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Microbial Solubilization of a Preoxidized Subbituminous Coal

Product Characterization

B. D. FAISON,* C. A. WOODWARD,[1] AND R. M. BEAN[2]

[1]*Chemical Technology Division, Oak Ridge National Laboratory, P.O. Box 2008, Oak Ridge, TN 37831-6194; and* [2]*Chemical Sciences Department, Battelle Pacific Northwest Laboratories, Richland, WA 99352*

ABSTRACT

Preliminary characterization of a microbial coal solubilization product has been performed. Submerged cultures of *Paecilomyces* TLi or *Candida* ML13 were grown in defined minimal media containing preoxidized Wyodak subbituminous coal. Culture supernatants contained high-molecular-weight, acid-precipitable material that was separated by gel permeation chromatography (GPC) in aqueous and polar organic solvents. Organic GPC also separated a low-molecular-weight (<2700 daltons) fraction that was converted to higher-molecular-weight material upon acidification. Elemental analysis of the acid-precipitated material indicated an increased oxygen content as a result of the biological treatment. The biosolubilized product may undergo further microbial modification.

Index Entries: Coal; coal solubilization; subbituminous coal; Wyodak coal; biosolubilization.

INTRODUCTION

The phenomenon of microbial coal solubilization, first described by Cohen and Gabrielle in 1982 (*1*), has become the focus of intense fundamental and applied research. Coal-solubilizing activity has been ascribed to a number of fungal (*2,3*) and bacterial (*4–6*) species. However, the bio-

*Author to whom all correspondence and reprint requests should be addressed.

chemistry underlying this activity is not fully understood. Coal solubilization has been widely associated with the production of nonenzymatic catalysts, particularly alkaline metabolites, in cultures of coal-solubilizing organisms (*4,5–8*). Enzymes, specifically oxidoreductases, have also been implicated in this activity both in vivo and in cell-free systems (*3,9–11*). The principal product of microbial activity against various lignite and bituminous coals is generally characterized as a heterogeneous, high-molecular-weight, acid-precipitable, polar material that is partially oxidized and has a high oxygen content (*1,3,5,12*). Little information is available on other product components or on the fate of biosolubilized coal in cultures.

The work presented here was focused on the characterization of a microbial solubilization product from a preoxidized subbituminous coal. The effects of culture conditions, including organism type and treatment time, and of the product fractionation procedure are noted. Results presented here suggest the production of low-molecular-weight, acid-polymerizable products from coal.

METHODS

Preparation of Biosolubilized Coal

Coal Substrate

Wyodak subbituminous coal pretreated with nitric acid (*13*) was the substrate used in all experiments. This pretreatment results in an increase in available surface area, introduction of nitrogen and oxygen, and a minor increase in carbonyl content (*14*).

Organism and Culture Conditions

The fungi *Paecilomyces* TLi, isolated from coal in this laboratory (*3*), and *Candida* ML13, isolated from an exposed coal seam (*2*), were used for coal solubilization experiments. Stock cultures were maintained on potato dextrose agar slants. Experimental cultures were grown in submerged culture in defined inorganic salts media from conidial inocula as described previously (*15*). Initial spore density was 10^6/mL.

Culture media consisted of Czapek's medium containing either 0.1% maltose or 0.1% benzoic acid as sole carbon source, or Minimal I medium containing 0.1% maltose (*14*). Minimal I medium is an acidogenic medium, developed previously in this laboratory, that is useful for minimizing nonspecific alkali-catalyzed coal solubilization, but supports growth on carbohydrates only. Czapek's medium, which is alkaligenic, supports growth on a variety of noncarbohydrate carbon substrates, including benzoic acid. Benzoic acid was previously shown to stimulate production of coal-solubilizing activity in surface culture in both *Paecilomyces* and *Candida* (*16*).

Experimental cultures were dosed with coal at the time of inoculation. Coal was added at a concentration of 0.5 g/30 mL culture; particle

size was 1–3 mm. Two sets of controls were prepared, containing either coal in uninoculated medium, or conidia in medium (10^6/mL) without added coal. Cultures were routinely incubated at 30°C with agitation at 50 rpm (2.5-cm stroke) for 14 d.

Recovery of the Biosolubilized Coal Product

Cultures were harvested by centrifugation at 7000 × *g* for 10 min. Culture supernatants were dark yellow to brown in color and tested positive in a previously described spectroscopic assay for solubilized coal (*15*). These supernatants were concentrated by lyophilization and stored at room temperature until use. In some cases, an aliquot of the supernatant from cultures grown in Czapek's medium, which was at pH 4.8–5.0 at the time of harvest, was acidified to pH 4.0 with HCl prior to lyophilization.

Molecular Weight Fractionations of Biosolubilized Coal

Preparative/Analytical Organic Gel Permeation Chromatography (GPC)

A polar organic (dioxane)-soluble fraction of the lyophilized product was prepared as follows: One hundred milligrams of the dried powder (acidified or nonacidified) was extracted with 10 mL of dioxane for 1 h at room temperature (with stirring). Dioxane-soluble material was fractionated on Bio-Beads SX-2 resin (Bio-Rad Laboratories, Richmond, CA; fractionation range, 100–2700 daltons) preequilibrated with dioxane. Column dimensions were 3 cm diameter × 10 cm high. A 1-mL sample was applied to the column, and material was eluted with dioxane under gravity flow. Column fractions (1.8-mL vol) were analyzed for absorbance at 280 nm with a Bausch & Lomb Spectronic 21 spectrophotometer.

Analytical Aqueous GPC

The lyophilized product was dissolved at 1 mg/mL in phosphate buffer at pH 11.4 and analyzed by aqueous GPC. Material was fractionated with two Linear Ultrahydrogel columns (Waters Associates, Milford, MA) eluted with phosphate buffer at a flow rate of 1 mL/min. Column effluent was analyzed for absorbance at 254 nm with a Waters model 440 detector.

Molecular Weight Analysis of Coal Substrates

Samples of native and nitric acid treated coal were dissolved in phosphate buffer at pH 11.5 and analyzed by aqueous GPC as described above. Only 5% of the native substrate and 50% of the nitric acid treated substrate were solubilized by this treatment. Weight-averaged molecular weight (M_w) of material eluted during GPC was calculated as described previously (*14*).

Other Analytical Procedures

Elemental Analyses of Coal Substrates and Biosolubilized Coal

Samples of native (untreated) Wyodak coal, nitric acid treated coal, and biosolubilized coal product were analyzed for organic elemental composition (Schwartzkopf Microanalytical, Woodside, NY), and for metallic elements by X-ray fluorescence on a Kevex model 0810A fluorescence spectrometer. The biosolubilized coal products (lyophilized supernatants) were first purified of contaminating inorganic medium constituents by acid precipitation, as follows: samples were dissolved in a NaOH solution at pH 11, filtered through a 0.45-μm filter, and precipitated by adding concentrated HCl to pH 2. The precipitate was centrifuged at $50{,}000 \times g$ for 1–2 h, the supernatant decanted, and the precipitate redissolved at pH 11. The acidification and centrifugation steps were repeated. The precipitate was dried under nitrogen at room temperature prior to analysis.

^{13}C-NMR Spectroscopy of Coal Substrates and Biosolubilized Product

Samples of native coal, nitric acid treated coal, and biosolubilized product were analyzed by ^{13}C cross-polarization/magic angle spinning NMR spectroscopy on a Varian VXR-300 NMR spectrometer with a solid probe (Doty Scientific, Inc., Columbia, SC).

RESULTS

Extent of Solubilization

Gravimetric analysis of washed acid-precipitated material suggested $<15\%$ coal solubilization in all experimental cultures.

Molecular Weight Fractionations

Organic GPC

The supernatants from *Candida* cultures grown on benzoic acid contained dioxane-soluble material that absorbed at 280 nm, a characteristic of aromatic compounds. In nonacidified samples, a low-molecular-weight fraction was evident (Fig. 1). This material coeluted with benzoic acid (mol wt, 122 daltons). Acidified samples contained additional amounts of low-molecular-weight material plus a higher-molecular-weight fraction that was beyond the resin's fractionation range (*i.e.*, >2700 daltons). This high-molecular-weight material was colored and absorbed light at both 450 and 280 nm. The low-molecular-weight fraction was acidic (pH 4.3 in 1% aqueous solution). Isolated low-molecular-weight material, when redissolved in water, acidified to pH 2, and rechromatographed, was con-

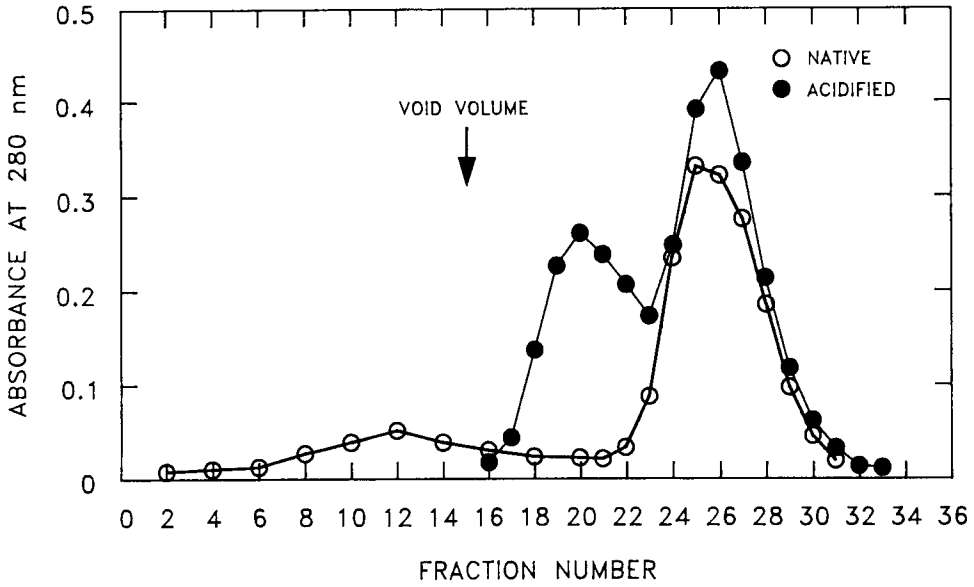

Fig. 1. Gel permeation chromatography of biosolubilized coal product from *Candida* (14-d cultures grown on Czapek's medium with benzoic acid).

verted to high-molecular-weight material. Benzoic acid was not converted by this treatment. Capillary gas chromatographic analysis of the low-molecular-weight fraction resolved a variety of unidentified compounds, but no benzoic acid.

Control cultures (acidified or nonacidified) of *Candida* without coal contained neither fraction. Capillary GC showed no residual benzoic acid in these cultures. Uninoculated controls (acidified or nonacidified) containing coal in medium with benzoic acid showed only the low-molecular-weight peak. Coal in medium without benzoic acid showed no low-molecular-weight peak.

Acidified samples prepared from *Candida* cultures grown on Czapek's medium containing 0.1% maltose contained minimal amounts of high-molecular-weight material (data not shown).

Cultures of *Paecilomyces* grown on benzoic acid and containing coal exhibited a low-molecular-weight peak in nonacidified samples; acidified samples contained predominantly high-molecular-weight material (Fig. 2). Control samples without coal (acidified or nonacidified) contained a small amount of low-molecular-weight material.

Fractionation of nonacidified 14-d-old *Paecilomyces* cultures grown in medium with maltose resolved primarily high-molecular-weight material with 280-nm absorbance (Fig. 3). Longer (120-d) incubation resulted in the apparent diminution of high-molecular-weight material in acidified samples, and the appearance of a low-molecular-weight fraction with 280-nm absorbance, which coeluted with benzoic acid (Fig. 4).

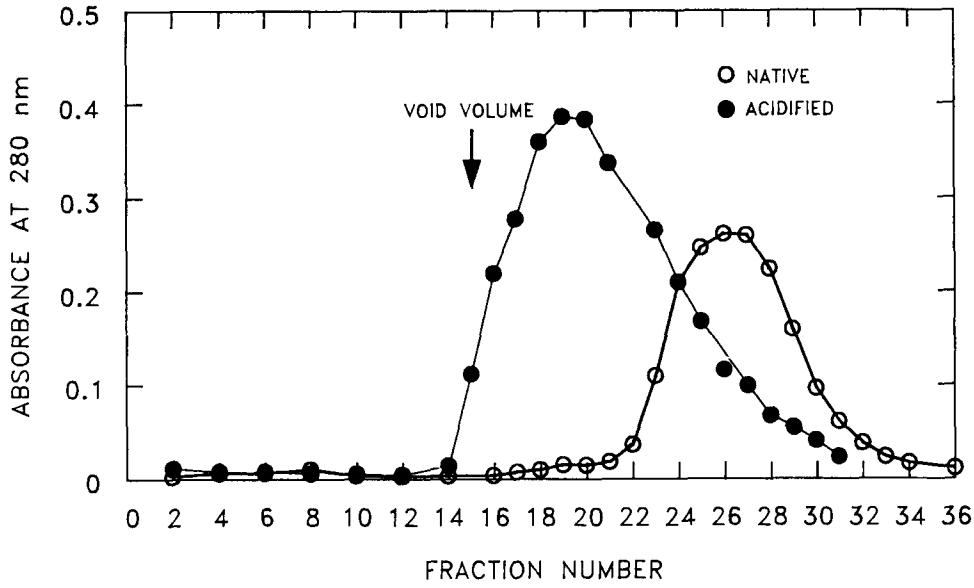

Fig. 2. Gel permeation chromatography of biosolubilized coal product from *Paecilomyces* (14-d cultures grown on Czapek's medium with benzoic acid).

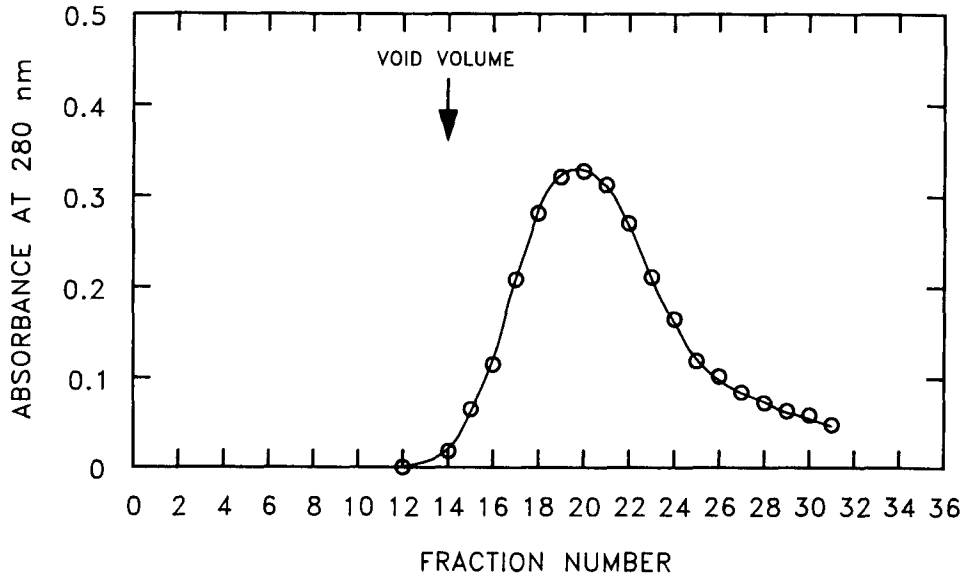

Fig. 3. Gel permeation chromatography of biosolubilized coal product from *Paecilomyces* (14-d cultures grown on Czapek's medium with maltose; sample pH=3.7).

Aqueous GPC

The base-soluble fraction of acid-precipitated biosolubilized coal from *Candida* cultures grown on benzoic acid contained high-molecular-weight material of M_w 4300 (range: 420–57,000; mode: 4600). The base-soluble fractions of native and pretreated coal were of M_w 8300 and 10,500, respectively.

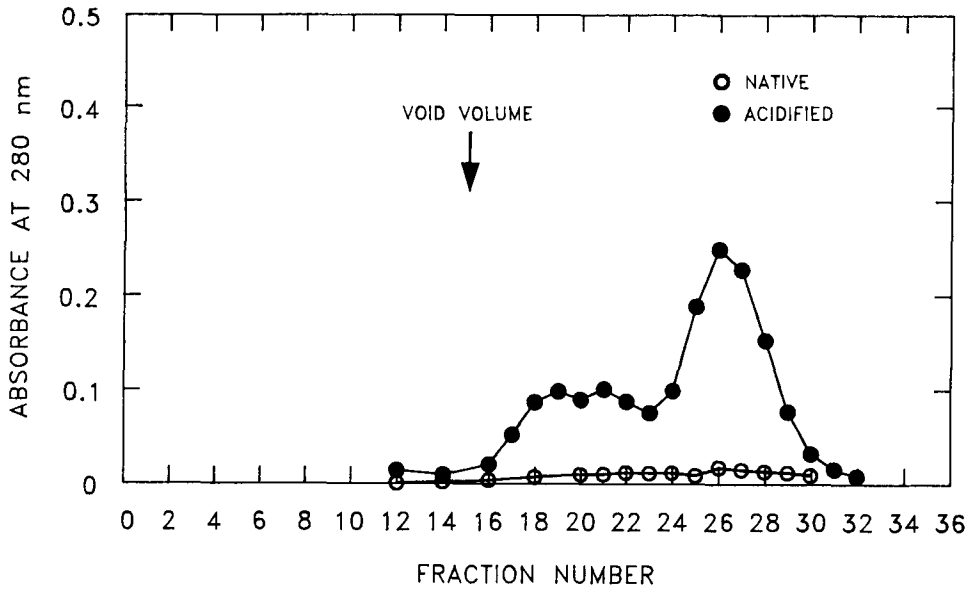

Fig. 4. Gel permeation chromatography of biosolubilized coal product from *Paecilomyces* (120-d cultures grown on Minimal I with maltose).

Elemental Analyses

Acid-precipitated biosolubilized coal products from *Paecilomyces* were substantially enriched in sulfur, sodium, and chlorine on a weight basis, relative to the native coal or the pretreated coal substrate (Table 1). Biological treatment caused an increase in the heteroatomic content of the preoxidized coal substrate, including a 10% increase in oxygen content relative to carbon (bioproduct #2 vs the pretreated coal). The hydrogen and oxygen content of the product showed an apparent increase with length of treatment.

^{13}C-NMR Analyses

The acid-precipitated biosolubilized coal product from *Candida* contained a substantial amount of carbon bound to oxygen (Table 2). Biological treatment had little apparent effect on aromaticity.

DISCUSSION

Cultures of either *Candida* or *Paecilomyces* grown in the presence of coal produced a material exhibiting UV absorbance at 450 nm, a presumptive test for coal solubilization. The overall yield of material was low (< 15%). Supernatants from these cultures contained a high-molecular-weight, oxidized, presumably polyaromatic fraction that was evident in aqueous samples prepared by acid precipitation. This material may be closely re-

Table 1
Elemental Analysis of Coal and Biosolubilized Coal Product

	Composition, %							
	C	H	O[a]	N	S	Ash	Cl	Na
Native coal	66.10	3.82	19.51	1.16	0.81	8.60	<0.01	0.025
Pretreated coal	53.69	3.08	28.82	4.93	0.68	8.80	0.03	0.020
Coal bioproduct #1[b]	53.55	2.91	29.30	5.26	1.26	7.72	1.72	2.29
Coal bioproduct #2[c]	54.87	3.06	32.41	5.41	1.51	2.74	1.15	0.91

Ash-free basis

	Composition, %				
	C	H	O	N	S
Native coal	72.32	4.18	21.35	1.27	0.89
Pretreated coal	58.87	3.38	31.60	5.41	0.75
Coal bioproduct #1	58.03	3.15	31.75	5.70	1.37
Coal bioproduct #2	56.42	3.15	33.32	5.56	1.55

Molar ratios

	C	H	O	N	S
Native coal	100.	48.0	22.1	1.5	0.5
Pretreated coal	100.	58.9	40.3	7.9	0.5
Coal bioproduct #1	100.	59.8	41.0	8.4	0.9
Coal bioproduct #2	100.	61.5	44.3	8.4	1.0

[a]Calculated by difference.
[b]Derived by acid precipitation of a supernatant from a 14-d culture of *Paecilomyces* grown in Czapek's medium containing benzoic acid.
[c]Derived by acid precipitation of a supernatant from a 120-d culture of *Paecilomyces* grown in Minimal I medium containing maltose.

lated to that separated by aqueous GPC at high pH. Dioxane extracts of the acidified, lyophilized supernatants contained high-molecular-weight colored material, detected by organic GPC. The amount of this material was greater in cultures grown on benzoic acid, a substrate previously shown to stimulate coal solubilization (*15*), than in cultures grown on maltose. These observations suggest that both the aqueous and organic GPC procedures employed here resolve a biosolubilized fraction from this preoxidized subbituminous coal that is analogous (based on spectroscopic and elemental analyses) to the product obtained by biological treatment of other coals with other organisms (*1,3,5*). These results are also consistent with those of Strandberg and Lewis (*12*), who obtained a high-molecular-weight product after incubation of this substrate with cultures of a streptomycete.

Table 2
^{13}C-NMR Analysis of Coal and Biosolubilized Coal Product

	Carbon type, %		
	Aromatic	Aliphatic	Bound to oxygen
Native coal	53	47	small
Pretreated coal	34	66	small
Bioproduct[a]	38	28	34

[a]Derived by acid precipitation of a supernatant from a 14-d culture of *Candida* grown in Czapek's medium containing benzoic acid.

The high-molecular-weight fraction found in the present work may be at least partially derived from lower-molecular-weight material detected in dioxane extracts of the nonacidified culture supernatants. This latter material was produced by both organisms when grown in medium containing benzoic acid plus coal. The identity and origin of this material are not known, but a contamination of the biosolubilized coal product with metabolites of benzoic acid cannot be ruled out. However, the appearance of this low-molecular-weight, presumably aromatic fraction in older *Paecilomyces* cultures grown on maltose suggests a coal origin. The biological production of low-molecular-weight, monoaromatic compounds from coal has been described previously by Gupta et al. (5). In that work, the compounds were extracted with moderately polar organic solvents from culture supernatants previously acidified to pH 6. Other, high-molecular-weight products were isolated by acid precipitation (pH 2). Possible relationships between the low- and high-molecular-weight fractions were not explored. Other workers have routinely used acid precipitation as a separation procedure (*17*), conceivably obscuring the presence of lower-molecular-weight biosolubilized components.

The basis for the apparent acid-catalyzed polymerization of the low-molecular-weight material found in benzoic acid cultures is not known. Since the low-molecular-weight material is acidic, enhanced hydrogen bonding at low pH may cause an agglomeration instead of a true polymerization. Acidification of *Candida* cultures yielded increased amounts of low-molecular-weight material (presumably through protonation of carboxylic acids with resultant enhanced solubility in the organic solvent) in addition to the high-molecular-weight fraction. This behavior was not seen in *Paecilomyces* cultures, suggesting differences in product composition between these organisms. *Paecilomyces* and *Candida* were previously shown to generate different products during solubilization of lignite (3).

The high-molecular-weight, polyaromatic material isolated by acid precipitation was enriched in heteroatoms, including oxygen. As in other systems (*3,5*), this material possessed a higher proportion of ash than did the coal substrate. This increased inorganic ion content may be caused in part

by the formation of carboxylic acid salts. The high chlorine content seen in the biological material may also derive from the preparation procedure.

The effect of the length of biological treatment on oxygen content was not studied extensively, although elemental analyses suggested increased substitution (oxidation) of the biosolubilized coal product during a 120-d incubation. The spectral characteristics of the culture supernatants changed over the course of a 36-d incubation, exhibiting a gradual shift in the wavelength of maximal absorbance (A_{max}) from 420 to 550 nm. This bathochromic shift, which is also consistent with increased substitution of the product, unfortunately complicates the validation of the spectroscopic assays for coal solubilization currently in use (*15,17*). Prolonged biological treatment may result in breakdown of the solubilized coal product through oxidative ring-cleavage and other reactions. Microbial processing of this sort has been discounted in other systems (*5*). However, the growth of coal-solubilizing organisms on alkali-solubilized coal has been demonstrated (*18*), as has the breakdown of high-molecular-weight alkali-solubilized coal components with fungal enzymes in vitro (*19*). The appearance of low-molecular-weight material in 120-d cultures in the present work suggests further metabolism of the biosolubilized product in vivo. In addition, both of the organisms studied here have been shown to metabolize a variety of polyaromatic coal substructure model compounds, including biphenyl, naphthalene, phenanthrene, and various heterocyclic compounds (data not shown).

The evidence presented above suggests that spectroscopic measurements of the high-molecular-weight, presumably polyaromatic material produced in these cultures may underestimate the extent of coal solubilization. Additional product characterization studies, including investigation of the fate of solubilized material in cultures, would be useful in the development of improved assays for coal solubilization.

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