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Thermophilic Microorganisms for Coal Biosolubilization

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ABSTRACT

Biological processing of coal at mild operating conditions could provide significant economic and environmental advantages over thermal/chemical coal conversion processes. Thermophilic microorganisms with the ability to biosolubilize Leonardite were isolated from geothermal waters. Biosolubilization correlated with final culture pH. The mechanism of solubilization was a microbially-produced alkaline compound. Metal ion sequestering agents (chelators) contributed to biosolubilization.

Index Entries: Biological coal liquefaction; coal biosolubilization; coal conversion; thermophiles.

INTRODUCTION

Coal liquefaction technology in the US has developed to the stage where successful, large-scale pilot plants are in operation. However, economic and environmental concerns have curtailed plans for commercial ventures. Natural populations of bacteria and fungi, isolated from decaying wood, forest soil, pond sediments, coal piles, and weathered coal outcrops, have been described that utilize coal as a carbon source or produce water soluble degradation products from coal (*1–4*). The degree of biosolubilization is dependent on the coal type, the oxidation state of the coal, and the microorganism (*3*).

In virtually all extreme environments, it has been found that under more demanding conditions, some unusual microorganisms have evolved (*5*). Organisms from hot, sulfurous, acidic waters have been used for

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pyrite sulfur removal from coal. The Yellowstone ecosystem, with nearly 10,000 geothermal features, has the most varied environmental habitats in a single location (*6*). Extremes are common. Water temperatures range from cold mountain streams to boiling geysers. Strongly acidic (pH below 1) and strongly basic (pH above 10) waters can be found in this region. High concentrations of certain ions present unique environments for evolutionary adaptations. A number of hot springs contain downed trees, branches, twigs, or straw that have fallen into them. Thermophilic microorganisms cover the biomass and some degrade the cellulose and lignin. At Calcite Springs (Yellowstone National Park, WY), oil emerges from the ground with the geothermal waters (*7*). Filamentous bacteria are growing in the outflow channel from the springs. Many springs have high levels of metals that would be toxic to most microorganisms, but organisms evolving here are able to tolerate these conditions.

Water samples were collected from these extreme environments, and thermophilic microorganisms were isolated and screened for their ability to biosolubilize coal. These microorganisms are able to function over a wide pH range, act rapidly owing to higher operating temperatures, and survive high levels of toxic metals commonly associated with coal. This report presents coal biosolubilization results, as well as data on the mechanism of solubilization.

METHODS

Sampling and Screening

Leonardite, a naturally weathered lignite from North Dakota, was used for all screening tests. Leonardite was provided by Gary Winbourn, Knife River Coal Mining Company (Gascoyne mine), Bismark, ND. For all tests, coal was ground to 12 mesh, autoclaved at 121°C for 30 min, and then dried overnight at 70°C in air. A culture of *Trametes* (*Polyporus*) *versicolor* (ATCC 12676) was supplied by David Quigley of the Idaho National Engineering Lab (INEL, Idaho Falls, ID). Donald Crawford, University of Idaho (Moscow, ID), provided a culture of *Streptomyces viridosporus* strain T7A. These two microorganisms have proven to biosolubilize coal and so were used as standards in screening tests (*1,8*).

Samples were collected from thermal waters of the Yellowstone ecosystem. All samples collected were raised on three low nutrient media: AX adapted from Castenholz (*9*), DYM, a dilution of yeast-malt extract (*10*), and C, a dilution of yeast extract (0.45% w/v)—mineral salts medium used by Crawford in his coal solubilization work (*8*). Gelrite (Scott Labs, Carson, CA), a gellan gum that gives a clear solid gel at temperatures up to 110°C, was used to replace agar. Calcium chloride was added to stabilize the gum. For cultures that required sulfur, 1.2 g ammonium sulfate was added per liter of media. Isolates were grown aerobically at a pH and in-

cubation temperature approximating their natural habitat. Media pHs were 2, 4, 6, 8, or 10. Incubator temperatures were 20, 50, 60, and 70°C. After significant growth was noted (6–12 d), 0.1 g Leonardite was spread on the surface. A positive screen (ranked +1 to +5, with +5 being maximum biosolubility) was indicated by discoloration of media during the 16-d culture period. Controls were run on all three media at the five pHs and four temperatures.

Biosolubilization Tests

Isolates were raised on solid media for 6 d. These cultures were used to inoculate 80 mL liquid cultures in 125 mL culture flasks. Medium C without Gelrite was used. Culture pH was adjusted to 2, 4, 6, 8, or 10 with dilute sulfuric acid or sodium hydroxide, and they were incubated at 20, 50, 60, or 70°C for 6 d without agitation. Then, 1.5 g sterilized Leonardite was added. Culture media controls were run with coal and without microorganisms. Cultures of *Streptomyces viridosporus* strain T7A and *Trametes versicolor* were incubated at 20 and 37°C and served as standards for later comparisons. Cultures and controls were sampled on d 0, 0.5, 1, 2, 4, 6, 8, 10, and 12. All samples were centrifuged for 5 min at 12,500*g* and diluted, as necessary. The absorbance at 400 nm (A_{400}) was measured spectrophotometrically against distilled water. Sample pH was recorded before dilution.

Mechanism Studies

Mechanism studies were designed to determine if enzymes were involved in coal biosolubilization. The approach was to inactivate any enzymes in the culture liquid and monitor the effect on coal biosolubilization. Isolates were grown for 6 d and filtered to remove microbial cells. Cultures were first filtered through a coarse glass fiber filter (Gelman, Ann Arbor, MI, No. 66078), then aseptically through a 0.45 micron filter (Gelman, Ann Arbor, MI, Supor-450). Filtrate samples were autoclaved in sealed containers for 5, 20, or 60 min or treated with 10 mg proteinase K (Sigma, St. Louis, MO, No. P8044, Type X1-S) for 5 d at 37°C. Media controls were treated the same as culture liquids. Sodium azide (.02%) was added to controls and treated media to inhibit microbial contamination. Leonardite (1.5 g) was added to each test culture. The A_{400} was read at 0, 1, 2, 4, 8, and 12 d.

A second set of mechanism studies was designed to determine if microorganisms were acting as metal ion sequestering agents (chelators) to enhance coal biosolubility. A chelating agent, ethylenediaminetetraacetic acid (disodium-EDTA), was autoclaved in water and added (1, 2, 5, 10, 25, 40, and 80 m*M* final concn.) to cultures and controls on d 0. Sterile ferrous sulfate or ferric citrate (0.1, 0.5, 2, 5, 10, and 15 m*M* final concn.) were added to a different set of cultures and controls on day 0. Biosolubility was measured at A_{400}.

Table 1
Leonardite Biosolubilization Screening Results[a]

Incubation temperature, °C[c]	Biosolubility[b]					
	+1	+2	+3	+4	+5	Total
20	3	1	3	0	0	7
50	7	7	7	7	14	42
60	3	3	1	13	2	22
70	6	0	2	1	0	9
Total	19	11	13	21	16	80
Medium pH[d]						
2	0	0	0	0	0	0
4	2	1	1	0	0	4
6	9	6	3	17	12	47
8	6	4	9	3	1	23
10	2	0	0	1	1	6
Total	19	11	13	21	16	80

[a]The values in the table are the number of isolates that gave positive Leonardite biosolubilization screens at the temperatures and pHs used to culture the microorganisms.
[b]Biosolubility [ranked +1 (minimum) to +5 (maximum)] was indicated by discoloration of the medium during the 16-d culture period.
[c]Temperature is incubation temperature closest to the natural habitat.
[d]The pH is the medium pH closest to the natural habitat.

RESULTS

Sampling and Screening

The Yellowstone geothermal region was sampled for thermophilic microorganisms exposed to lignin and hydrocarbons. Sixty-three samples, ranging from 23 to 80°C and pH 0.9–9.4, were collected. From these 63 samples, 99 isolates were obtained. These 99 isolates, plus additional isolates from the J. K. Research's culture collection (21 isolates with high peroxidase and 38 with high amine oxidase), were screened for coal biosolubilization activity.

Results of screening tests, showing the number of positive isolates at each ranking for the temperatures and pHs tested, are presented in Table 1. All controls were zero except those for medium C at pHs 6, 8, and 10. Medium C control values were subtracted from the screening test values. The 80 organisms that tested positive in screens were aerobes. The temperature distribution of the results in Table 1 reflects the fact that most samples were collected between 40 and 65°C. No positives were recorded at pH 2 despite the large number of isolates screened at this pH. The pHs ranged

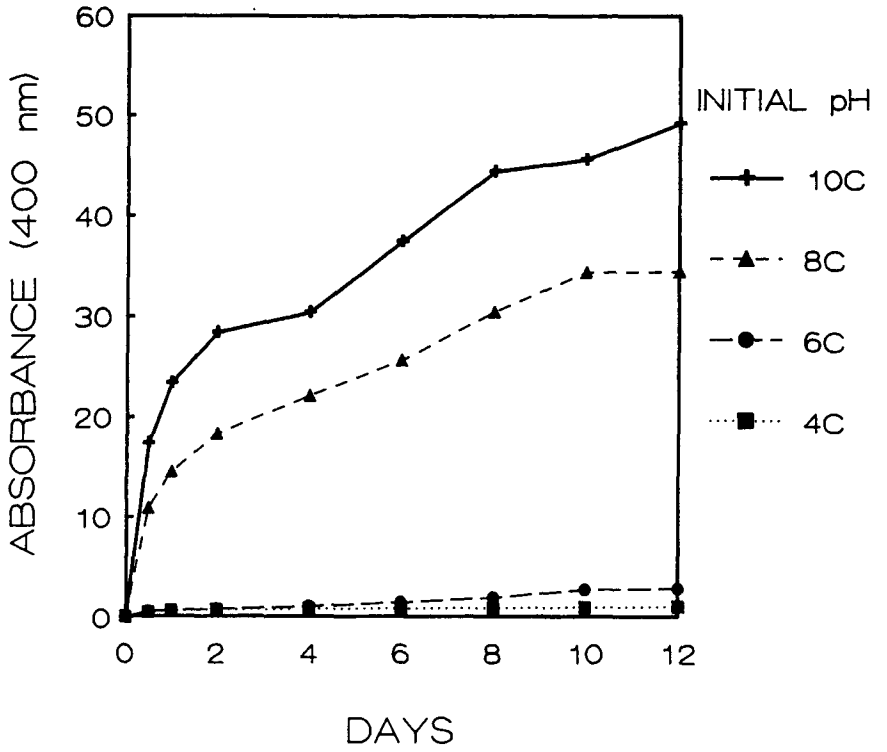

Fig. 1. Leonardite solubility (absorbance at 400 nm) in C medium controls (no microorganisms) adjusted to pHs 4, 6, 8, or 10. Controls were incubated at 50°C, and samples were removed, at indicated times, over a 12-d period to determine amounts of coal solubilized.

from 4 to 10 for positive screens. Biosolubilization tests in liquid culture gave a clearer distinction between isolates.

Biosolubilization Tests

The 80 cultures that were positive for coal biosolubilization in initial screening were tested in liquid culture. Faison demonstrated that liquid cultures gave biosolubilization results equivalent to solid-state cultures (*11*). Solubilization of Leonardite was affected by culture conditions. Figure 1 is a graph of Leonardite solubilization for controls (no microorganisms) at varying initial medium pH and 50°C over a 12-d period. Solubilization (A_{400}) directly correlated with initial pH. There was little solubility at pH 4 or 6, substantial solubility at pH 8, and even more at pH 10. Controls were run with all biosolubilization tests. The effect of temperature on Leonardite solubility, with C medium controls at an initial pH of 8, is shown in Fig. 2. As the incubation temperature was increased from 20 to 70°C, solubility increased. This trend follows classical chemical solubility by increasing solubility with an increase in temperature. The con-

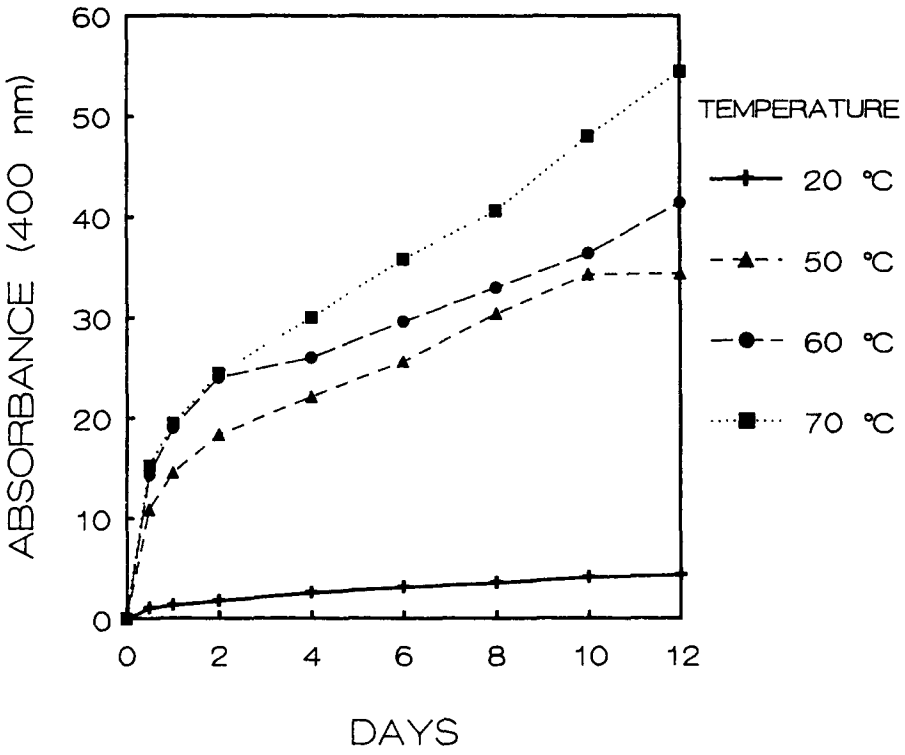

Fig. 2. Leonardite solubility in C medium controls (no microorganisms), pH 8, with varying temperatures over a 12-d period.

trols started with the same initial pH of 8; however, final culture pH decreased with an increase in biosolubility and temperature (i.e., after 12 d at 50°C, biosolubility was 34.4 and final pH was 6.06, whereas at 70°C, biosolubility was 54.4 and final pH was 5.66). This illustrates that, as the coal is solubilized, media pH decreases.

Leonardite biosolubility results are graphed over the 12-d culture period for the most active cultures at 20, 50, 60, and 70°C (Fig. 3). Control solubility results were subtracted from biosolubility values. At 20°C, *Streptomyces viridosporus* strain T7A produced biosolubility results higher than any isolates. *Trametes versicolor,* the other microorganism used as a standard for biosolubilization tests, gave results less than T7A at 20 and 37°C (data not shown). Thermophile 90B, grown at pH 8 and 50°C, showed the greatest biosolubilization activity. Uncorrected biosolubilization values at 60 and 70°C were similar to those at 50°C, but control values were higher at higher temperatures (*see* Fig. 2).

Biosolubilization results and culture pH for isolate 90B (control values subtracted) and medium C controls at pH 8 are graphed in Figs. 4A and B. For culture 90B, the pH dropped from an initial value of 8 to 6.47 at d 1, increased to pH 6.61, then gradually decreased to pH 6.47 over the next

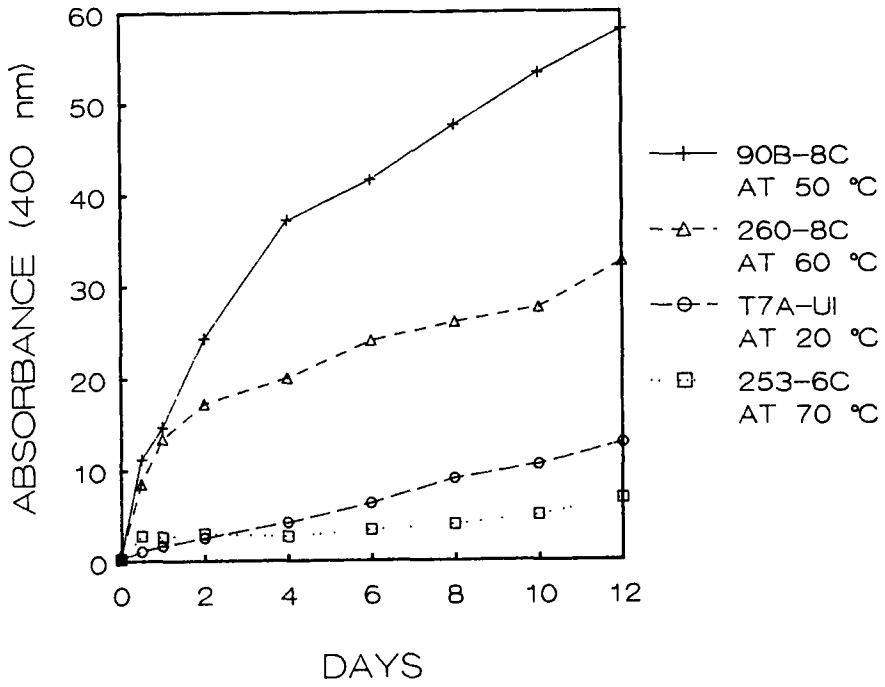

Fig. 3. Leonardite biosolubility for the most active isolates at incubation temperatures of 20, 50, 60, and 70°C. Isolates were grown for 12 d in C medium at pH 6 (–6C) or pH 8 (–8C). Control values for C medium were subtracted from biosolubility values.

10 d. The pH values for the control dropped from an initial value of 8 to 6.48 after d 1, then gradually decreased to 6.06 at the end of the 12 d. Not all isolates on C medium at pH 8 were able to biosolubilize Leonardite. Cultures with high biosolubilization activity gave a pH profile similar to the one shown for 90B in Fig. 4A. Cultures with minimal or no biosolubilization activity gave a pH curve similar to the control in Fig. 4B. The best cultures for biosolubilization are probably producing an alkaline compound(s), as shown by higher pH.

Microbiology

Four aerobic isolates showed the most coal biosolubilizing activity, 24B, 90B, 260, and 268C. Isolate 24B was collected from the runoff channel of Surging Spring (Yellowstone National Park, WY). Microbial life in the runoff channel is subjected to temperature fluctuations of 37–54°C on a 3-to 4-min cycle. Phototrophs were present in the microbial mat found here although this isolate was not phototrophic. The water pH was 8.5. On isolation media, 24B formed cream-colored colonies. The gram positive rods were obligate heterotrophs.

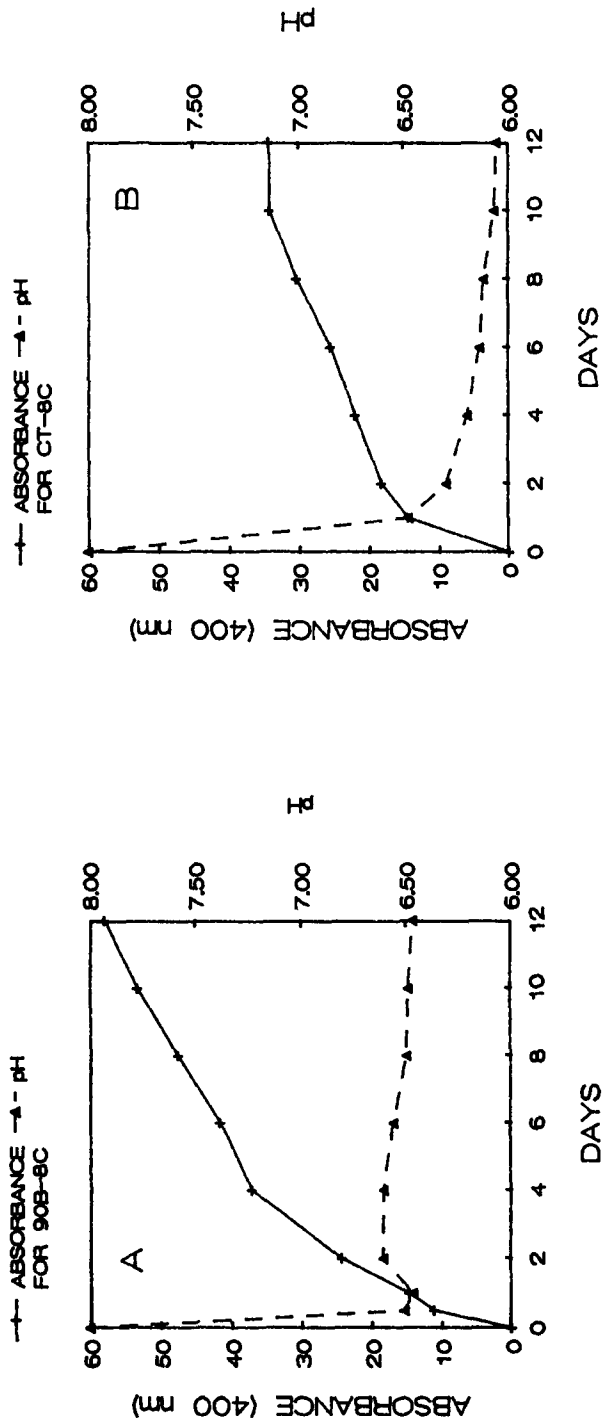

Fig. 4. (A) Leonardite biosolubility and pH values for isolate 90B in C medium at pH 8 (90B–8C) and 50°C. Abiotic control values for C medium were subtracted from biosolubility values. (B) Leonardite solubility and pH values for C medium control at pH 8 (CT–8C) and 50°C.

Isolate 90B was collected from pH 8.8, 44°C water in the Witch Creek (Yellowstone National Park, WY) area. The organism was part of a laminated mat community dominated by phototrophs producing substantial amounts of gases (presumably including oxygen) that were trapped within the mat. Isolate 90B was not phototrophic. On solid medium, 90B formed cream-colored colonies, with red colonies being observed under certain cultural conditions. The long, thin, gram-positive rods grew in the presence of small amounts of organic carbon, but growth was completely inhibited in the presence of moderate amounts of organic carbon in gelled media, indicating it is obligately oligotrophic.

One organism with coal solubilizing activity (260) was isolated on an autotrophic medium although it was later shown to also grow on heterotrophic medium. This bacterium came from an unnamed hot spring in the Upper Geyser Basin (Yellowstone National Park, WY). The water temperature was 65°C and the pH 8.8. There was a substantial amount of yellow, green, and orange filamentous growth in this water. Here, microorganisms often have high levels of carotenoid pigments for protection against the intense UV radiation occurring at this altitude. On solid media, 260 formed brown to tan colonies. The long, thin, gram-positive rods often appeared in pairs.

The most active coal biosolubilizing microorganism from Calcite Springs (Yellowstone National Park, WY) came from a pool with an oily scum on the surface. The water was at 54°C, pH 6.3. Like many organisms isolated from Calcite Springs, 268C had an absolute requirement for sulfur. These short, gram-positive rods formed white, glistening colonies on solid medium.

Mechanism Studies

Proposed mechanisms of coal biosolubilization include enzymatic, alkali solubility, chelating agents, and combinations of the three (*12*). Proponents of base solubilization contend that biosolubilization correlates with culture pH (*13*). Leonardite biosolubilization (A_{400}) for cultures and controls were graphed vs final pH at the four incubation temperatures of 20, 50, 60, and 70°C. Controls were not subtracted from biosolubility values to determine if culture pH from medium (control values) or microbially-produced alkaline compounds correlated with Leonardite biosolubilization. Figure 5 shows biosolubility results at 50°C for isolates and controls in C medium at pH 8. The line on the graph represents a least-squares regression analysis. A direct relationship was shown between biosolubilization (A_{400}) and final culture or control pH. Similar results were obtained at 20, 60, and 70°C. These results are consistent with the data presented by Quigley, suggesting a similar mechanism for coal biosolubilization that he proposed of microbially-produced alkaline materials (*14*). Three triangles on Fig. 5 represent screening results for 24B, 90B, and 268C. Biosolubility values of the three isolates were much higher than

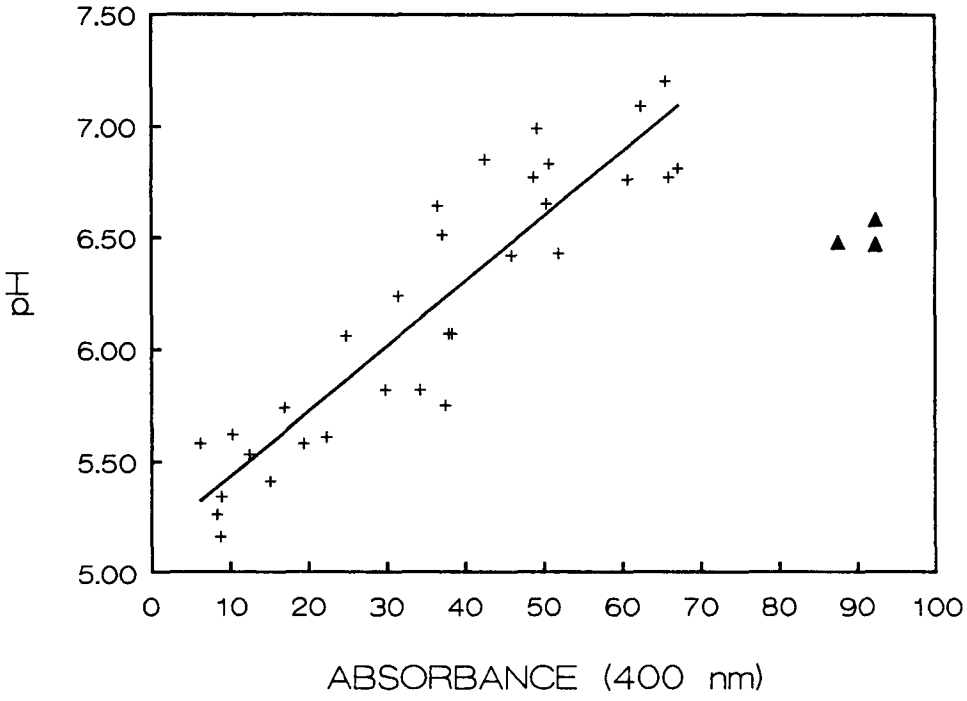

Fig. 5. Relationship between Leonardite solubility and biosolubility vs pH, 12-d cultures in C medium at pH 8 and 50°C. The line represents a least-squares regression analysis. Controls were not subtracted from biosolubility values. Triangles represent values for 24B, 90B, and 268C.

predicted from culture pH. Two of the isolates, 24B and 90B, were selected for mechanism studies.

The first mechanism studies were designed to determine if enzymes were involved in the observed enhanced biosolubility results for 24B and 90B. Cell-free culture broths were subjected to treatments with proteinase K or autoclaving to destroy enzyme activity. None of the treatments tested resulted in a loss of biosolubilization activity (data not shown).

The second mechanism study was designed to look for a metal ion sequestering agent (chelator). Researchers at Battelle (PNL) and INEL have indicated that chelating agents are active in some cultures that biosolubilize coal (*15,16*). If the removal of metal ions is required for biosolubilization, the addition of metal ions in excess could saturate microbially-produced chelating agents. EDTA, a chelating agent, or an iron compound was added in varying amounts to cultures of 24B, 90B, and C medium controls at pH 8 and 50°C. At the levels tested, EDTA had no additional effect on biosolubilization results. Ferric citrate and ferrous sulfate should inhibit biosolubilization by reacting with microbially-produced chelating agents. Ferric citrate at concentrations greater than 2 m*M* decreased biosolubilization. Figure 6 shows 12 d culture results after adding ferrous sulfate to isolate 24B. Control values were not subtracted. At 0.1 m*M* ferrous sulfate,

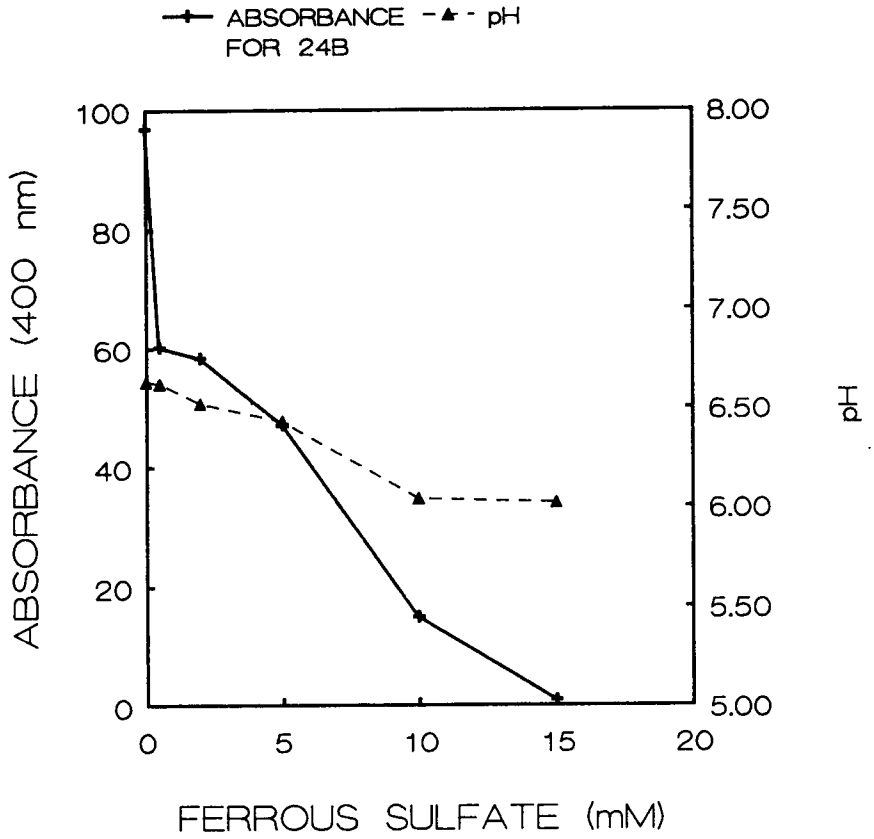

Fig. 6. Leonardite biosolubility and pH values for isolate 24B, with the addition of varying concentrations of ferrous sulfate. Cultures were grown for 12 d in C medium at pH 8 and 50°C. Controls were not subtracted from biosolubility values.

38% of the activity was lost. At higher concentrations, the Leonardite biosolubility dropped to near zero. Final pH values are presented on the same graph. The initial loss of activity did not correspond with a pH decrease. At higher concentrations of ferrous sulfate, some of the activity loss may be attributed to a drop in pH.

DISCUSSION

Thermophilic microorganisms capable of biosolubilizing Leonardite were isolated from the Yellowstone ecosystem. Coal biosolubilization was achieved for cultures grown from pHs 4 to 10 and temperatures from 20 to 70°C, expanding pH and temperature ranges of coal biosolubility reported in the literature. Isolates with the highest biosolubilization activity came from habitats between pHs 6 and 9 and temperatures between 45

and 65°C. Cultures above pH 9 were difficult to grow on the rich media needed for biosolubilization. Below pH 6, isolates showed minimal or no biosolubilization activity. Few samples were collected below 45°C since the objective was to look for thermophiles. Many isolates collected above 65°C were anaerobes, a reflection of low oxygen solubility in water at these temperatures.

The best isolates produce a compound that solubilized coal. Viable thermophiles were recovered from liquid cultures 12 d after coal had been added. A medium that buffered the culture as coal went into solution was also important. Cultures and controls showed a direct correlation between coal biosolubility and pH. The mechanism of biosolubilization seemed to be alkaline or base mediated. Biosolubilization by isolates 24B and 90B were not enzymatic. Metal ion sequestering agents (chelators) contributed to biosolubilization. Additional work is required to determine the role played by elevated temperatures in coal biosolubilization.

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