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Evidence that Microbially Produced Alkaline Materials are Involved in Coal Biosolubilization

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ABSTRACT

The biosolubilities (susceptibility to being attacked and solubilized by microbes) of 17 coals by 9 microbes were evaluated, as were solubilities of these coals in dilute alkaline buffers. A direct relationship between coal alkali- and biosolubilities was observed suggesting a common mechanism of coal solubilization. These data are consistent with the hypothesis that a mechanism by which microorganisms solubilize coal is by production of alkaline materials that raise medium pH and effect solubilization.

Index Entries: Biosolubilization; coal; alkaline.

INTRODUCTION

The ability of microorganisms to solubilize coal was first reported in the early 1980s (*1,2*) and, since then, a large number of coal solubilizing microbes have been isolated (*3*). It has been shown that microbial coal solubilization occurs more readily with weathered lower-ranked coals than with unweathered samples. Oxidative pretreatments of coals (e.g., nitric acid, ozone, hydrogen peroxide), which are thought to mimic the weathering process to some degree, have been shown to significantly enhance both rates and quantities of coal solubilized (*4,5*).

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Recent work in our laboratories has involved investigating mechanisms of microbial coal solubilization. Initial work using nitric acid oxidized coal revealed that microbial coal solubilization occurred in conjunction with microbial production of alkali. Little measurable change in coal molecular structure, as determined using UV-Vis and FTIR spectroscopy, either during or after solubilization, was observed, indicating that microbial coal solubilization was not primarily directed by enzymes (*6,7*). In this paper we report that microbial production of alkali appears to be a mechanism for microbial solubilization of coals that have not been oxidatively pretreated.

METHODS

Streptomyces viridosporus T7A (ATCC 39115) (*8*), *Streptomyces setonii* 75Vi2 (ATCC 39116) (*8*), and *Streptomyces badius* 252 (ATCC 39117) (*9*) were maintained on yeast extract-malt extract-dextrose agar (YEMED). Spores from YEMED slants were used as inocula for experiments described below. Fungal strains DML-12 (*Acremonium* sp.), ML-13 (*Candida* sp.), RWL-40 (unidentified basidiomycete), YML-1 (*Cunninghamella* sp.), and YML-21 (*Cunninghamella*-like sp.) isolated from weathered coal seams (*3,10*) were maintained on Sabouraud Dextrose Agar (SDA) supplemented with powdered Wilcox coal (1 mg/mL). Bacterial strain BHB-1 (*Bacillus* sp.) isolated from weathered coal was maintained on Tryptic Soy Agar (TSA) supplemented with powdered Wilcox lignite (1 mg/mL).

Texas lignite was provided by Northwestern Resources, Jewett, TX. Illinois #6 was supplied by the Consolidation Coal Co., St. Louis, MO. Vermont (Brandon Seam, no PSOC #) and North Dakota (PSOC 1482) lignites were provided by A. Davis, Coal Research Station, Pennsylvania State University, University Park, PA. Remaining coals were collected at those sites listed in Table 1 as described (*3*) and varied in degree of weathering. Different samples of Mississippi Wilccox and Alabama York lignites collected from different areas of the seams were included in tests. Each coal was ground to a uniform mesh and mixed to homogeneity. Prior to biosolubilization experiments using either fungi or BHB-1, coals were soaked for 24 h in a dilute mineral salts solution (*3,10*). All coals were autoclaved (15 min, 121°C) before being added to culture media.

Tests to determine the ability of *Streptomyces* to solubilize various coals was performed at the University of Idaho. Spores were inoculated onto plates of either YEMED or 0.6% (w/v) yeast extract—mineral salts agar of pH 7.2-7.4 (*11*). Plates were incubated (37°C) until confluent growth had developed (2-4 d). Sterile coal particles of known dry wt (70-250 mg) and uniform size (5-10 mesh) were then aseptically placed on culture lawns. Coal solubilization was followed visually and, after one, two, three, or four weeks, solubilized coal present in surface droplets was harvested

Table 1
Sources of Coals for Alkali and Biological Solubility Testing

Source	Trivial name	Locality
Wilcox Group (Tuscahoma Formation)	Wilcox	I20 roadcut near Russell, MS
Oak Hill Lignite (Naheola Formation)	York	I20 roadcut between York and Cuba, AL
Kiowa Bed	Kiowa	Outcrop near Kiowa, CO
North Park Field (Coalmont Formation)	Brownlee	Outcrop north of Walden, CO
	TM	Mine spoils, east of Walden, CO
	Kerr	Run of mine, east of Walden, CO
Green River Region (Yampa Field)	Black Dan	Abandoned strip mine, Hayden, CO
Lower Hartshorne Bed (McAlester Formation)	Hartshorne	HWY 7 roadcut, Dardanelle, AR

using a micropipet. Residual coal was removed from the surface, washed with distilled water, dried at 100°C (2 h under vacuum), and weighed. Controls consisted of sterile coal particles incubated on sterile agar and treated as described above. Biosolubility was estimated by the volume of pigmented material in surface droplets and the weight loss of coal. Final medium pH was measured by harvesting the agar medium, melting it using steam, and measuring the pH of the molten agar (50°C).

Investigations of coal solubilization by fungi or BHB-1 were performed at the University of Mississippi. Sterile coal particles (35-60 mesh) were placed in mounds (4 mm dia) on either SDA, pH 5.6, or TSA, pH 7.3. Media were inoculated with either streaks of BHB-1 (TSA), fungal spores (SDA), or hyphal fragments (SDA). Cultures were incubated in the dark (25°C, 50–70% relative humidity) for 14 d. Controls consisted of either coal-only or organism-only replicates. Average biosolubilization ($n > 10$ per organism) was estimated by measuring the zone diameter (*11*) of pigmented material diffusing into the medium (as corrected for controls). Relative biosolubilities, in all cases, were expressed on a "0" (no detectable solubilization) to "5" (maximum soluble product formation) scale.

Tests for coal solubilities in alkali were performed at the Idaho National Engineering Laboratory. Coals were from those lots used for biosolubility assays and were tested for alkali solubility as received. Aliquots of coal (0.5 g) were placed in a 250 mL Erlenmeyer flask containing 50 mL 50 m*M* Tris buffer, pH 8.0. Flasks were sealed and shaken (140 rpm) for 24 h at 25°C. Resulting suspensions were centrifuged (10,000×g, 10 min) and supernatants filtered through membrane filters (type GA-8 SUPOR, 0.2 μM pore dia, Gelman Sciences, Inc., Ann Arbor, MI). The pH of filtrates

were determined, filtrates were diluted as necessary and absorbances at 400 nm measured, the region of maximum absorbance for base solubilized coals (*6*). Absorbances were multiplied by their respective dilution factor to obtain calculated absorbances.

The source of microbially produced alkaline materials was determined at the Idaho National Engineering Laboratory by growing microorganisms in media that had varied concentrations of either nitrogen source or phosphate. Nitrogen source concentration was varied by altering the concentration of YMP (yeast extract 0.3%, malt extract 0.3%, proteose peptone (0.5%) in growth media. YMP was used in low (0.1X), medium (1X), or high (5X) concentrations. Monosodium phosphate was used in low (0 g/L), medium (2.5 g/L), or high (25 g/L) concentrations. Sodium hydroxide was used to adjust the pH of solutions containing 25 g/L phosphate to 6.9. Glucose concentration in all cases was 10 g/L. Microorganisms were incubated 14 d at 25°C while shaken (140 RPM) before pH values were measured.

Moisture and ash analyses of coals were determined according to ASTM methods #D3173 and D3174. Ultimate analyses of coals were performed at the Fuel Characterization Laboratory, University of Utah, Salt Lake City, UT. Ammonia assays were performed using Hach test kit #22669 (Hach Co., Loveland, CO).

RESULTS

The results of tests for alkali- or biosolubilities of coals are presented in Table 2. Considerable differences in degree of alkali- or biosolubility for these coals were observed, yet general agreement was apparent for duplicate samples of coals tested in different laboratories against different organisms.

Comparisons of biosolubilities with alkali solubilities for coals tested are presented in Fig. 1. Biosolubilities were plotted against the natural log of the calculated absorbances of respective alkali solubilized coal solutions in an effort to condense the latters' wide ranging values (Fig. 1) into a more easily visualized form. This plot indicates that there was a direct relationship between the alkali solubility and biosolubility for each coal. Coals having low biosolubilities had low alkali solubilities and coals having high biosolubilities had high alkali solubilities. A plot of coal oxygen content against alkali solubility yielded a curve similar to that observed in Fig. 1 (data not shown). When coal biosolubility was plotted against coal oxygen content, a linear relationship was observed for oxygen concentrations between 17% and 28% (Fig. 2). The point that corresponds to 11% oxygen is that for Illinois #6; we are unable to explain this apparent anomaly.

When coal was solubilized by treatment with alkaline buffer there was a decrease in buffer pH from the original value of 8. The degree of pH

Table 2
Coal Biosolubility Rankings

Coal[a]	Rank	Biosolubility[b]	Alkali solubility[c]
(W) Hartshorne	Bituminous	2.5	0.960
(C) York	Lignite	3	6.800
(W) Wilcox	Lignite	4	16.36
(C) York	Lignite	5	4.15
(C) North Dakota	Lignite	1	0.196
(W) Kerr	Submituminous	0	0.022
(C) Illinois #6	Bituminous	2	0.073
(W) TM	Subbituminous	4	1.160
(C) Vermont	Lignite	3	0.860
(W) Black Dan	Subbituminous	0	0.074
(W) York	Lignite	3	9.510
(W) Wilcox	Lignite	4	16.910
(C) Wilcox	Lignite	4	38.00
(C) Texas	Lignite	1	0.191
(W) Kiowa	Lignite	1	0.191
(W) York	Lignite	3	3.050
(W) Brownlee	Subbituminous	5	55.50

[a]Coals were obtained and tested as described in methods. "W" indicates work performed at the University of Mississippi and "C" indicates work performed at the University of Idaho.
[b]Biosolubility is given as the average value obtained from all cultures.
[c]Alkali solubility is given as the absorbance at 400 nm.

change was dependent upon the amount of coal solubilized, i.e., the greater the amount of coal solubilized the greater the buffer pH decrease (Fig. 3). As more coal was solubilized buffer pH appeared to asymptotically approach a value of 5.5–6.5. If equivalents of base needed to cause a buffer pH decrease are plotted against the alkali solubility of the coal that caused that pH decrease, then a straight line is obtained (Fig. 4). The extinction coefficient for solubilized coal (1 mg/mL coal solution yields an absorbance of 9) indicates that for every 30 mg of coal solubilized, one milliequivalent of base was consumed.

Growth media pH values were found to increase as coal was solubilized for all cultures tested (data not shown). Experiments indicated that culture medium pH increases were dependent upon nitrogen concentration and independent of phosphate concentration (Table 3). Ammonium concentration increased as pH increased in microbial cultures (Fig. 5) whereas both remained constant in abiotic controls (data not shown). Efforts to detect other amines (using gas chromatography) that might have been produced during coal solubilization were unsuccessful.

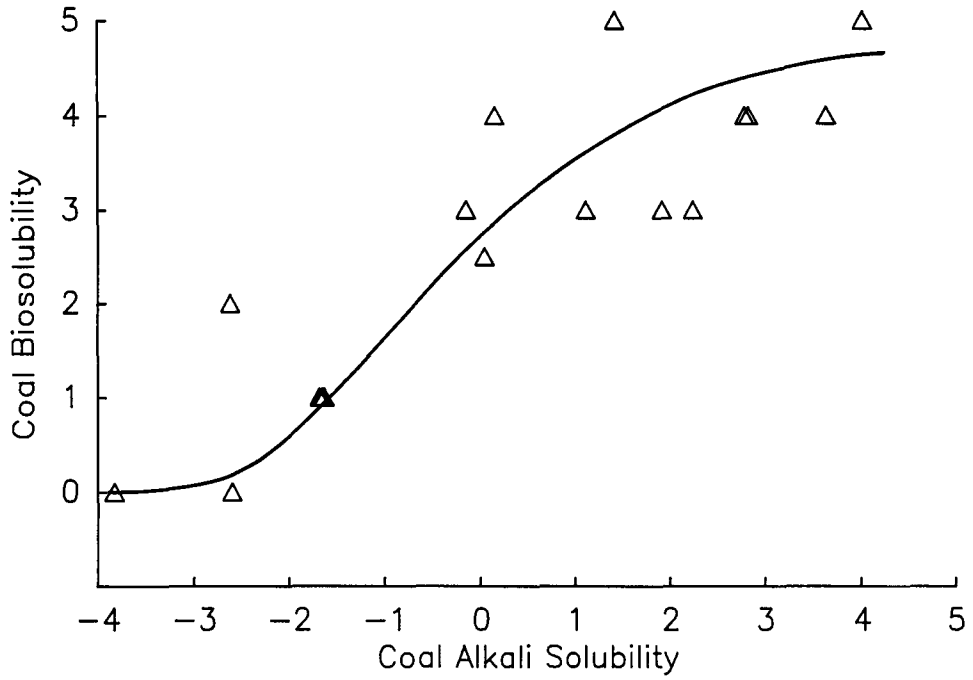

Fig. 1. Relationship between alkali and biosolubilities of coals. Alkali and biosolubilities of coal were determined as described in Methods. Biosolubilities were subjectively rated on a scale of 0 (low) to 5 (high). Alkali solubilities are expressed as the natural log of the absorbance (400 nm) of coal buffer supernatants.

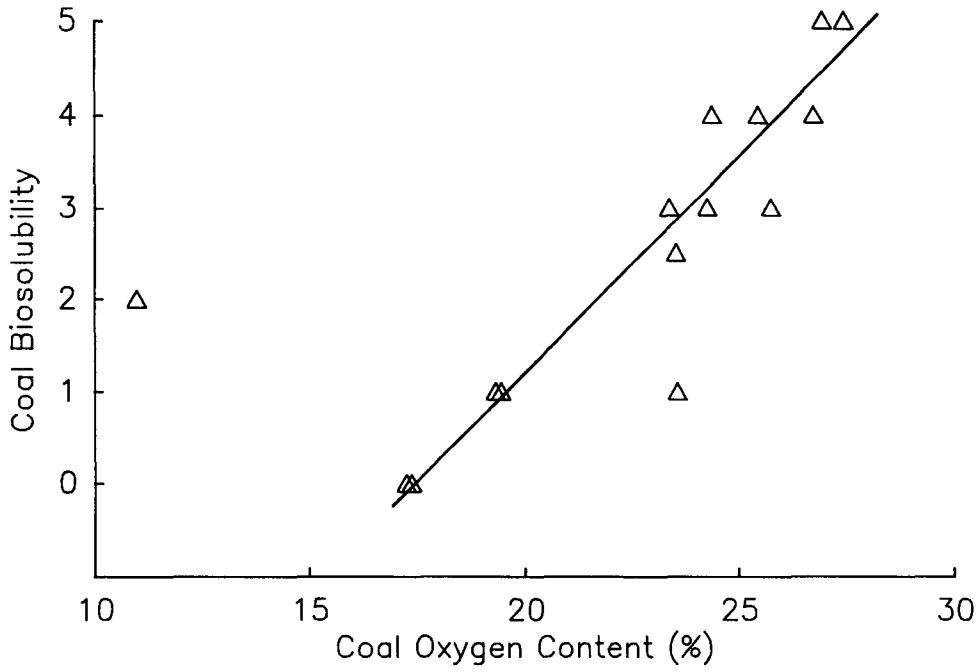

Fig. 2. Relationship between coal biosolubility and oxygen content. Coal biosolubilities and oxygen contents were determined as described in Methods.

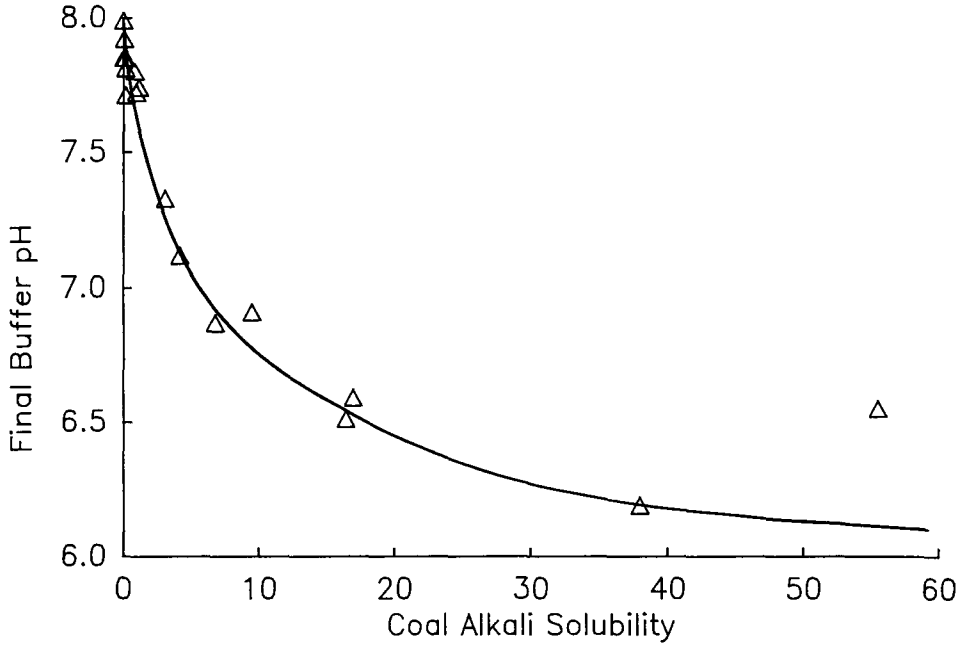

Fig. 3. Effect of coal solubilization on buffer pH. Coal alkali solubilities were determined as described in Methods. Final pH of the buffer solution was determined prior to dilutions required for alkali solubility measurements.

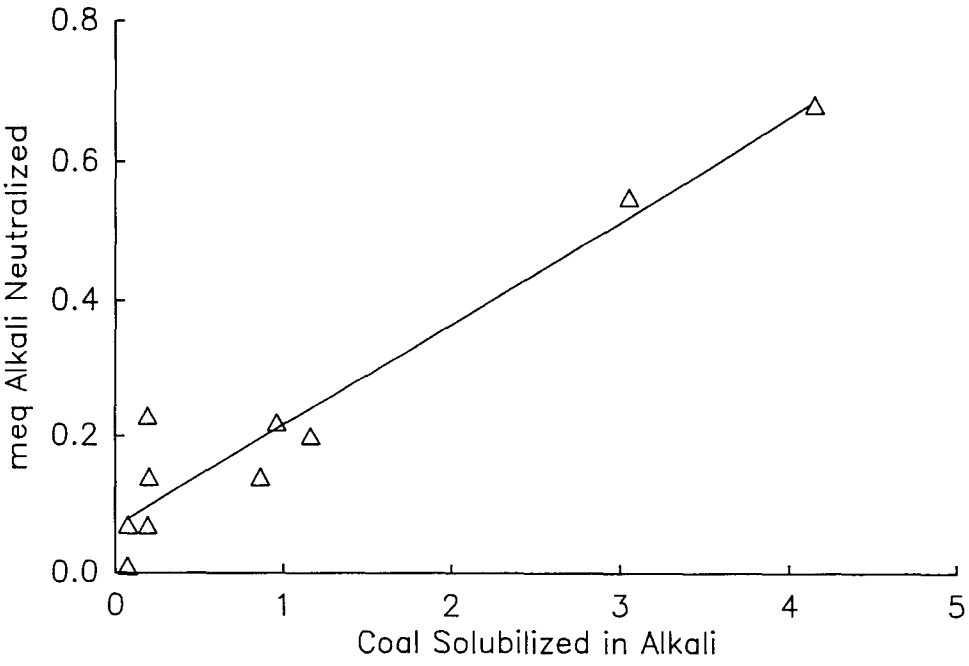

Fig. 4. Relationship between milliequivalents of buffer neutralized and amounts of coal solubilized in alkaline buffers. Amounts of coal solubilized are given as absorbance (400 nm) of coal solutions.

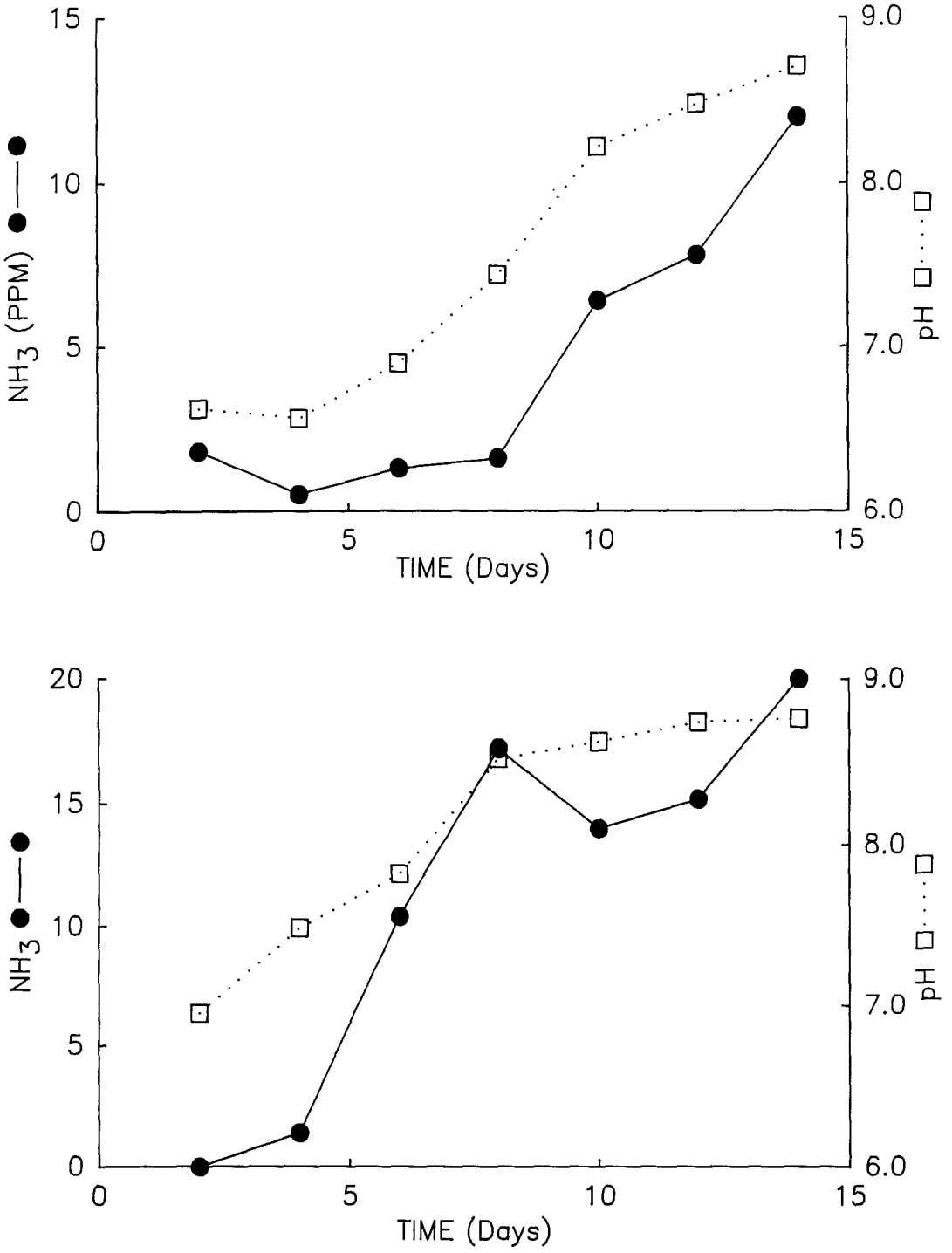

Fig. 5. Ammonia concentration and pH variances during microbial coal solubilization. *Streptomyces setonii* was grown in YMB medium (50 mL) until late log phase (~3 d). Texas lignite (1 g) was added to some cultures and pH and ammonia concentration were monitored at times indicated. A) *S. setonii* B) *S. setonii* + coal.

Table 3
Dependence on Medium for Microbial Production of Alkali[a]

		Organism	
$[PO_4^{=}]$	[YMP]	YML-21, pH	*S. setonii*, pH
Low	Low	4.98	5.02
Low	Medium	8.17	6.59
Low	High	8.48	8.97
Medium	Low	6.34	5.72
Medium	Medium	7.79	6.55
Medium	High	8.53	8.79
High	Low	6.89	6.69
High	Medium	6.98	6.74
High	High	8.09	6.38

[a]Organisms were grown in media listed as described in methods. Culture fluid pH was measured after 14 d growth.

DISCUSSION

The ability of microbes to solubilize coal was related to oxygen content and confirms earlier work (*4,5*). Since this relationship was observed in experiments that had so many variables (e.g., 17 coals, 9 organisms, 3 media, 2 laboratories), it appears that the mechanism of biological coal solubilization in these experiments is independent of microbial species or coal used. A mechanism that has been previously reported (*6,7*) and agrees with data presented here is that of alkali coal solubilization.

Low rank coals are known to contain carboxylic acid functional groups and that the concentration of these groups increases as oxygen content in the coal increases (*12*). It is also known that increasing a coals oxygen content due to weathering or chemical oxidation (e.g., nitric acid, ozone, permanganate...) increases the concentration of carboxylic acid functional groups thereby increasing the coals solubility in strong alkali (*13–17*). It would seem that during the solubilization process carboxylic acids present in the coal are neutralized resulting in the coal being solubilized. Observations that the pH of the buffer solution decreases during solubilization would support this hypothesis. Decreases in buffer pH observed during coal solubilization were directly related to both the number of equivalents neutralized and the amount of coal solubilized. Furthermore, pH values for buffers containing various solubilized coals in increasing concentration decreased and asymptotically approached pH 5.5–6.5. A similar pH range was observed for both the buffering range of oxidized Texas lignite and the range within which the most oxidized Texas lignite was solubilized (*6*); this is also the buffering range of carboxylic acids. Taken together, it would appear that coal that contains greater concentrations of oxygen

would also contain greater concentrations of carboxylic acid functional groups and that coal solubility at biologically relevant pHs would increase.

It is characteristic of all reported coal solubilizing microorganisms to increase medium pH to values greater than 6 (usually 8) during the coal solubilization process (*4–7,18*). These pH increases are observed with *Streptomyces*, bacteria, and fungi and supports the hypothesis of microbial solubilization of coal being mediated by alkali. Microbially produced ammonia appears to be one source of alkalinity, but other sources are likely to be present. Strandberg and Lewis (*19*) have observed large molecular weight (i.e., 1,000–10,000) molecules of possibly polyamines or basic polypeptides. These observations are consistent with those presented here, which indicate that the source of alkaline materials is nitrogen based.

From this report and others (*5–7,18*) it would appear that microbial production of alkaline materials is a mechanism involved in coal biosolubilization. Although we have not yet been able to show that direct enzyme attack is involved during solubilization, it may be that microbes, using enzymes, may be able to alter the molecular structure of soluble coal. This will be the subject of a subsequent manuscript.

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