# BIODEGRADATION OF COAL-RELATED MODEL COMPOUNDS

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## INTRODUCTION

Since 1982 when Cohen and Gabriele (1) first reported that fungi could grow directly on and metabolize naturally occuring coal, biological conversion of low-rank coals by bacteria, fungi, or preparations of the enzymes they produce has been the subject of intensive research. Because these processes occur at ambient temperatures and pressures, they represent a potential savings in the processing of certain coals and lignites. Current technology for coal conversion requires both high temperatures and pressures which may result in the production of components that are more toxic than the original starting material.

Cohen and Gabriele (1) reported that fungi could metabolize leonardite, a naturally oxidized form of lignite coal. Wilson et al. (2) have shown that the leonardite-biodegraded product from *C. versicolor*, a white-rot fungus, was water soluble and contained no detectable polycyclic aromatic hydrocarbons (PAH) having three to six rings. In addition, the bioconverted material was inactive in the microbial histidine reversion assay for mutagenic activity. Linehan et al. (3) determined by gel permeation chromatography that the average molecular weight of the biodegraded product was 1800 daltons.

The details of the specific reactions of lignin biodegradation, and the biochemistry involved, have been primarily based on the use of low molecular weight compounds representing specific substructures rather than the complex, polymeric lignin material. As an example, Gold et al. (4) utilized ß-aryl ether model compounds to examine oxidation by lignin peroxidase from *P. chrysosporium*. We have studied the reactions of model compounds having coal-related functionalities (ester linkages, ether linkages, PAH) with the intact organism, cell-free filtrate, and cell-free enzyme of *C. versicolor* to

better understand the process of biosolubilization. Many of the degradation products have been identified by gas chromatography/mass spectrometry (GC/MS).

## EXPERIMENTAL

Preparation of the Enzyme (5). The fungus was grown in a Chemap CF-20 fermentor at 25° C, and was supplied with 4 L of filtered air per minute at atmospheric pressure to 15 L of growth medium. The growth medium used was that published by Fahraeus and Reinhammar (6). A two-stage Rushton turbine (6 blade) agitator system was used at a speed of 400 rpm. The inoculum used for the fermentation was three 50 mL cultures of *C. versicolor* grown on Sabouraud-maltose broth (1) for 7 to 10 days at 25° C with no agitation. The fungi grown under these conditions form a mycelial mat. The mats were then transferred to a stoppered vessel containing 250 mL of distilled water and 50 mL of 3-mm glass beads. This vessel was shaken vigorously and the mycelial fragments were transferred to the fermentor. After three days growth in the fermentor, 0.3 mL of 2,5-xylidine (Aldrich Chemical Co., Milwaukee, Wi) was added to increase levels of extracellular laccase (5).

The extracellular fluid formed during the growth of *C. versicolor* was separated from the cell mass by filtration through several layers of cheesecloth. This fluid was termed the cell-free filtrate. The resulting fluid was then filtered through an ultrafiltration membrane with a molecular weight cutoff of 100,000 (Amicon H5P100-43). The higher molecular weight material in aqueous fluid was concentrated using an ultrafiltration membrane with a molecular weight cutoff of 10,000 (Amicon H5P10-43). The remaining fluid was further washed (diafiltered) with three 300-mL portions of water while being continually passed through the H5P10-43 membrane to maintain a constant volume of 1 L. The concentration and diafiltration steps caused precipitate formation which was removed by either filtration or centrifugation. The concentrated, extracellular fluid was then chromatographed on a 2 cm X 30 cm DEAE-cellulose (Amicon cellufine DEAE-AM) column which had been previously equilibrated to 0.01 M sodium phosphate, pH 7.0 buffer. This final fluid was termed the cell-free enzyme.

Model Compound Preparation. All of the model compounds were prepared in the same manner. Approximately 10 mg of each was dissolved in 10 mL of methanol. Suitable volumes of this stock

solution were added to the incubation system to make the desired concentration in water.

*C. versicolor* was cultured on Kirk's minimal medium (10 mL) for ten days in 125-mL Erlenmeyer flasks. One hundred microliters of benzylbenzoate and methoxybenzophenone were added to the culture. Controls consisted of (a) cultures with the same volume of methanol added as with the model compounds and (b) benzyl benzoate and methoxybenzophenone were added to the sterile minimal medium. Hyphal growth of fungal cultures was evident following addition of methanol indicating that the volumes of methanol added were not inhibitory. The model compounds and intact organism were incubated for 1 week. The cultures were then filtered through 0.2 μm filters and extracted with three equal volumes of chloroform. The extracts were combined, concentrated to 1 mL, and analyzed by gas chromatography/flame ionization detection (GC/FID) and GC/MS.

Dibenzothiophene, indole, and bibenzyl were incubated with *C. versicolor* for three weeks. The samples were extracted and concentrated in the same manner as discussed above. Controls, as discussed previously, were also obtained and analyzed.

Benzylbenzoate, bibenzyl, phenylbenzoate, methoxybenzopheneone, and benzyl ether were added (100 μL) to a mixture of 1 mL of cell-free filtrate and 1 mL of buffer solution (pH 5.2). After one day, the solutions were extracted with three equal volumes of chloroform. The extracts were combined and concentrated to 1 mL for subsequent GC/FID and GC/MS analysis. Controls were made with the model compounds and the buffer solution. The controls were extracted, concentrated, and analyzed in the same manner. Anthracene was incubated with the cell-free filtrate for three days. The solution was then extracted, concentrated, and analyzed by GC/FID and GC/MS.

Benzylbenzoate and methoxybenzophenone were introduced (100 μL) to the cell-free enzyme and a buffer solution at pH 5.2. After equilibration of the enzyme and buffer for one day, the solution was extracted, concentrated, and analyzed in the same manner as discussed previously.

Extraction efficiencies were determined by GC analysis of a solution of the model compound in the medium that did not contain

enzyme. The peak areas were then compared with the standard to determine the efficiency of extraction and possible reaction of the model compound with the medium.

Molecular Weight Separation of Cell-Free Filtrate. A culture filtrate was passed through ultrafiltration membranes with molecular weight cutoffs of 10,000, 5000, 1000 , and 500 using an Amicon ultrafiltration apparatus.

Analytical Instrumentation. A HP 5880 gas chromatograph with a flame ionization detector was used to determine the amount of degradation for each model compound . The GC was equipped with a DB-5 30m column and appropriate flow rates were utilized as recommended by the manufacturer. The temperature program used was as follows: 50° C for 1 minute, 50-250° C at 8°/min, and 250° C for 10 minutes. The temperature of the injection port was 250° C and the temperature of the detector was 275° C. A HP 5985 GC/MS/DS was used in the electron impact mode at 70 eV to identify specific degradation products. The same type of column and temperature program were used. The temperature of the source was 200° C, the interface temperature was 275° C, and the electron multiplier voltage was 2500 eV.

## RESULTS

Intact Organism. The gas chromatograms obtained from the degradation of benzylbenzoate and methoxybenzophenone by *C. versicolor* are illustrated in Figure 1. Figure 1a is a chromatogram of a 100 ppm standard of benzylbenzoate and 1b is a chromatogram after degradation by the intact organism. Figure 1c is a chromatogram of a 100 ppm standard of methoxybenzophenone and 1d is after degradation. These results clearly indicate that both compounds were degraded by the total organism. The data obtained from the degradation of model compounds by the intact organism is summarized in Table 1.

The extracts were analyzed by GC/MS. For benzylbenzoate, the primary degradation products included benzyl alcohol and benzoic acid.

Cell-Free Filtrate. The results for the degradation of benzylbenzoate, methoxybenzophenone, phenylbenzoate, and benzyl ether by the cell-free filtrate of *C. versicolor* are summarized in Table 1. Phenyl

benzoate was degraded the most; methoxybenzophenone was degraded only slightly. This may reflect the difference in reactivity of the two compounds was well as the selectivity of the enzymes.

Analysis of the degradation of anthracene by the cell-free filtrate indicated the presence of 9.10-anthracenedione (anthraquinone) as a degradation product in about 10% yield. Since anthracene can be oxidized to anthraquinone by a number of non-microbial mechanisms, it is important to note that the controls showed no indication of any quinones or any other degradation products.

Cell-Free Enzyme. For benzylbenzoate, the amount of degradation after one day was approximately 50% but methoxybenzophenone was degraded very little. It should be noted that 85-90% of the model compounds were recovered by extraction from the buffer solution, thereby eliminating reaction with the buffer as a possibility for model compound degradation. The results are summarized in Table 1 and are corrected for extraction efficiencies.

Molecular Weight Separation of Cell-Free Filtrate. The molecular weight separations indicated no reaction with the model compounds with the <10,000 MW fraction while the >10,000 MW fraction completely degraded the model compounds. It is interesting to note that the <10,000 MW fraction is primarily responsible for the biosolubilization of coal.

## DISCUSSION

The results obtained from degradation studies of oxygen-containing compounds by *C. versicolor* and its extracts, shown in Table 1, indicate that the two compounds tested with the intact fungal organism were completely degraded. Complete degradation refers to no recovery of model compound. We can probably assume that the other two would also be totally degraded, since we have not yet found a simple compound that will survive long-term exposure to the intact fungus. The ease of degradation with the cell-free filtrate appears to be in the order: phenylbenzoate > benzylbenzoate > benzyl ether > methoxybenzophenone. Esters and ethers that are activated by aromatic rings appear to be susceptible to the fungal extract; however, aromatic ketones are not affected by the extract. From the limited results we have obtained from the isolated enzyme, it appears that the activity may parallel the cell-free filtrate. When

the cell-free extract was tested with the model compounds indole, dibenzothiophene, and bibenzyl, no degradation with the enzyme was noted; however, exposure of these compounds to the intact organism resulted in complete degradation.

Our results with anthracene indicate that its degradation by *C. versicolor* may occur through a radical cation mechanism similar to that proposed for trichlorophenol by *P. chrysosporium* (7). Related research has shown that there is a relationship between the ionization potential of the polycyclic aromatic hydrocarbon and the amount of degradation observed (8). Horseradish peroxidase has been shown to degrade polycyclic aromatic hydrocarbons to quinones, but only in the presence of hydrogen peroxide (9). It is of interest to note that our cell-free filtrate tests positive for peroxidase, although no peroxide is added to any of our biodegradation experiments.

The results of the molecular weight separations of the cell-free filtrate by ultrafiltration indicate the possibility of at least two mechanisms taking place during the biosolubilization process, one of which may be hydrolysis. The >10,000 MW fraction has been very reactive with the model compounds we have tried. The <10,000 MW fraction, where most of the biosolubilization occurs, has been found to be fairly unreactive toward most of the model compounds. Some preliminary results indicate the p-methoxyphenol may be a good substrate for this fraction.

Other preliminary results of further molecular weight separations indicate that the fraction of cell-free filtrate responsible for the majority of the biosolubilization activity is between 500 and 1000 MW. We are presently trying to characterize this fraction to determine the source of biosolubilizing activity.

## REFERENCES

(1) Cohen, M. J.; Gabriele, P. D. Appl. Environ. Microbiol. (1982), 44, 23-27.

(2) Wilson, B. W.; Bean, R. M.; Franz, J. A.; Thomas, B. L.; Cohen, M. S.; Aronson, H.; Gray, E. T. Jr. Energy and Fuels, (1987), 1, 80-84.

(3) Linehan, J. C.; Campbell, J. A.; Franz, J. A.; Bean, R. M. Submitted to Energy and Fuels.

(4) Miki, K.; Renganathan, V.; Gold, M. H. Biochemistry, (1986), 25, 4790-4796.

(5) Pyne, J. W.; Stewart, D. L.; Fredrickson, J. K.; Wilson, B. W. Appl. Environ. Microbiol., (1987), 53, 2844-2848.

(6) Fahraeus, G.; Reinhammar, B.; Acta. Chem. Scand., (1967), 21, 2367-2378.

(7) Zurer, P. S. Chem. and Engineering News, (1987), 65, 17-23.

(8) Hammel, K. E.; Kalyanaraman, B.; Kirk, T. K. In: American Chemical Society, Division of Environmental Chemistry Preprints, (1987), 23, 624-626.

(9) Cavalieri, E.; Rogan, E. Environ. Health Perspect., (1985), 64, 69-75.

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Table 1. Percent Model Compound Degradation by Coriolus Versicolor and its Cell-Free Isolates

| Compound | Total Organism | Cell-Free Filtrate | Cell-Free Enzyme |
|---|---|---|---|
| benzylbenzoate | 100 (a)(b) | 56 | 55 |
| methoxybenzophenone | 100 (b) | 1 | 2 |
| dibenzothiophene | 100 (c) | | |
| phenylbenzoate | | 100 | |
| indole | 100 (c) | | |
| bibenzyl | 100 (c) | | |
| benzyl ether | | 45 | |

(a) 100% = no model compound detected after incubation
(b) incubated one week with total organism
(c) incubated three weeks with total organism

Figure 1. Gas chromatograms of (a) benzylbenzoate, (b) after degradation, (c) methoxybenzophenone, and (d) after degradation by *C. versicolor*.