

## RECENT PROGRESS IN CELL-FREE SOLUBILIZATION OF COAL

Martin S. Cohen<sup>1</sup>, Harold Aronson<sup>1</sup>, Kathleen Feldman<sup>1</sup>,  
Cynthia Brown<sup>1</sup> and Edward T. Gray, Jr.<sup>2</sup>,  
Department of Biology<sup>1</sup> and Department of Chemistry<sup>2</sup>,  
University of Hartford, West Hartford, CT 06117.

### ABSTRACT

Low rank coal has been solubilized using cell-free filtrates separated from cultures of Polyporus versicolor. Solubilization has been obtained with neat filtrates and with fractions collected from the neat filtrates after gel permeation chromatography. The coal solubilizing enzymes have been collected in enriched fractions with gpc. This increased relative purity has allowed the determination of the average molecular weight of this enzyme by gel permeation chromatography and by polyacrylamide gel electrophoresis. Rates of coal solubilization are dependent on the size of coal particles, mass of coal, temperature, pH, concentration of the cell-free filtrate, and the concentration of several inorganic ions.

### INTRODUCTION

The focus of experimentation at the beginning of this project was to produce a solubilized product from coal which had been exposed to the fungus Polyporus versicolor. Initially, Polyporus was routinely grown in dishes containing Sabouraud maltose agar and incubated at 30°C. Leonardite was routinely solubilized by adding small pieces directly to the surface of the growing mycelium. In a second stage, Polyporus was grown in a liquid medium consisting of Sabouraud broth. After a suitable period of fungal growth, the hyphae were separated from the culture medium by filtration and aliquots of the cell-free broth were added to samples of leonardite. The general characteristics of the observed (cell-free) solubilization were reported in the open literature (1). The research presented here has the ultimate aim of isolation and purification of the active agent(s) in the solubilization process, which are present in the neat cell-free broth. Ultimate success does, of course, require a knowledge of the presence of those agents and their relative concentrations. Thus, this paper reports the optimization of the characteristics of the solubilization process, and a standard set of conditions for the testing of the solubilization phenomena. From this base, a discussion of the progress towards isolation and purification of the species involved in the solubilization process is presented.

### OPTIMIZATION AND STANDARDIZATION OF THE SOLUBILIZATION PROCESS.

Having identified the scope of the solubilization process, it became necessary to determine an optimal set of conditions for the study of the process itself. A standard set of conditions is necessary in order to develop an assay for the solubilization ability of the broth, with some form of assay being required in order to compare the activity of various samples of broth with each other. This set of conditions is also necessary to be able to systematically avoid any inhibitors of the process.

An initial series of tests showed that some ionic strength in the solubilization medium was helpful in improving the solubilization action of the Polyporus broth. While nitrate, perchlorate, and acetate showed little effect at pH = 5.5 (even

though acetate has buffering capacity at this pH), the presence of phosphate (Figure 1) and, to some degree, chloride, did influence the solubilization of coal. The explanation at the time centered around the ability of these latter two bases to coordinate ferric ion, especially phosphate. From this work, a standardized buffering system of 0.050M phosphate and 0.050M acetate (all sodium salts) was established.

Using this medium, it was determined that the broth did not simply act as a catalyst for coal dissolution, but that the broth had a capacity, and that this capacity could be measured (Figure 2). This was the first standardization procedure for the solubilization ability of the broth. Knowing that the action of the broth was not being limited by its inherent capacity, and the fact that the constant medium being used and the constancy of the procedures was dramatically reducing the scatter in the data which was being obtained, it was possible to obtain a solubilization yield of the broth for the first time. This was accomplished by comparing the lyophilized bioextract from the agar plates against the liquid culture solubilizations. A 70% yield of coal solubilization was obtained, and that particular batch of broth had a capacity of 0.32mg/mL at pH = 5.5. At pH = 5.25, the yield was only 32% but the capacity was above 1.5mg/mL.

In a parallel investigation, an increasing amount of the broth was added to a fixed amount of coal. Figure 3 shows that the absorbance of solubilized coal is a function of the concentration of broth at low broth concentrations, and becomes independent of the amount of broth at higher concentrations. It should be noted that undissolved particles were present in all the test mixtures. This supports the concept of enzyme capacity discussed above.

The pH effect mentioned above prompted the generation of a pH profile (Figure 4). The maximum effect appears between pH = 5.3 and pH = 5.5. It is interesting to note that the activity does not rapidly fall to zero as pH decreases, but appears to fall to zero as pH increases by one unit. This rapid drop at higher pH is at least partially due to the much higher background of solubilization by the buffer at higher pH values.

In an attempt to understand the nature of the active agent in the broth, and, thus, to better be able to handle the system, ascorbic acid (a reducing agent) was added to inactivate any oxidative enzymes which might be present in the broth. The addition of ascorbic acid decreased the effectiveness of the broth by 90%, while the addition of oxygen after the ascorbic acid (to oxidize the ascorbic acid and show that the oxidized ascorbate was not responsible for the inactivity of the broth) restored the activity. The addition of oxygen to the broth alone showed a slight increase in the activity of the broth.

A temperature profile of the rate of solubilization activity was developed. Figure 5 shows a steady increase of the rate of cell-free solubilization with increasing temperature until 60°C after which the rate of solubilization turns up very quickly. The active agent for solubilization must be very rugged, and any 3-dimensional property of the agent must not be important in solubilization. Another critical point in the analysis is the presence of cations which are poor Lewis acids (i.e. cations which tend to be "soft" and basic in solution). It was noticed early in 1987 that virtually all the iron which the coal contained was brought into the solution phase during the solubilization process. When various eluents were being used in the HPLC separation of the broth, it was noticed that the presence of added Fe(III) as well as cations such as ammonium ion and

tetraethylammonium ion inhibited the solubilization process. This initial nuisance has become a potential analytical probe for the activity of the broth. In Figure 6, the intersection of the negative slope and the horizontal baseline is a direct function of the concentration of active agent, since the iron content eventually stops the solubilization process. Thus, for the same coal sample and time of contact, the slope of the absorbance vs. Fe(III) line should remain constant as the x-axis intercept changes to reflect the activity of the particular enzyme sample.

Furthermore, the active agent has a very strong binding constant toward iron, which presents itself as an irreversible process in the simple sense. This is seen when EDTA is added to a sample of coal, broth, and excess iron. If the iron is added to the broth before the EDTA, the broth is inhibited under conditions where the EDTA is able to coordinate all the iron. If the EDTA is added before the broth, it coordinates to the iron and the solubilization continues normally. Not all cations act in the same manner as Fe(III). Figure 7 shows the effect of the addition of ammonium ions on the solubilization process. As seen in the Figure, the concentration of  $\text{NH}_4^+$  needed for a loss of enzyme activity equivalent to that seen in the Fe(III) system is much greater. The far greater effectiveness of Fe(III) in inhibiting the solubilization process implies that the binding constant of ammonium ion with the active enzyme is clearly much lower than that with Fe(III). Even at 0.5M, the ammonium ion presence has not totally inhibited the solubilization process, although it is significantly decreased.

#### ISOLATION OF THE ACTIVE AGENT(S) IN THE SOLUBILIZATION PROCESS.

With a set of standard analytical procedures in place, the process of separating the active agent(s) from the broth was begun.

Using a  $\text{C}_{18}$  column with the standard phosphate/acetate buffer as eluent, two of the nine discernable peaks in the chromatogram showed activity toward coal (Figure 8). In order to further resolve these peaks, the eluent polarity was varied from that of methanol to 5% (w/w) NaCl. Resolution increased with polarity, but the peaks of interest always partially overlapped the void volume. Thus, another separation technique was necessary. Even so, gel electrophoresis separations on the broth produced patterns of protein which mirrored those of the  $\text{C}_{18}$  chromatography, indicating that the number of large molecules from which a separation had to be effected was not enormous.

Anion exchange initially showed some potential. The compound(s) of interest apparently have weak bases which have  $\text{pK}_a$ 's in the  $\text{pH} = 5.5$  region, since the placement of peaks coming off the anion column was quite sensitive to  $\text{pH}$  and  $[\text{Cl}^-]$  in this  $\text{pH}$  region. The work carefully led to ever further refinements of procedure from step gradients to gradient elution through various concentrations of  $\text{Cl}^-$  and  $\text{H}^+$ .

The difficulty with all these methods is the high  $[\text{Cl}^-]$  which was part of the anion-exchange process. Since coordinating agents which might ordinarily be of use in removing the  $\text{Cl}^-$  are not available to the work because of the inhibition of the system, the method of separation turned to exclusion chromatography. With an analytical gel permeation column (Waters Protein-Pak 125), the active solubilizing agent can be isolated to a single fraction of the chromatogram (Figure 9). A similar pattern of separation can be accomplished on a preparative gpc column (Sephadex, G-25-150) as shown in Figure 10.

In order to determine the molecular weight of the active fraction, the analyticalgpc column was used with six compounds of known molecular weight. The active fraction eluted from the column in an elution volume of 11.9 mL (Figure 11). This corresponds to an average molecular weight of 25,000 Daltons.

#### FUTURE EXPERIMENTATION

Experiments aimed at purification of the proteins responsible for solubilization of coal will be continued, with the goal of the formulation of a large-scale process of enzyme separation.

#### REFERENCES

1. Cohen, M.S., W.C. Bowers, N. Aronson, and E.T. Gray, Jr. 1987. "Cell-Free Solubilization of Coal by Polyporus versicolor". Appl. Environ. Microbiol. 53:2840-2843.

#### ACKNOWLEDGEMENT

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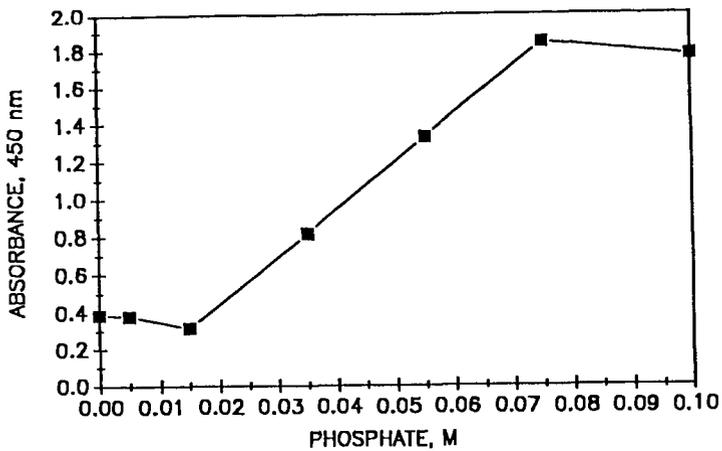


Figure 1. Effect of phosphate concentration on cell-free solubilization of leonardite coal.

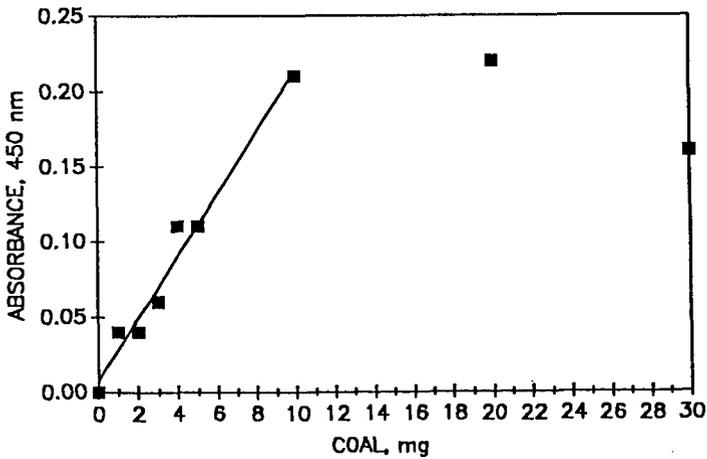


Figure 2. Effect of mass of coal added to a constant amount of cell-free filtrate on extent of solubilization of leonardite.

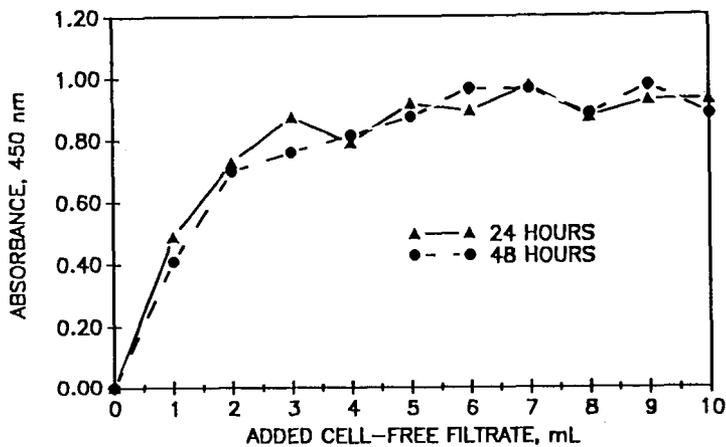


Figure 3. Effect of various amounts of cell-free filtrate added to a constant amount of leonardite on extent of solubilization of leonardite.

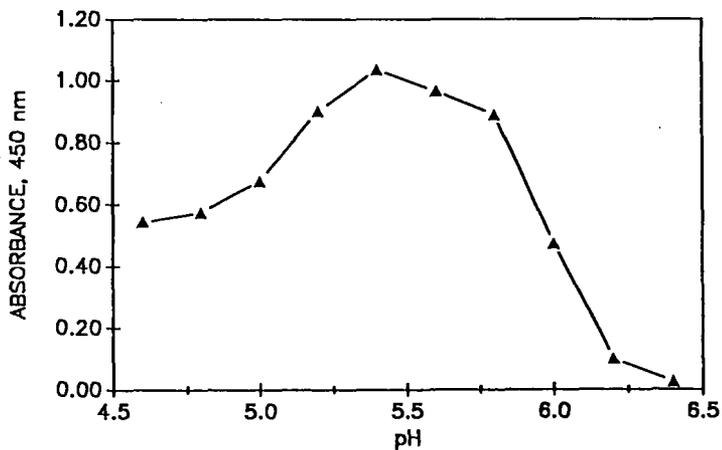


Figure 4. Effect of pH on activity of the cell-free filtrate separated from the SMB in which *Polyporus* had grown.

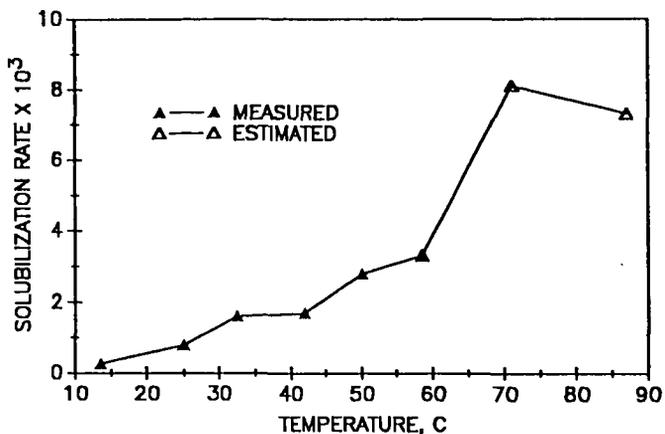


Figure 5. Effect of temperature on the rate of cell-free solubilization of leonardite coal.

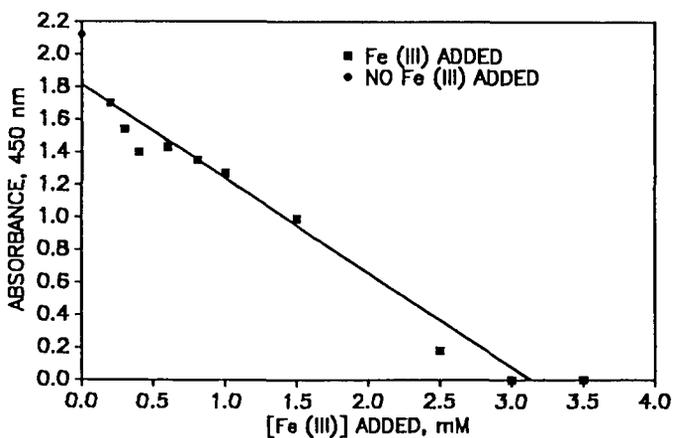


Figure 6. Inhibition of cell-free solubilization of leonardite coal by addition of Fe(III) to the filtrates. The inhibition can be reversed by mixing EDTA with the Fe(III) before addition of the iron solution to the filtrates.

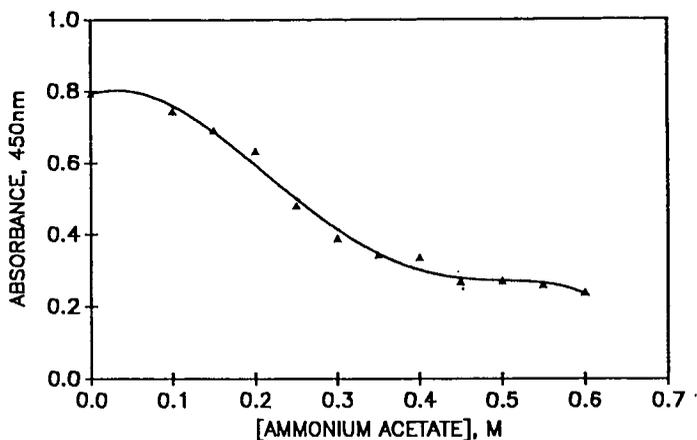


Figure 7. Inhibition of cell-free solubilization of Leonardite coal by addition of ammonium ion to the filtrates.

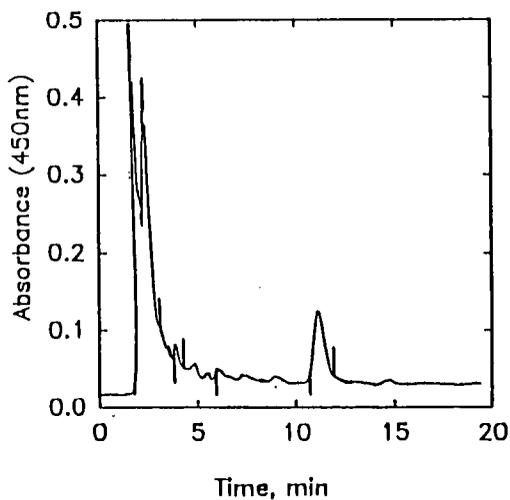


Figure 8. Separation of cell-free filtrate from Polyporus on a C<sub>18</sub> column.

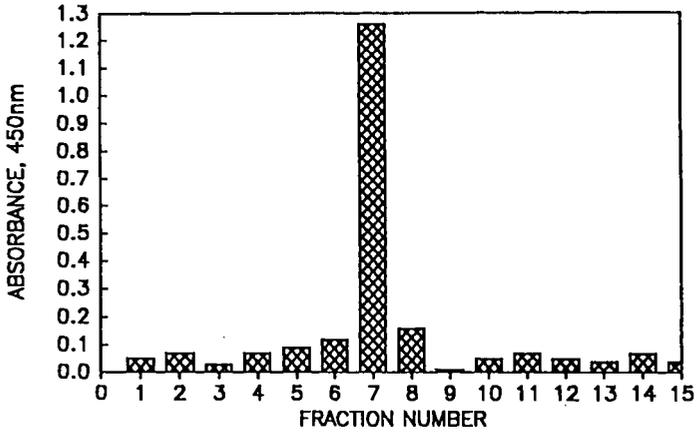


Figure 9. Fractionation of the neat cell-free filtrate on a gel permeation column. The leonardite-degrading activity has been separated into a single fraction.

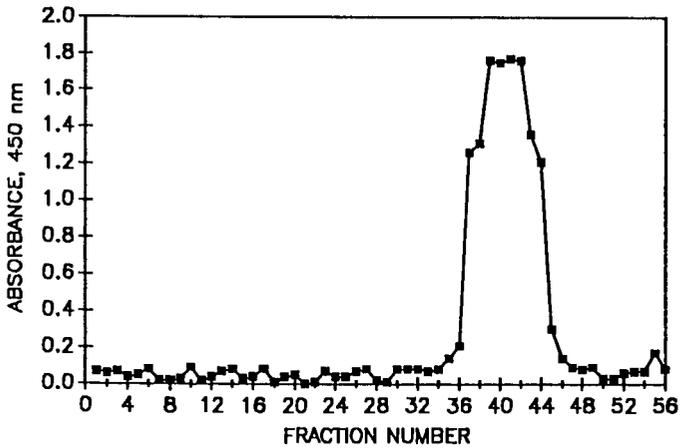


Figure 10. Separation of the coal solubilizing enzyme on a preparative column (1.5 cm X 25 cm) packed with Sephadex G-25-150 resin.

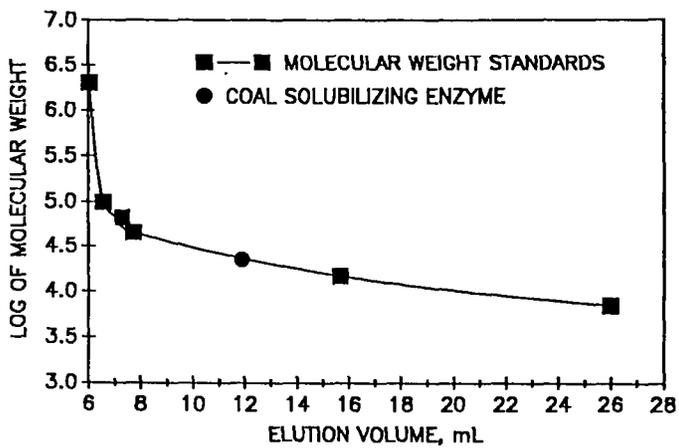


Figure 11. Molecular weight determination of the coal-solubilizing enzyme.