0273-2289/88/1800-0403/$02.00

Biological Solubilization of Coal Using Both In Vivo and In Vitro Processes

Scientific Note

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Index Entries: Coal solubilization; enzymes, microbial.

INTRODUCTION

Several researchers have shown that low-ranked coals can be solubilized by many different fungi and a few bacteria (*1–5*). Thus far, this solubilization process has been carried out in an aqueous, aerobic environment (suspension culture or humid air) to yield a product that is water soluble and of relatively high molecular weight (*3*). Additional tests are being made to investigate additional microorganisms and to optimize the parameters.

Some of these early tests indicated that the microbial solubilization of coal is predominantly an extracellular process (*3,4*). Therefore, similar solubilization processes can be considered in which appropriate enzymes are used in vitro.

Anaerobic biological processes are also being considered. For such concepts, either anaerobic microorganisms or appropriate enzyme systems are being studied with operation in an environment in which free oxygen is absent. The products resulting from anaerobic interactions should have properties that are significantly different from those of the products of aerobic solubilization.

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†Operated by Martin Marietta Energy Systems, Inc., under contract No. DE-AC05-84OR21400 with the U.S. Department of Energy.

BIOCHEMICAL PROCESSES OF SOLUBILIZATION

Two primary processes must be carried out to solubilize or liquefy coal: (1) some of the chemical bonding that creates a stable, three-dimensional, solid matrix must be broken (*6*); and (2) hydrogen and/or oxygen must be transferred between the coal and the reaction medium or between portions of the coal itself. Thus, biocatalysts that can enhance the degradation of various chemical linkages in coal (e.g., ester, ether, or aliphatic) or that can aid in the addition or removal of hydrogen and oxygen should materially affect the conversion of coal to a liquid product.

The work to date on aerobic solubilization of coal has resulted in aqueous products of high molecular weight, thus, indicating that only limited cleavage of chemical linkages has occurred (*3*). This might be expected since it is likely that mild oxygenation will primarily affect only the aldehyde linkages. As also indicated in lignin degradation, an oxidase enzyme must be an important catalytic agent for this type of chemical transformation (*7*).

Additional chemical bonding must be broken in order to obtain a lower-molecular-weight product. Such a process may be enhanced by the use of appropriate bond-breaking enzymes such as *esterases* or *etherases*, especially where hydrogen addition is possible. In general, the production of nonpolar compounds (the type produced by conventional thermal/chemical processes) (*8*), will require an increase in the hydrogen content of the product with a concomitant reduction in oxygen content. Biocatalysts such as hydrogenase, dehydrogenase, and oxygen-transferring enzymes should be important here. An anaerobic environment that includes a hydrogen-donor for the supply of transferable H_2 may be necessary for this type of conversion process.

It may also be desirable to utilize a less polar, organic solvent instead of water as the coal solubilization agent, since this would also allow easy removal of the nonpolar product. In some limited cases, in vitro processes utilizing enzymes dissolved in an organic solvent have been shown to be very effective (*9,10*). Such an approach could prove to be useful in the anaerobic solubilization of coal.

MATERIALS AND PROCEDURES

Both surface cultures and suspension cultures have been used in evaluating microbial activity. The former was used primarily with fungi in an aerobic environment, while the latter was used with bacteria and with in vitro systems for both aerobic and anaerobic environments.

Microbial Strains

As previously reported, several fungi and a few bacteria have been shown to interact with coals in an aerobic environment (*3,5*). These have

included *Trametes versicolor, Poria placenta, Pencillium waksmanii, Candida* sp., *Aspergillus* sp., *Paecilomyces* sp., *Sporothrix* sp., *Streptomyces setonii,* and *Streptomyces viridosporous.* Recent investigations have also included *Phanerochaete chrysosporium,* a fungus that has been studied for lignin degradation. A strain (BKMS-1767) of the fungi was obtained from Dr. B. D. Faison, Procter and Gamble Co., who has carried out pioneering research on lignin solubilization.

Coal Samples

A variety of lignite coals (with primary emphasis on a North Dakota leonardite), one subbituminous coal, and one bituminous coal were studied for biological interactions (*see* Table 1). In some cases, the higher-ranked coals were subjected to pretreatment with 8*N* HNO_3 at ambient temperature for 24 h in order to further oxidize the coal for easier biological interactions in aerobic systems (*11*). The coal was first size-reduced to 1 mm diam, then sterilized at 120°C for 45 min, and subsequently dried before use.

Enzymes

The preliminary in vitro solubilization tests were made with various oxidases, hydrogenase, and dehydrogenase. The oxidases (either Type II horseradish peroxidase or laccase from *Pyricularia oryzae*) were obtained from the Calbiochem Company, La Jolla, CA, and alcohol deydrogenase, grade A from yeast, was obtained from the Sigma Chemical Company, St. Louis, MO. Crude hydrogenase, a more unstable biocatalyst, was extracted from *Clostridium pasteuranium* (grown under N_2 in a nitrogen-free

Table 1
Coal Samples That Have Shown Some Susceptibility to Biological Interactions

Type of coal	Source
Mississippi lignite (probably Type B)	Mississippi Mineral Resources Institute via H. B. Ward, University of Mississippi
North Dakota lignite (Type A)	Coal Research Section, Pennsylvania State University, University Park, PA
North Dakota leonardite (a highly oxidized lignite)	American Colloid Company, Skokie, IL
Texas lignite	Courtesy of Exxon Research and Development Company, Baytown, TX
Vermont lignite (probably Type B)	Coal Research Section, Pennsylvania State University, University Park, PA
Wyodak subbituminous	Amax Coal Company, Indianapolis, IN
Illinois no. 6 bituminous	Illinois State Geologic Survey

medium at 25°C (*12*) by rupturing the cells via sonication, followed by centrifugation to remove cell fragments.

Enzyme activity for the hydrogenase was determined by the method of Hoffman et al. (*13*), while the values supplied by the manufacturers were used for those enzymes that were purchased. Units of enzyme activity represent the conversion rate of a particular chemical species stated in μM/min.

Organic Solvents

Several different organic solvents were used in conjunction with dissolved enzymes for the study of coal solubilization. In all cases, small quantities of water were dissolved in the solvents, an apparent necessity to maintain enzyme activity in an organic medium. The solvents included: pyridine, a conventional coal solvent (*14*) (spectrophotometric grade; Burdick and Jackson Laboratories, Muskegon, MI); tetralin, a hydrogen-donor solvent frequently used in the study of coal chemistry (*14*) (99% pure; Aldrich Chemical Company, Milwaukee, WI); and dioxane, an organic that had been previously investigated for enzyme interactions (*10*) (spectrophotometric grade; MCB Reagents, Cincinnati, OH).

Surface Cultures

Some additional screening tests were made with microorganisms by surface culture techniques in which the organisms were cultured at a humidity greater than 80% at 30°C in the presence of air on the surface of Sabouraud maltose agar. Typically, 30 mL of the sterile medium was inoculated with the microorganism in a covered 0.25-L glass jar and incubated in air. After a fungal mat had developed on the agar surface (6–7 d), sterile coal particles were placed on top of the microbial mat and the jar was reincubated (>80% humidity, 30°C). Interactions were observed (*3*).

Suspension Cultures

Suspension cultures were carried out in air at 25°C in 250-mL shake flasks using 100 mL of the growth medium previously developed for lignin metabolism (*15*). Seven days after inoculation, coal particles were introduced into the shake flask and an absorption scan was periodically carried out on a spectrophotometer for several additional days.

DISCUSSION

Additional screening tests are being made to add to the list of microorganisms that can contribute to the solubilization of low-ranked coals. Several preliminary tests have also been made on the solubilization of various types of coal by the use of in vitro enzyme systems.

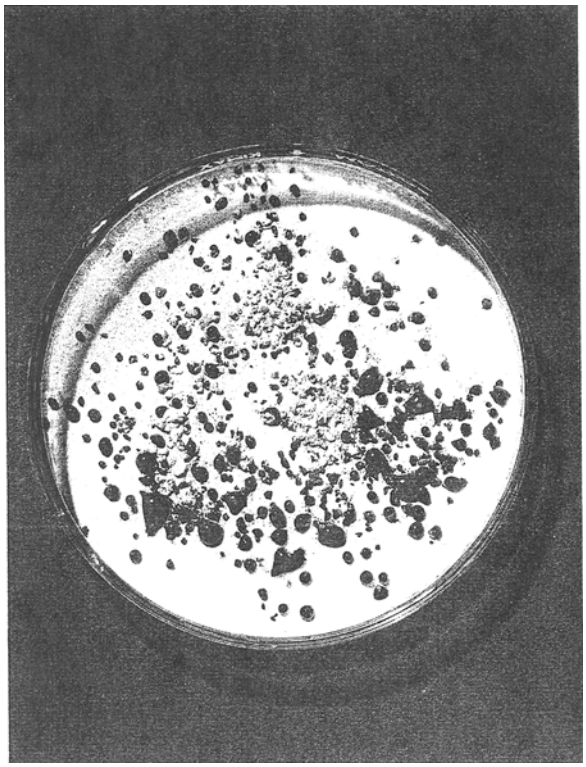

Fig. 1. Particles of North Dakota leonardite undergoing solubilization on a fungal mat of *Phanerochaete chrysosporium*.

Microbial Solubilization of Coal

Preliminary surface culture tests have shown that *Phanerochaete chrysosporium* is one of the more effective fungi for solubilizing low-ranked coal in an aerobic environment. As in past tests (*3*), it was found that after the coal particles were placed on the surface of the formed fungal mat, small, colored liquid droplets began to form on the top surface of the coal. During the course of an additional 7–10 d, the liquid tended to engulf the particle with a progressive diminution of the solid mass (Fig. 1). Microscopic examination indicated no growth of microorganisms in the liquid product, and no liquid droplet formation was observed in control tests where sterile coal was used in the absence of the fungi.

When a biological mat of *P. chrysosporium* was established on Sabouraud maltose agar, it was found to significantly solubilize particles of all the lignites previously tested, except the Vermont lignite (*see* Table 1), as well as nitric acid-treated Wyodak subbituminous coal. In one series of tests with North Dakota leonardite, the liquid product was removed by capillary pipet at the end of each of several test periods of varying lengths, and the coal residues were recovered by collecting the particulates from the distilled water wash used to remove particulates from the

fungal mat surface. These particulates were then dried and weighed to determine the degree of solubilization as a function of time. As observed with other fungi, a total of 10–14 d was required for significant solubilization of coal. The most rapid solubilization occurred during the latter part of the test.

P. chrysosporium was also cultured in suspension and several tests were made in which acid-treated Wyodak subbituminous coal particles were introduced. There was only limited solubilization with the best results when the organism was activated by 1 m*M* veratryl alcohol in the culture broth. This technique was previously developed during tests with this organism for interaction with lignin (*7*). Typically, the veratryl alcohol was added to the growth broth 5 d after inoculation. In that case, after 2 d of activation, the coal particles were introduced to the shake flask. Within 1 d there was an indication of solubilization by an increase in light-absorbing substances. The solubilization process persisted for the course of the test (Table 2).

Solubilization of Coal by Enzyme Systems

Two approaches have been made to the use of enzymes for enhancing the solubilization of coal. In one case, the enzymes were dissolved in a buffered aqueous solution that contacted the coal particles in suspension in an aerobic environment. In a second approach, the enzymes were incorporated into a hydrous organic solvent that was then used to contact the coal particles in either an aerobic or an anaerobic environment. In the latter case, there was specific interest in producing a solubilization product that would be relatively nonpolar and would have significant dissolution in the organic.

Aqueous Solvents

A few coal solubilization tests were made with oxidizing enzymes in a dilute aqueous buffer. Approximately 700 units/mL of laccase, or 1600/mL of peroxidase was introduced into 10 mL of a 0.05*M* potassium phosphate buffer in 50-mL shake flasks. The pH of the buffer was either

Table 2
Solubilization of Acid-Treated Wyodak Coal in a Suspension Culture of *P. chrysosoporium* Activated by Veratryl Alcohol

Run time (d)	Absorption of broth at 400 nm	
	With microorganisms	Without microorganisms
0[a]	0	0
1	1.52	1.42
2	1.94	1.50
3	2.10	1.61

[a]Approximately .5 g of coal particles were introduced to 100 mL of a growth broth in a 250-mL shake flask in which the fungi was inoculated 7 d earlier with 2 d of activation by 1 m*M* veratryl alcohol.

6.0 or 7.0, and 0.3 to 0.5 g of nitric acid-treated subbituminous coal particles were added to each flask. Solubilization tests were conducted under filtered air for 48 h at ambient temperature (22 ± 1°C) with agitation by an orbital shaker operating at 80 rpm with a 1-in. stroke.

With a buffer pH of 7.0, there was little difference in the degree of solubilization between a control system with no enzyme and systems in which either enzyme was present. However, at a pH of 6.0, a significant enhancement of solubilization occurred when laccase was used, although the extent of solubilization was still somewhat low (Table 3). The peroxidase also enhanced solubilization, but to a smaller extent.

Organic Solvents

Preliminary coal solubilization tests have also been made with enzymes in organic solvents. The solvents chosen were those conventionally used for coal solubilization or those previously used with enzymes for other purposes. In all cases, the organics had adequate water solubility to support appreciable enzyme activity. Since we expect the major solubilization processes to be associated with hydrogen and/or oxygen transfer, these scouting tests were made with alcohol dehydrogenase, horseradish peroxidase, and/or bacterial hydrogenase. The tests were carried out in 50-mL shake flasks to which 20 mL of liquid and .3 to .5 g of −10 +30 mesh coal particulates were added. Tests were made either under filtered air (aerobic) or hydrogen (anaerobic) and at temperatures ranging from 22 to 50°C. Several different types of coal, ranging from highly oxidized lignite (leonardite) to subbituminous and bituminous, were also studied.

Aerobic tests were made with horseradish peroxidase dissolved in hydrous dioxane (a dioxane-water solution). The method of Dordick et al. (*10*) was used for adding the enzyme to the organic. As in the case of enzymatic solubilization in an aqueous solution, subbituminous coal was used as the feed material. Untreated coal was minimally soluble in the hydrous organic solvent, but the presence of the peroxidase did double the degree of solubilization (Table 4). Preoxidation of the coal with nitric acid led to a significant increase in the degree of solubilization by the sol-

Table 3
Enzymatic Solubilization of Acid-Treated Wyodak Subbituminous Coal in the Presence of 0.05*M* Phosphate Aqueous Buffer at pH 6.0[a]

Aqueous constituents	Weight loss (%)
Buffer only	2.5
Buffer + 1625 units of peroxidase/mL in 2 m*M* H_2O_2	4.4
Buffer + 700 units of laccase/mL	6.8

[a]A nominal 0.3 g of dried coal in the form of ~1.0-mm-diam. particulates was added to a 50-mL shake flask containing 10 mL of aqueous solution under filtered air at 22°C. Afer 48 h, the coal residue was removed by centrifugation, vacuum dried, and weighed.

Table 4
Aerobic Solubilization of Coal in Air by Ambient Temperature and Pressure by Peroxidase in Organic Solvents[a]

Liquid phase	Type of coal	Enzymes	Weight loss of coal (%)
Dioxane + buffer	Subbituminous	None	1.9
Dioxane + buffer	Subbituminous	Peroxidase	3.8
Dioxane	Preoxidized subbituminous[b]	None	8.1
Dioxane + buffer	Preoxidized subbituminous[b]	None	44.7
Dioxane + buffer	Preoxidized subbituminous[b]	Peroxidase	85.4

[a]The tests were carried out for 48 h with 20 mL of liquid and ~0.3 g of coal size-reduced to a range of −10 to +30 mesh in 50-mL shake flasks operating aseptically with a controlled gas environment in the flask headspace. When the enzyme was used, it was introduced at a concentration of 400 U/mL. The 0.01*M* aqueous acetate buffer containing 2 m*M* H_2O_2 at a pH of 5.6 constituted 5% (v/v) of the mixture. The coal was dried in a vacuum oven at 110°C for 2 h before being introduced to the reaction vessel. At the end of the test, the coal was recovered by centrifugation and then washed and dried again in a vacuum oven.

[b]Preoxidation of the coal was carried out by contact with 8*N* HNO_3 for 48 h at ambient temperature, followed by washing with distilled water.

vent, particularly when aqueous buffer was added. The addition of peroxidase to this system again increased the degree of solubilization by a factor of almost 2, for a total value of 85.4%. This is significantly greater than the case where the enzyme was used in aqueous solution.

Preliminary tests were also made in which hydrogen gas was maintained in the headspace of the shake flask in order to ensure anaerobic conditions. Either dioxane or pyridine was used as the organic solvent and an aqueous phosphate buffer at pH 5.6 was used in conjunction with the solvents. A mixture of 400 U/mL of peroxidase, 0.325 U/mL of hydrogenase, and 3640 U/mL of dehydrogenase (0.1 w/v% of each enzyme preparation) was employed. The solubilization of leonardite was significantly enhanced by the inclusion of the enzymes, and the degree of solubilization approached that previously found with microbial systems after many days of exposure (Table 5) (3). In tests with bituminous coal and either hydrous dioxane or hydrous pyridine, there was a small positive effect on solubilization with the addition of a mixture of the enzymes; however, the degree of solubilization was relatively low.

A 1:1 mixture of pyridine and tetralin was also used as the organic solvent in contact with bituminous coal under anaerobic conditions. In this case, the tetralin was included since it is a known hydrogen donor that is frequently used with coal at higher temperature. Dehydrogenase, which was used with the mixed solvent, was shown to have a small posi-

Table 5
Anaerobic Solubilization of Coal in a Hydrogen Environment at Ambient Temperature and Pressure by Enzymes in Organic Solvents[a]

Liquid phase	Type of coal	Enzymes	Weight loss of coal (%)
Dioxane + buffer	Leonardite	None	19.6
Dioxane + buffer	Leonardite	Mixed[b]	86.4
Dioxane + buffer	Bituminous	None	1.2
Dioxane + buffer	Bituminous	Mixed[b]	3.7
Pyridine + buffer	Bituminous	None	3.0
Pyridine + buffer	Bituminous	Mixed[b]	.6.7
Pyridine + tetralin + buffer	Bituminous	None	4.6
Pyridine + tetralin + buffer	Bituminous	Dehydrogenase	6.0

[a]The tests were carried out for 48 h with 20 mL of liquid and 0.3 g of coal size-reduced to a range of −10 to +30 mesh in 50-mL shake flasks with a controlled gas environment in the flask headspace. When enzymes were used, they were introduced with an activity of 400 U/mL for peroxidase, 0.325 u/mL for hydrogenase, and 3640 U/mL for dehydrogenase. A 0.1*M* aqueous phosphate buffer, pH 5.6, that constituted 5% (v/v) of the liquid solution was used. The coal was dried in a vacuum oven at 110°C for 2 h and introduced to the reaction vessel. At the end of the test, the coal was recovered by centrifugation and then washed and again vacuum oven-dried.

[b]The enzyme mixture included peroxidase, hydrogenase, and dehydrogenase in equal proportions.

tive effect on the degree of solubilization, even though the total amount of solubilization was still quite low.

Liquid Product

Only qualitative observations have been made concerning the characteristics of the product from the enzymatic solubilization of coal by use of an organic solvent. At the end of the tests, the color of the organic solvent phase varied from a light yellow to a deep brown, presumably in accordance with the amount of solubilization products included. In order to determine the solubility properties of the product, 1.0 mL of the product solvent from the test with pyridine plus buffer plus mixed enzymes and bituminous coal was contacted with a mixture of 5 mL of benzene and 5 mL of the same aqueous buffer used in the experiment. This resulted in a distribution of the colored substances between the two phases, organic and aqueous. As indicated by absorption measured by a spectrophotometer at 600 nm, the organic phase contained the predominant fraction of the material, and there was an appreciable increase when the enzyme was added (Table 6). Thus, a major portion of this solubilization product is relatively nonpolar with limited water solubility; a striking difference between the enzymatically derived product and the product obtained via aerobic microbial action.[3]

Table 6
Characteristics of the Enzyme-Solubilized Product in a Hydrous Organic Solvent

Liquid product	Absorbance at 600 nm[a]	
	Benzene fraction	Aqueous fraction
Pyridine and buffer	0	0
Pyridine, buffer, and coal	0.16	0.020
Pyridine, buffer, coal and mixed enzymes	0.23	0.095

[a]One mL of the liquid product was contacted with 5 mL of benzene and 5 mL of buffer.

ACKNOWLEDGMENT

Research supported by the Fossil Energy Advanced Research and Technology Program, managed by the Pittsburgh Energy Technology Center, U.S. Department of Energy, under Contract No. DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc. The submitted manuscript has been authored by a contractor of the U.S. Government under contract No. DE-AC05-84OR21400. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or allow others to do so, for U.S. Government purposes.

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