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Colonization and degradation of oxidized bituminous and lignite coals by fungi

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SUMMARY

A *Penicillium* sp. previously shown to grow on lignite coals degraded an air-oxidized bituminous coal (Illinois #6) to a material that was more than 80% soluble in 0.5 N NaOH. Scanning electron microscopy of the oxidized Illinois #6 revealed colonization of the surface by the *Penicillium* sp., production of conidia, and erosion of the coal surface. The average molecular weight (MW) of Illinois #6 degraded by the fungus and base-solubilized was approximately 1000 Da. The average MW for base-solubilized Illinois #6 that was not exposed to the fungus was 6000 Da, suggesting solubilizing mechanisms other than base catalysis. A spectrophotometric assay to quantify the microbial conversion of biosolubilized coal was developed. Standard curves were constructed based on the absorbance at 450 nm of different quantities of microbe-solubilized coal. An acid precipitation step was necessary to remove medium and/or microbial metabolites from solubilized coal to prevent overestimation of the extent of coal biosolubilization. Furthermore, the absorption spectra for different coal products varied, necessitating construction of standard curves for individual coals.

INTRODUCTION

Microbial solubilization of coal is a recent development in solid fuel processing that may allow wider utilization of coal, the most abundant fossil fuel resource in the United States. Current methods for coal liquefaction are relatively energy-intensive and costly, given the current market value of crude oil [7]. Microbial solubilization of coal has potential as an initial processing step prior to conversion to methane (S. Barik, R. Wyza, G. Anspach, and J. Isbister, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, O-61, p. 271) or microbial removal of sulfur [6]. Cohen and Gabriele [3] reported that the white rot fungus *Coriolus versicolor* could convert leonardite, a highly oxidized lignite coal, to a dark, water-soluble product. Several investigators [2,8] have since shown that an extracellular product from *C. versicolor* is involved in solubilization of leonardite. The term biosolubilization was coined [8] to emphasize the biochemical origin of the water-soluble product. Since the initial studies with *C. versicolor*, other microorganisms have been identified that can grow on lignite coals [12], which are typically highly oxidized lignites or coals pretreated with nitric acid [11]. This article reports the colonization and degradation of air-oxidized Illinois #6, a bituminous coal, by a *Penicillium* sp.

Most studies regarding microbial solubilization of coal have reported coal conversion in qualitative terms, although some investigators [3,11] have reported coal conversion using gravimetric analysis (i.e., the change in weight of the solid coal before and after conversion). This method of quantifying coal conversion is cumbersome and does not account for coal which may be metabolized by the microorganism. Consequently, we report on the development of a spectrophotometric method for the quantitative determination of coal conversion.

MATERIALS AND METHODS

Qualitative screening. Sabouraud maltose agar plates were inoculated with 2 ml of suspensions from 7 to 10-day fungal Sabouraud maltose broth (SMB) cultures prepared by filtering the hyphal mat, resuspending in sterile deionized water, and shaking with glass beads to break up the hyphae. Agar plates were incubated for 7 days at 25–30 °C or until the surface of the agar was covered with a fungal mat. Approximately 0.3–0.4 g of coal was placed

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on the surface of the fungal mat and observed over time for coal solubilization. The amount of dark-colored liquid product on the surface of the coal or in the medium was visually estimated and reported qualitatively as the degree of biosolubilization.

Microbial strains. Coriolus (Trametes) versicolor was a gift from Martin Cohen (University of Hartford). *Candida* sp., *Penicillium* sp. RWL-5, and *Cunninghamella* sp. were gifts from Bailey Ward (University of Mississippi) and *Phanerochaete chrysosporium* was a gift from Ron Crawford (University of Idaho).

Coals and pretreatment. Both high- and low-ranked coals were assayed for biosolubilization. Lignites included Beulah standard #3, Beulah Zap from North Dakota, and leonardite [5], obtained from the American Colloid Co. (Skokie, IL). The latter three lignites were supplied by Bailey Ward. The bituminous coals included Pennsylvania Upper Freeport, Pittsburgh #8, Illinois #6, and Pocahontas. These higher-ranked coals were supplied by Argonne National Laboratory or the Illinois Department of Energy and Natural Resources. Only one subbituminous coal, Wyoming, was evaluated.

Coals were pretreated by placing a layer of coal approximately 1 cm deep in a forced-air oven at 150 °C for 7 days with periodic mixing. Coal was sieved to obtain particles between 0.5 and 5 mm diameter. After pretreatment, the coals were stored in airtight containers in the dark until used.

Spectrophotometric and gravimetric quantification of coal conversion. Analytical standards of biosolubilized coals were prepared from two different biosolubilized coal samples and used to construct standard curves for quantifying coal biosolubilization. The leonardite standard material was derived from leonardite solubilized by *C. versicolor*. Water-soluble coal was removed by pipette directly from the agar plates, filtered through 0.22-μm membrane filters, freeze-dried, and placed in a drying pistol over P_2O_5, using toluene as the refluxing solvent. The biosolubilized Illinois #6 standard was prepared from coal which had been airoxidized and exposed to *Penicillium* sp. RWL-5 for 5 weeks, solubilized in 0.5 N NaOH, acid-precipitated with 0.1 N HCl, centrifuged and dried as described above. All solubilized coal standards were dissolved in water. Standards produced in this way were contaminated, to some extent, by fungal exudate and/or medium, which may influence the accuracy of the method. Solubilized coal standards were also prepared using biosolubilized coal precipitated with 0.1 N HCl. Precipitated coal was centrifuged at 50 000 × *g* for 1 h, the supernatant decanted, and the coal residue thoroughly dried.

After exposure of coal to a given fungus (3–5 weeks at 27 °C), the entire contents of an agar plate were homogenized by mortar and pestle and placed into two 50-ml centrifuge tubes. The tubes were vortexed after adding 25 ml of deionized water and centrifuged at 15 000 × *g* for 10 min. The supernatant was decanted, and the pellet extracted with water. This step was repeated until the supernatant was nearly colorless (approximately 750 ml). All extracts were combined, and the final volume measured. A subsample was filtered through a 0.45-μm membrane filter (Acrodiscs, low protein binding, Gelman Sciences, Inc., Ann Arbor, MI). Appropriate dilutions were made, and the absorbance at 450-nm measured spectrophotometrically (Beckman model DU-8, Beckman Instruments Inc., Fullerton, CA) and compared with the standard curve.

Microbial conversion of coal was determined gravimetrically by carefully removing coal particles from agar plates, rinsing the coal in distilled water, and air-drying until a constant weight was obtained.

Electron microscopy. Samples of coal particles exposed and unexposed to the fungus were placed on aluminum stubs, coated with gold pallidium, and dried overnight at room temperature. These preparations were viewed and photographed with a JEOL 25 S-II scanning electron microscope.

Molecular weight determination. The molecular weight (MW) range and average MW of the various solubilized coal products were determined by high-performance gel permeation chromatography (GPC). Two linear Ultrahydrogel aqueous GPC columns (Water Associates, Inc., Milford, MA) in series were eluted with distilled, deionized water at neutral pH or at pH 11.5 (maintained with a sodium phosphate buffer) at a flow rate of 1 ml/min. Detection was accomplished using a Waters absorbance detector (model 440) at 254 nm and a Waters refractive index detector (model R401). Calibration was accomplished using polyethylene glycol standards from 20 000 to 200 Da and ethylene glycol, 67 Da. The resulting log molecular weight versus retention-time data were fitted using a linear least squares program and routinely yielded correlation coefficients greater than 0.99.

TABLE 1

Microbial solubilization of coals with and without heat pretreatment[a]

Type of coal	Microorganism	Untreated	Exposed to 150 °C for 7 days
Beulah Zap II (lignite)	*C. versicolor*	−	+
	P. chrysosporium	+	−
	Candida sp.	−	−
	Penicillium sp.	−	−
	Cunninghamella sp.	−	+ + +
Beulah Std. #3 (lignite)	*C. versicolor*	−	−
	P. chrysosporium	+	−
	Candida sp.	−	−
	Penicillium sp.	−	−
	Cunninghamella sp.	−	+ + +
Leonardite (oxidized lignite)	*C. versicolor*	+ + +	ND[b]
	P. chrysosporium	+ + + +	ND
	Candida sp.	+ + +	ND
	Penicillium sp.	+ + +	ND
	Cunninghamella sp.	+ + + +	ND
Pennsylvania upper freeport (bituminous)	*C. versicolor*	−	−
	P. chrysosporium	+	−
	Candida sp.	−	−
	Penicillium sp.	−	+ + +
	Cunninghamella sp.	−	+ + +
Pittsburgh #8 (bituminous)	*C. versicolor*	−	−
	P. chrysosporium	−	−
	Candida sp.	−	−
	Penicillium sp.	−	+ + +
	Cunninghamella sp.	−	+ +
Illinois #6 (bituminous)	*C. versicolor*	−	−
	P. chrysosporium	−	+
	Candida sp.	−	−
	Penicillium sp.	−	+ + + +
	Cunninghamella sp.	+	+ + +
Wyoming (subbituminous)	*C. versicolor*	−	−
	P. chrysosporium	−	−
	Candida sp.	−	−
	Penicillium sp.	−	−
	Cunninghamella sp.	−	−

+ + + +, Extensive microbial solubilization; + + +, Moderate microbial solubilization; + +, Low microbial solubilization; +, Traces of microbial solubilization.

[a] Pretreatment: layer of coal particles (0.5 to 5 mm in diameter) was placed in oven at 150 °C for 7 days with intermittent mixing of the layer.

[b] Not determined.

RESULTS

Evaluation of pretreatment and coal type on biosolubilization

The fungi *C. versicolor*, *Candida* sp., *Penicillium* sp. and *Cunninghamella* sp. were chosen because of their demonstrated ability to grow on lignite coal [12]. The influence of coal type and pretreatment on biosolubilization was evaluated using these fungi. *P. chrysosporium* was chosen for its ability to degrade lignin, a material which is believed to be a precursor of coal and contains many of the same structural units and linkages. The influence of coal type and air-oxidation pretreatment on coal solubilization by the fungi *C. versicolor*, *P. chrysosporium*, *Candida* sp., *Penicillium* sp. and *Cunninghamella* sp. is shown in Table 1. With the exception of leonardite, none of the coals was solubilized in more than trace quantities by any of the fungi examined.

Oxidation of the coal by heating in a forced-air oven at 150 °C for 7 days increased the susceptibility of the lignites and bituminous coals to solubilization (Table 1) but did not affect the subbituminous coal (Wyoming). The oxygen content of the Illinois #6 bituminous coal increased from 12% before treatment to 23% after treatment [1]. By analogy, it is likely that all of the coals oxidized in this manner increased in oxygen content. *Cunninghamella* sp. and *Penicillium* RWL-5 were the only fungi active in solubilizing the oxidized coals. The combination of *Penicillium* RWL-5 and oxidized Illinois #6 was chosen for further study because of the extensive solubilization observed with this combination. Much of the previous work regarding coal solubilization has been with lignites or nitric-acid-pretreated lignites [11].

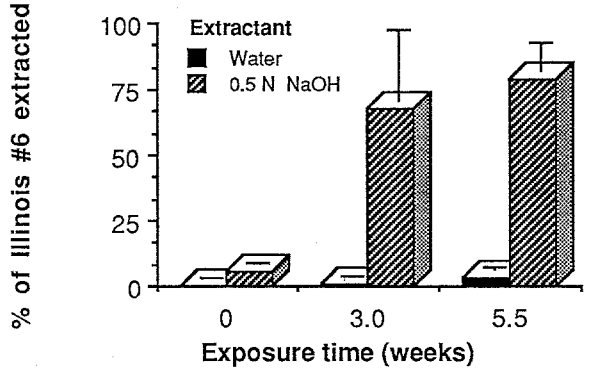

Fig. 1. Water and base solubility of air-oxidized Illinois #6, with and without exposure to *Penicillium* RWL-5. Whole plates were extracted with water or NaOH, and the percent coal extracted determined spectrophotometrically. (Error bars indicate 1 S.D.)

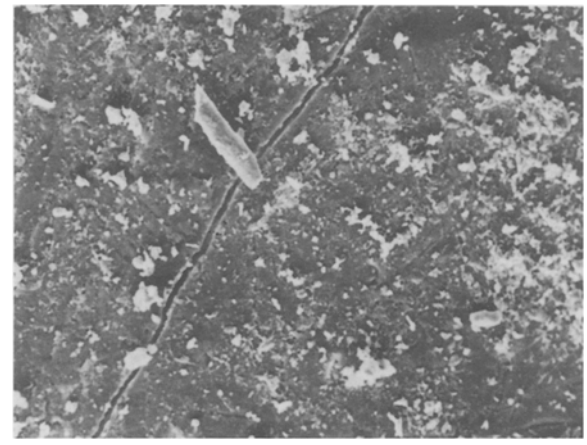

Fig. 2. Scanning electron micrograph of air-oxidized Illinois #6 before exposure to *Penicillium* RWL-5 × 300.

Degradation of Illinois #6 by Penicillium RWL-5

Despite the appearance of extensive biosolubilization of air-oxidized Illinois #6 by *Penicillium* RWL-5 in agar-plate assays, less than 10% of the coal was converted to a water-soluble material (Fig. 1). However, there was a significant increase in the solubility of the coal in 0.5 N NaOH after exposure to the fungi: from less than 6% to greater than 80%. In contrast, non-oxidized Illinois #6

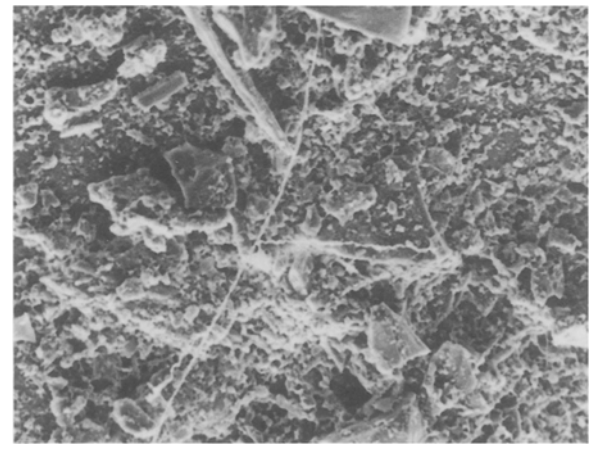

Fig. 3. Scanning electron micrograph of untreated Illinois #6 after exposure to *Penicillium* RWL-5 on agar plates for 6 weeks. Hyphae (arrows) are present on the surface, but there is little evidence of colonization or degradation of the coal. × 300.

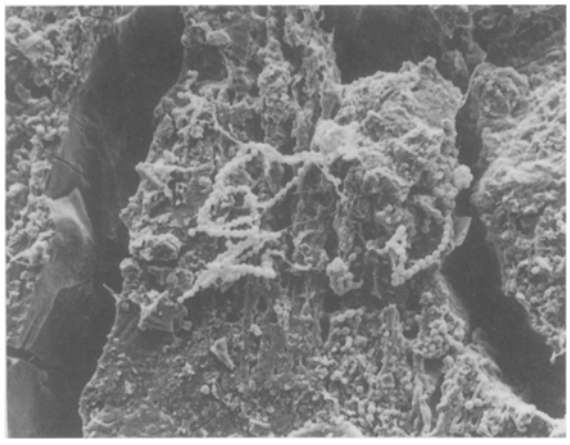

Fig. 4. Scanning electron micrograph of air-oxidized Illinois #6 after exposure to *Penicillium* RWL-5 for 6 weeks. Extensive surface colonization as well as production of conidia (arrows) are evident. The surface of the coal has been eroded, and fractures incurred during gentle handling are apparent. ×300.

exposed to *Penicillium* RWL-5 was only slightly soluble (0.8 ± 0.4%) in 0.5 N NaOH. In addition to the change in base solubility, there was a change in the physical state of the coal, which increased in brittleness and in ease of fracturing.

The surface of the air-oxidized Illinois #6 before exposure to the fungus was relatively unmarred, with some surface debris and few fractures (Fig. 2). The surface of untreated Illinois #6 exposed to *Penicillium* RWL-5 showed a limited amount of fungal hyphae on the coal surface, with few or no fractures (Fig. 3). *Penicillium* RWL-5 colonization of the air-oxidized Illinois #6 was more extensive, resulting in erosion of the coal surface (Fig. 4) and a change in the physical nature of the coal. The latter was evident from stress fractures (Fig. 4), which were easily induced in the coal particles. In addition to colonizing the air-oxidized coal, numerous conidia were present on the surface.

The MW range and the average MW were determined for the various water- and base-soluble fractions from air-oxidized Illinois #6, exposed and unexposed to *Penicillium* RWL-5 (Table 2). The MW range was identical for all the coal fractions, while the average MW for the *Penicillium*-exposed coal fractions was considerably lower than for base-soluble, air-oxidized Illinois #6, suggesting that the mechanism of coal degradation by *Penicillium* is other than base solubilization.

Spectrophotometric assay

A quantitative spectrophotometric assay was developed for routine analysis of coal biosolubilization. Absorption spectra of *C. versicolor* biosolubilized leonardite and for the same material that had been acid-precipitated, dried, and dissolved in deionized water are shown in Fig. 5. Also shown are the absorption spectra for *Penicillium*-degraded Illinois #6 product, which had been acid-precipitated. The spectra for the acid-precipitated and non-acid-precipitated coals differ considerably, probably due to absorption by medium and/or fungal metabolic products. The spectra are also dependent on coal type, shown by the fact that the traces for leonardite and Illinois #6 vary. The absorption at 450 nm was chosen for routine analysis for coal biosolubilization because of the minimal absorption by protein and buffer and strong absorption by solubilized coal at this wavelength [8]. Also, the absorbance (450 nm) of Sabouraud maltose agar cultures of *C. versicolor*, that were extracted as described earlier, was 20-fold less than cultures where leonardite had been added.

TABLE 2

Molecular weight (MW) distribution and MW average of Illinois #6 water- and base-soluble fractions

Illinois #6 sample	Extractant	Average MW (Da)	MW range (Da)
Air-oxidized, *Penicillium*-exposed			
Base-soluble	0.5 N NaOH	900	200–100,000
Water-soluble	water	2000	200–100,000
Water-soluble/acid-precipitated	water	1000	200–100,000
Air-oxidized			
Strong base	2.4 N NaOH	6000	200–100,000

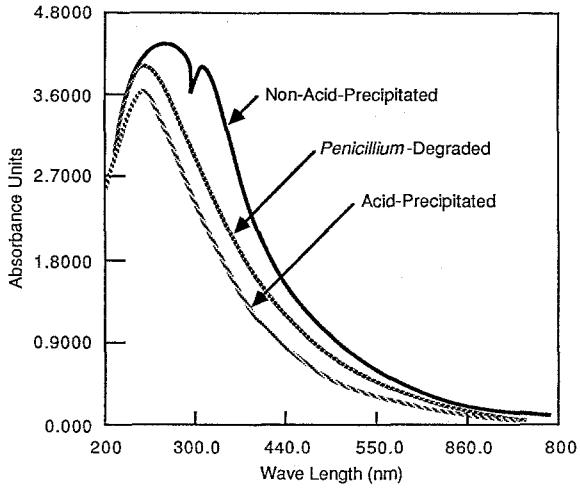

Fig. 5. Absorption spectra of *C. versicolor*-biosolubilized leonardite, both non-acid-precipitated, acid-precipitated, and *Penicillium*-degraded Illinois #6.

Standard curves constructed from non-acid-precipitated and acid-precipitated biosolubilized leonardite and Illinois #6 are presented in Fig. 6. The slope of the acid-precipitated product is less than those for the non-acid-precipitated leonardite or Illinois #6 products, indicating that material absorbing at 450 nm was removed by acid precipitation. The slope and position of the non-acid-precipitated leonardite and Illinois #6 products versus absorbance lines are similar, while the slope and position of the acid-precipitated materials are different. This indicates that the same or similar components in both are removed by the acid-precipitation step. Since both organisms were cultured in SMB, the most likely component removed is the medium.

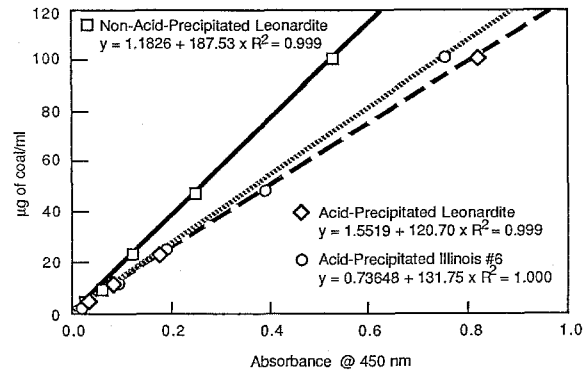

Fig. 6. Standard curves of *C. versicolor*-biosolubilized leonardite and *Penicillium*-degraded Illinois #6 with and without acid precipitation. R^2 values were greater than 0.99 for both curves.

Biosolubilization of leonardite by agar plate cultures or culture filtrates [2,8] of *C. versicolor* was quantified spectrophotometrically and gravimetrically. The values for the weight percent of leonardite converted by *C. versicolor* culture filtrate to water soluble material using the spectrophotometric assay (46 ± 5) were in close agreement with the values obtained by direct gravimetric analysis (47 ± 6). However, the percent of leonardite solubilized by whole cultures was 32 ± 14 as determined by the spectrophotometric assay compared to 7 ± 3 as determined by gravimetric analysis. The gravimetric measurement of coal biosolubilization consistently gave higher conversion values, likely because of incomplete coal recovery from agar plates.

To determine the efficiency of extraction of biosolubilized coal, agar-plate cultures of *C. versicolor* were amended with 89 mg of solubilized Texas lignite, extracted, and the concentration of recovered coal product determined spectrophotometrically. The average recovery of coal product was 102% (S.D. = ± 2, $n = 3$).

DISCUSSION

The results from this study show that air oxidation rendered bituminous and lignite coals susceptible to microbial solubilization, thus confirming previous observations [9,10]. However, biosolubilization was organism-dependent, with *Penicillium* sp. RWL-5 and *Cunninghamella* sp. being the most active of the five cultures examined (Table 1).

The air-oxidation of Illinois #6 increased the oxygen content of the coal, allowing degradation by *Penicillium* RWL-5. Biodegradation was evident from the increase in base solubility of the coal and from the extensive deterioration of the coal surface, which was colonized by the fungus, as exhibited by the electron micrographs. The need to develop a method to quantify coal biosolubilization was reinforced by the discrepancy obtained between visual observations of coal biosolubilization and actual measurement of mass converted. The appearance of air-oxidized Illinois #6 after contact with *Penicillium* RWL-5 agar-plate cultures indicated that significant quantities of the coal had been solubilized, yet quantitative analysis indicated that less than 10% of the total coal mass had been converted to a water-soluble product.

Although the exact relationship is not clear at this time, it may be that the fungus is growing on the coal and

that the production of enzymes or metabolites required for biosolubilization is linked to the growth stage associated with production of spores, or that the solubilizing activity is associated directly with the spores. Faison and Kuster [4] found that ligninase protein in *P. chrysosporium* was associated with the exteriors of asexual spores.

The microbial solubilization of coal has been hypothesized to be due, at least in part, to base solubilization [9,10]. The results presented in this study suggest the involvement of additional mechanisms. The air-oxidized Illinois #6 had limited solubility in 0.5 N NaOH, while the biodegraded coal was highly soluble in dilute base (Fig. 1). Although the molecular weight range of the various *Penicillium*-derived Illinois #6 products and that of 2.4 N NaOH-solubilized coal were the same, the weight average molecular weight of the *Penicillium*-derived product was significantly lower than the strong base-solubilized Illinois #6. Other than molecular weight, there is relatively little chemical difference between biosolubilized Illinois #6 and the original air-oxidized coal [1]. This suggests that microbial solubilization results in a greater degree of depolymerization of the coal, and that mechanisms other than base-solubilization were acting on the coal. Previous studies with extracellular fractions from *C. versicolor* cultures which solubilize leonardite suggest that an enzymatic mechanism may be involved [2,8].

Studies of the microbial solubilization of coal have traditionally relied on qualitative or gravimetric analyses to determine the extent of microbial coal conversion [11]. This information is crucial for scaling up, increasing the efficiency of the process, and for investigations into the mechanism of coal biosolubilization. By developing standard curves from the absorption (at 450 nm) of known quantities of biosolubilized coal, a quantitative method of coal solubilization was developed. The difference in absorption spectra between acid-precipitated and non-acid-precipitated solubilized coal indicated that medium (SMB) and/or fungal metabolites were being removed. Therefore, it was necessary to remove these components prior to construction of standard curves to avoid overestimating the extent of conversion.

The absorption spectra of microbe-solubilized or degraded coal also varied as demonstrated by the different standard curves obtained with *C. versicolor*-solubilized, acid-precipitated leonardite and *Penicillium* RWL-5-degraded, base-solubilized, acid-precipitated Illinois #6. Therefore, for accurate measurements of the extent of microbial coal solubilization by spectrophotometric analysis, standard curves had to be constructed for each coal type.

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