APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2840–2843
0099-2240/87/122840-04$02.00/0

Vol. 53, No. 12

Cell-Free Solubilization of Coal by *Polyporus versicolor*

MARTIN S. COHEN,[1]* WILLIAM C. BOWERS,[1] HAROLD ARONSON,[1] AND EDWARD T. GRAY, JR.[2]

Departments of Biology[1] and Chemistry,[2] University of Hartford, West Hartford, Connecticut 06117

Received 9 September 1987/Accepted 15 September 1987

Solubilization of coal was demonstrated with filtrates (0.45-μm-pore-size filters) obtained from the broth in which *Polyporus versicolor* had grown. The rate and extent of solubilization were dependent on the age of the fungal cultures, the particle size of the coal, the pH of the filtrates, and the presence of proteins in the filtrates. The rate of solubilization of coal was significantly reduced after proteins in the filtrates were denatured by acid hydrolysis.

Biological solubilization of coal has become a subject of rapidly increasing interest because it occurs at virtually ambient temperatures and pressures and changes solid coal particles to a liquid with very little loss in total energy content (M. S. Cohen, H. Aronson, and E. T. Gray, Proceedings: Eleventh Annual EPRI Contractors' Conference on Clean Liquid and Solid Fuels, 1986, p. 2-63–2-79). When this process is compared with established methods of coal liquefaction, pyrolysis, and gasification, the potential energy savings that would result from biological processing of coal become evident.

Rene Fakoussa (Ph.D. thesis, Friedrich-Wilhelms University, Bonn, Federal Republic of Germany, 1981) first demonstrated that fungal and bacterial species could metabolize coals, but no products for analyses were recovered. Direct formation and recovery of a water-soluble liquid product produced from coal by microbial action were first reported by Cohen and Gabriele (1). Since that time, there have been a number of reports repeating and extending these results to several fungi (4, 6) and bacteria (5). In a recent report, the products of the solubilization of leonardite by *Polyporus versicolor* (*Coriolus versicolor*) were characterized (7).

Wilson et al. (B. W. Wilson, J. A. Pyne, R. M. Bean, J. A. Fredrickson, D. L. Stewart, E. Sass, M. Burnside, and M. S. Cohen, Proceedings: Eleventh Annual EPRI Contractors' Conference on Clean Liquid and Solid Fuels, 1986, p. 2-45–2-62) first demonstrated that leonardite coal could be solubilized by partially purified cellular enzyme preparations. We are now able to extend this observation and demonstrate solubilization of leonardite coal by neat cell filtrates derived from cultures of *Polyporus versicolor* grown in nutrient broth solutions.

MATERIALS AND METHODS

Maintenance of fungal cultures. *P. versicolor* was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 12679). Stock cultures were routinely maintained in 5% Sabouraud maltose broth (SMB) and handled as previously described (1).

The experimental cultures were inoculated with 2 ml of the hyphal suspension and grown in stationary 250-ml flasks containing 50 ml of SMB. We observed that shaking the fungal cultures resulted in an increase in mycelial growth (as compared with that in similar unshaken cultures) coupled with a dramatic decrease in the ability of the broth to solubilize coal. The inoculum consisted of bead-disrupted hyphae from 14-day-old stock cultures grown in 25 ml of 5% SMB. Two weeks after inoculation, sterile pieces of leonardite coal (2) (approximately 0.5 to 2.5 cm) were added to the surfaces of the mycelial mats. The mycelial mats supported the coal above the SMB. The day on which coal was added to the mycelial mats was designated day zero. Ten flask cultures were grown in a similar manner but without the addition of coal. The media in which the fungi were grown was harvested before (days −2 and −1), on, and after the day of coal addition. The cell filtrates were collected by vacuum filtration (0.45-μm-pore-size filters; Millipore Corp., Bedford, Mass.) and used immediately or frozen at −20°C until use. Solubilization of coal was observed on the surfaces of the mycelia at about the same time that it was observed in the broth filtrates. The fungal mycelia were discarded.

Coal preparation. Five groups of different-sized leonardite coal (American Colloid Co., Reeder, N.D.) were separated by sieving for use in this study. Group 1 consisted of the largest pieces, approximately 0.5 to 2.5 cm. One or two pieces of this size group were added to the continuous mycelial mats on day zero. Group 2 was made up of all particles which passed through a 500-μm-pore sieve. Additional sieves were used to obtain particles of <250 to >149 μm (group 3), <149 to >105 μm (group 4), and <105 μm (group 5). The remaining particles (<500 to >250 μm) were discarded. After sieving, all coals were rinsed to remove water-soluble components on the surfaces and dried before use.

Spectrophotometry and leonardite solubilization. The solutions in which solubilization of leonardite occurred consisted of 1.5 ml of sodium dihydrogen phosphate monohydrate (pH 5.5, 0.1 M) and 1.5 ml of cell filtrate from cultures of *P. versicolor* grown in SMB. The solubilization reaction was initiated by the addition of 3 ml of the buffer-cell filtrate mixture to 30 mg of coal. The flask contents were mixed with a Pasteur pipette and transferred into Ultra-Vu disposable cuvettes (1-cm path length; VWR Scientific, Boston, Mass.). Each cuvette was loaded into a clinical desk-top centrifuge and spun at 2,000 × *g* for 5 min. After centrifugation, absorbance measurements were made with a model DMS90 spectrophotometer (Varian, Palo Alto, Calif.). Solubilization rates of culture filtrates were determined by monitoring the increase in the A_{450} over time. This wavelength proved to be sensitive to the appearance of solubilized products while minimizing the background interference from the cell filtrates. Absorbance measurements were recorded, and the contents of each cuvette were mixed again with a Pasteur pipette. The process of centrifugation, absorbance measure-

* Corresponding author.

ment, and mixing was repeated at selected time intervals during the experiments. Protein quantification was accomplished by using the biuret reaction (3).

Effect of pH on the solubilization of leonardite. The pH values of phosphate buffer in eight tubes, each containing 3 ml of buffer, were adjusted with 1.0 M HCl or 1.0 M NaOH as appropriate to obtain a range of pH values from pH 4.0 to pH 7.5 in increments of pH 0.5. To each of these tubes, 30 mg of leonardite coal (<250 to >149 μm) was added, and the A_{450} was recorded hourly over a 4-h period.

Acid hydrolysis. Filtrates from days 5, 6, and 7 were pooled, and 15.0 ml was added to a Pyrex tube with 4.0 ml of 12 M HCl. The solution was frozen in a dry ice-acetone slurry, and the tube was sealed under vacuum. The resulting sealed ampoule was thawed at room temperature and placed in an oven at 110°C for 47 h.

The contents of the tube (the hydrolysate) were freeze-dried by using a cold-finger condenser (Bellco Glass, Inc., Vineland, N.J.). The hydrolysate was removed from the cold-finger condenser after 6 h and placed in a vacuum desiccator containing 50 ml of concentrated H_2SO_4. On the following day, the hydrolysate was removed, reconstituted with deionized water to a volume of 15 ml, and adjusted to pH 5.6.

Standard addition of laccase. Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) from *Pyricularia oryzae* was obtained from Sigma Chemical Co., St. Louis, Mo. (activity, 40 to 120 U/mg). A standard laccase solution was prepared at a concentration of 1.0 mg/ml. One milliliter of pooled filtrates from day 8 of the culture period was added to each of six tubes. To each tube, 0, 0.2, 0.4, 0.6, 0.8, or 1.0 ml of the standard laccase solution was added, and the volume of each tube was adjusted to 3 ml by the addition of the 0.1 M phosphate solution described above. In addition to the appropriate blanks, two tubes, each containing freshly prepared broth in place of the filtrates, were prepared. These tubes contained either no laccase or 1.0 ml of laccase, with the final volume adjusted to 3 ml with the phosphate solution (pH 5.5). The coal particles added to each of these tubes were <250 to >149 μm. The assays were run in triplicate and repeated once.

RESULTS

Leonardite coal was solubilized by neat cell filtrates from cultures of *P. versicolor* grown in liquid. Relative amounts of coal solubilization by different cell filtrates were measured and quantified by spectrophotometry.

Effect of pH on the rate of solubilization of coal without the addition of cell filtrate. Thirty milligrams of coal (<500 μm) was added to 3 ml of the phosphate solution initially adjusted to pH values from 4.0 to 7.5. Table 1 shows the rates of the change in absorbance of these solutions over a period of 4 h. The rate of background solubilization of leonardite was low below pH 6.0. At pH 6 the rate dramatically increased, and beyond pH 7 the solution was opaque before it could be placed in the spectrophotometer. Fungal growth was rapid at pH 5.5, and it is likely that enzymes secreted by the fungus remained active in the cell filtrates. Thus, since the background solubilization was low at pH 5.5, further experimentation was conducted at this pH.

TABLE 1. Initial rates of solubilization of leonardite coal as a function of pH

pH	Initial rate (A_{450}/h at 21°C) of solubilization
4.0	0.015 ± 0.002
4.5	0.026 ± 0.004
5.0	0.027 ± 0.007
5.5	0.039 ± 0.008
6.0	0.098 ± 0.005
6.5	0.9 ± 0.1
>7	Too fast to measure

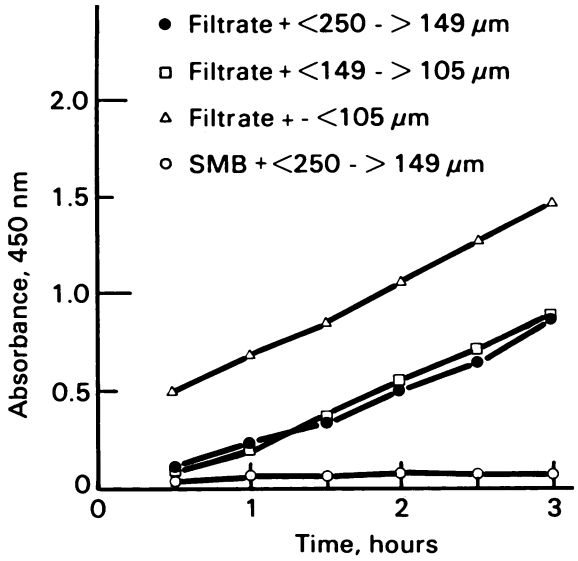

FIG. 1. Effect of particle size on the rate of solubilization of leonardite by filtrates from SMB cultures of *P. versicolor* and by freshly prepared SMB.

Effect of coal particle size on solubilization rates. Figure 1 shows the rates of solubilization of three defined coal particle size groups, 3, 4, and 5. The study was done twice with filtrates from day-8 cultures, with freshly prepared SMB samples serving as a control group. It is evident that the two groups containing particles of <250 to >149 μm (group 3) and <149 to >105 μm (group 4) had a lower extent of solubilization than did the group containing particles of <105 μm (group 5). The rates of solubilization of coal from groups 3 and 4 were approximately the same as and slightly lower than the rate of solubilization of coal from group 5. The amount of within-group variability was larger with coal particles of <105 μm than with those of <250 to >149 μm or <149 to >105 μm, which were similar.

The mixtures containing only SMB had background solubilization rates which were similar for all three particle size groups. Coal from group 4 was used for all experiments unless indicated otherwise.

Rates of solubilization by cell filtrates. Figure 2 shows the rates of solubilization of leonardite by cell filtrates of *P. versicolor* cultures harvested over a 12-day period following the addition of leonardite to the cultures. Solubilization rates for days −2 to 5 were similar to each other and to solubilization rates of the controls. The rates of solubilization of leonardite were highest on days 6 and 9. The rates of solubilization of leonardite by cell filtrates derived from cultures grown without coal addition were similar to those shown in Fig. 2.

Protein quantification. The results presented in Fig. 3 show the protein content of culture filtrates from days 2 to 8 of the experiments described above and shown in Fig. 2. On day 1,

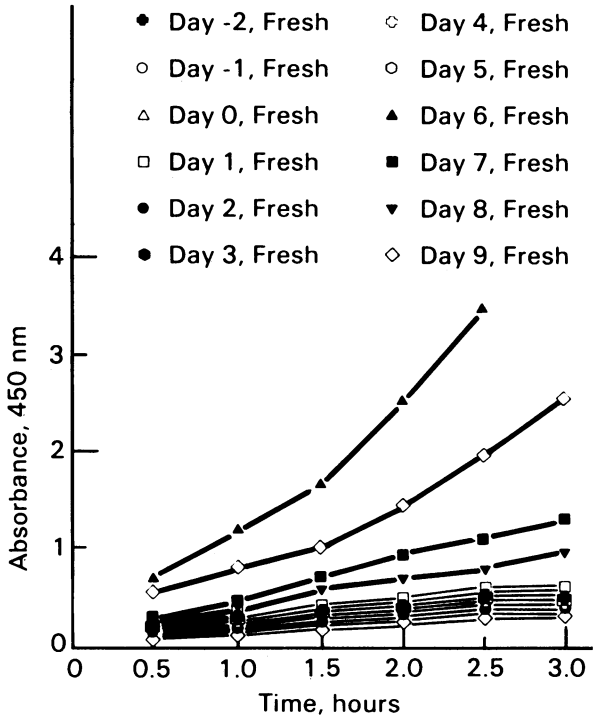

FIG. 2. Rates of leonardite solubilization by cell filtrates from SMB cultures of *P. versicolor* over a 12-day period.

the protein content of the filtrates was below the limit of detection of the test. From days 2 to 8, the rate of increase in the protein content was essentially linear (slope, 0.109 ± 0.001; *y* intercept, 0.017 ± 0.002).

Effect of acid hydrolysis on coal solubilization. Figure 4 shows the effect of acid hydrolysis of a cell filtrate on the rate of solubilization of leonardite coal. The acid-hydrolyzed filtrate produced substantially lower rates of solubilization than did the nonhydrolyzed filtrate. The hydrolyzed filtrate was tested with coal particles of <105 μm, which have been shown to yield higher rates of solubilization than larger coal particles. The nonhydrolyzed filtrate was tested with coal

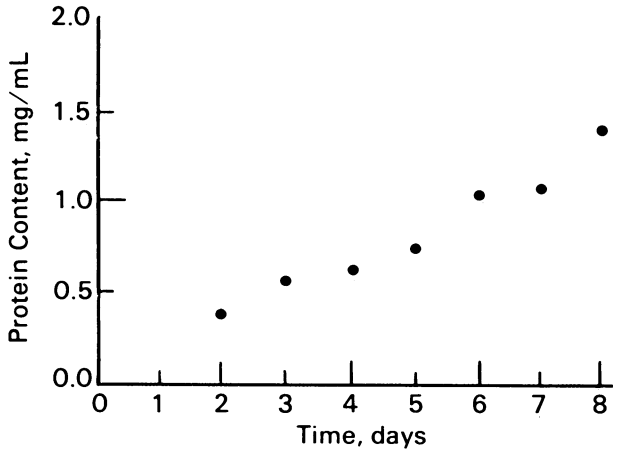

FIG. 3. Total protein content of cell filtrates from SMB cultures of *P. versicolor*.

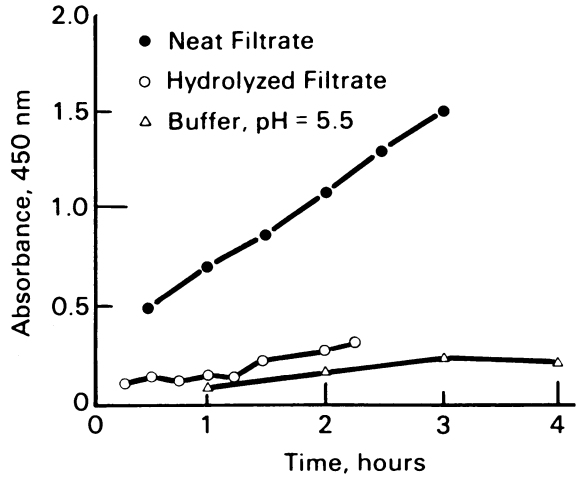

FIG. 4. Solubilization of leonardite by nonhydrolyzed (neat) and acid-hydrolyzed cell filtrates from SMB cultures of *P. versicolor* and by a pH 5.5 buffer.

particles of <149 to >105 μm. However, the rate of solubilization produced by the hydrolyzed filtrate was much lower than that produced by the nonhydrolyzed filtrate and similar to that produced by the buffer alone.

Standard addition of laccase. The results presented in Fig. 5 show a comparison of the extent and rates of coal solubilization by cell filtrates and by 5% SMB, each with or without standard additions of laccase. The additions of either 0.5 or 1.0 ml of laccase to the day-8 filtrate resulted in approximately the same rates and possibly a lower extent of solubilization than did the neat filtrate. The addition of laccase to freshly prepared SMB did not increase either the rate or the extent of solubilization of leonardite by the neat filtrate.

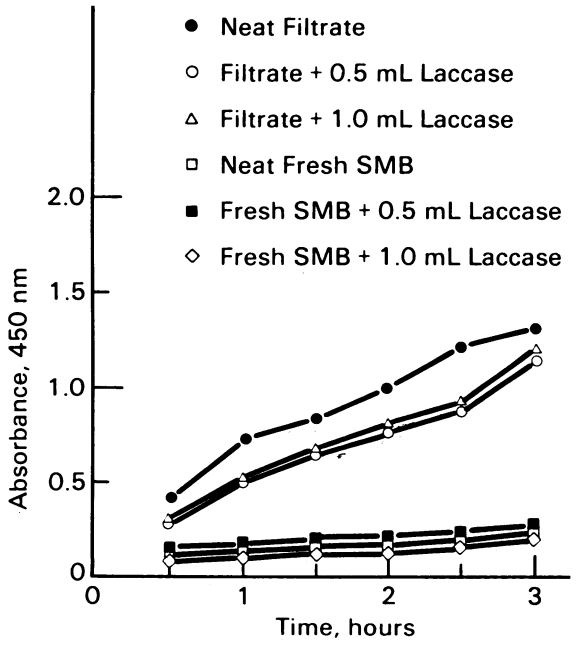

FIG. 5. Solubilization of leonardite by cell filtrates from SMB cultures of *P. versicolor* and by freshly prepared SMB, each with or without additions of laccase.

DISCUSSION

Solubilization of leonardite coal was demonstrated with neat cell filtrates of SMB cultures in which *P. versicolor* had grown. Coal particle size affected the rate of solubilization. Smaller particles (<105 μm) were degraded faster than larger particles during the first 0.5 h of exposure to the cell filtrates but at the same rate over the remainder of the 3-hour measurement period. This result was interpreted to mean that the smallest particles, those much smaller than 105 μm, were solubilized very rapidly, while those closer to 105 μm were solubilized more slowly. Therefore, coal particles in the smaller size group were degraded more extensively early in the experiment but at the same overall rate as those in the larger size group subsequent to that early spurt of solubilization.

The pH of the buffer solution also affected the rate and extent of nonenzymatic leonardite solubilization. Solubilization caused exclusively by chemical effects was slower at lower pH values and faster at higher pH values. Leonardite coals can be dissolved in solutions of 1 M sodium hydroxide (2), and we have shown that they are partially soluble in 1 M HCl. Solubilization of leonardite was very slow over the pH range from 4.0 to 5.5. The pH used in these experiments (pH 5.5) allowed a combination of rapid enzymatic solubilization and negligible chemical dissolution of the leonardite coal samples.

The relative rates of solubilization produced by filtrates from cultures of different ages varied. Solubilization activity was not present in broth on the day of inoculation with the fungus but developed near the end of a 12-day period of growth and differentiation. Cell filtrates from cultures grown without added coal produced leonardite solubilization at rates similar to those described above. This result strongly suggests that exposure to leonardite during the growth phase did not serve as an inducer of solubilization activity. Protein content did not increase rapidly during the time of the most rapid coal solubilization. Instead, protein content increased steadily during the 12-day period of fungal incubation. This result suggests that proteins necessary for growth and differentiation were synthesized early in the development of the fungal mycelium. Toward the end of the period of most rapid hyphal growth, the production of enzymes apparently shifted to those involved in coal solubilization.

The addition of exogenous laccase to cell filtrates did not result in an increase in the rate of leonardite solubilization. However, we have not established that laccase produced by *P. versicolor* has no role in the solubilization process. Hydrolysis of proteins in the broth reduced the rate of the solubilization process to background levels. This result strongly suggests that the solubilization of coal resulted from enzymatic action.

ACKNOWLEDGMENT

This research was supported by Electric Power Research Institute contract RP8003-6.

LITERATURE CITED

1. **Cohen, M. S., and P. D. Gabriele.** 1982. Degradation of coal by the fungi *Polyporus versicolor* and *Poria monticola*. Appl. Environ. Microbiol. **44**:23–27.
2. **Fowkes, W. W., and C. M. Frost.** 1960. Leonardite: a lignite byproduct. Report of investigations 5611. Bureau of Mines, U.S. Department of the Interior, Washington, D.C.
3. **Robinson, H. W., and C. G. Hogden.** 1940. The biuret reaction in the determination of serum proteins. II. Measurements made by a duboscq colorimeter compared with the values obtained by the Kjeldahl procedure. J. Biol. Chem. **135**:727–773.
4. **Scott, C. D., G. W. Strandberg, and S. N. Lewis.** 1986. Microbial solubilization of coal. Biotechnol. Prog. **2**:131–139.
5. **Strandberg, B. W., and S. N. Lewis.** 1987. Solubilization of coal by an extracellular product from *Streptomyces setonii* 75Vi2. J. Ind. Microbiol. **1**:371–376.
6. **Ward, B.** 1985. Lignite-degrading fungi isolated from a weathered outcrop. Syst. Appl. Microbiol. **6**:236–238.
7. **Wilson, B. W., R. M. Bean, J. A. Franz, B. L. Thomas, M. S. Cohen, H. Aronson, and E. T. Gray, Jr.** 1987. Microbial conversion of low rank coal: characterization of biodegraded product. Energy Fuels **1**:80–84.