.

We used three approaches for isolation of microorganisms.

(1) From weathered coal, we prepared pulverized samples(by grinding using a mortar and pestle),

”peppered" these onto the surface of Sabouraud dextrose agar (SDA) or nutrient agar, ánd incubated the plates at 23-25°C until good growth occurred (2-5 days). From spores, hyphae, or colonies growing on the coal particles, we obtained uni-microbial cultures.

(2) At the outcrop site, we located areas under exposed roots, within crevices, etc., where cotton-like tufts of fungi were growing directly on the exposed coal. These organisms were inoculated directly onto sterile SDA at the site and incubated at 23-25°C after return to the laboratory. Successive transfers resulted in unifungal isolates.

(3) A third technique involved sealing (loosely) chunks of exposed lignite in plastic bags. After incubation for 1-2 weeks at 23-25°C,numerous surface colonies of fungi appeared from which we ultimately obtained unifungal isolates on SDA. All cultures were grown in the dark. Microbial isolates were identified to genus (when possible) by use of standard manuals and taxonomic keys. We have not yet been able to identify several of the lignite-degrading microorganisms isolated.

Although we obtained approximately 40 isolates total (combined methods), any number of these could have been

utilizing organic carbon and minerals derived from sources other than the exposed lignite coal. To demonstrate direct utilization of the lignite, we used the following technique. Large chunks of subsurface lignite were collected at the site and processed in the lab into small, irregularly-shaped pieces of 0.5-1.0 cm width. Care was taken to use only pieces of lignite which had not previously been exposed or otherwise available to contamination with exogenous materials. Only metal tools were used to avoid contamination with body oils, etc., which might occur with direct finger contact. The lignite pieces were sterilized by autoclaving, after which individual pieces were placed in pre-sterilized polystyrene tissue culture flasks. A small amount of glass-distilled or chemically pure water (HPLC grade, Mallinckrodt, Inc.) was added to the bottom of each flask to maintain high humidity. For inoculation, a few spores or small amounts of mycelium or cells from each isolate were placed directly onto the surface of a lignite piece. All lignite cultures were maintained in the dark at 23-25°C. Growth was assessed visually using a stereoscopic dissecting microscope.

From the over 40 isolates of bacteria and fungi, at least 12 exhibited growth at the expense of lignite as a sole source of organic carbon and minerals and each of these is a fungus-type microorganism. Table 1 lists the

coal-using isolates according to isolation method and relative rate of growth. This latter necessarily is quite subjective and "fast" represents a case in which the lignite piece was covered with fungal growth within 5-7 days. However, the considerable variations in natural growth habitats and reproductive structures among the several genera make direct growth rate comparisons tentative, at best. We have not yet been able to identify the LW-2, LW-5, and LW-30 isolates.

We noted several features of the growth on lignite which might reflect the nature of biodegradation and point to promising avenues for further work.

(1) Most organisms grew steadily until the entire surface of the coal was covered with mycellium after which no additional (obvious) growth occurred. It appeared that surface nutrients were depleted and that the organisms were not able to utilize subsurface materials.

(2) In the case of LW-20, a thin surface layer of the coal became changed in physical texture in that it became soft, "gummy," and could be scraped off using a metal probe. Control pieces treated as were experimentals remained unchanged in texture.

Table I

Lignite-Degrading Fungal Isolates

Method		Organism	Relative Growth Rate
D	Weathered Particles	LW-2* (Yeast)	Slow
		LW-5 (Fungus)	Slow
		LW-13 Candida Sp.	Fast
		LW-18 Pénicillium Sp.	Fast
		LW-20 Pénicillium Sp.	Fast
2)	Site	LE-24 Mucor Sp.	Moderate
		LE-24 Mucor Sp.	Slow
		LE-27 Mucor Sp.	Moderate
3)	Plastic Bag	LE-30 (Unident. Fungus)	Fast
		LE-31 Pénicillium Sp.	Fast
		LE-33 Paecilomyces Sp.	Fast
		LE-34 Pénicillium Sp.	Fast

*LW = Isolated by H.B. Ward

LE = Isolated by T.E. Enslin

(3) In the case of LW-13, (but not for all samples) *a* viscous, liquid material resembling a bacterial colony formed on the surface of the coal but microscopic observation revealed that no cellular material was present. We interpret this phenomenon to indicate some degree of bioconversion of lignite organic compounds. Further work on this phenomenon is in progress.

(4) The unidentified LW-5 grew as minute hyphal threads connecting isolated green clumps of spore-producing structures. Manipulation of the green structures revealed that the lignite immediately underneath the clumps had become degraded (pulverized) such that pits formed when the spore-producing structures were removed.

In summary, at the time of this report, we have isolated several different kinds of fungal microorganisms which are capable of utilizing lignite coal as sole source of organic carbon and all minerals required for growth. We also have performed several experiments designed to enhance growth on the lignite. Inasmuch as all micro-organisms display requirements for several mineral elements and some growth factors (which catalyze chemical reactions) we have been able to increase growth by supplementing with a basal mineral solution containing most inorganics usually required by most microorganisms. Table II illustrates the type of growth enhancement achieved for a few of the

Table II

Growth on Lignite with Basal Mineral Supplement

isolate	Basal Concentration 0	0.0001	0.01	0.1	1.0
Pénicillium Sp. (LW - 2 0)	+1	4-4-	+++	4-4-	-|-I-
Candida Sp. (LW-13)	4—b	+++	-H-	4—h	4—(-
Mucor Sp. (LE-27)	4-	4-	4+	4—H-	+4-
(Unidentified (LE-30) Fungus)	+	4-4-4-	+i-	4-	4-

1 + = relative degree of growth after one week.

fungal isolates. Moreover, we observed that deletion of sulfur (as sulfate) did not affect growth. We interpret this observation to mean that the organisms were obtaining all required sulfur from the coal. Thus, our isolates offer a potential for removal of sulfur by biological activity. We are pursuing this promising lead. I should report that a research paper addressing the potential of our isolates for demineralization of lignites was presented at a symposium on the Biological and Chemical Removal of Sulfur and Trace Elements in Coal and Lignite held Nov. 11-12, at Louisiana Tech University, Ruston. The paper will appear later in a Proceedings of the symposium published by the sponsor, Department of Natural Resources, State of Louisiana. I am impressed that Louisiana (DNR) is focusing attention on lignite coal research and funds basic research in this area.

We also have obtained from the American Type Culture Collection pure cultures of the two fungi reported (3) to transform a North Dakota lignite coal and are now in the process of testing these for activity on the Mississippi lignite.

Other investigations currently in progress or planned are studies

(1) on the potential use of our isolates for removal of various minerals from lignite (in progress),

(2) of growth on ethanolic and aqueous extracts of lignite (in progress) ,

(3) designed to characterize the organic and inorganic nutrient requirements of the isolates (in progress) ,

(4) aimed at precise identification to species level of the most active isolates (in progress) ,

(5) on application to slurry pipeline technology (biological pretreatment of lignite) (in progress) , and

(6) designed to identify the organic lignite compounds attacked by the fungi and to identify bioconversion products (in planning stage).

This important phase of the research requires access to a gas-liquid chromatograph and we will need to make arrangements to use such an instrument housed in another lab.

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