DOE/PC/94209--T4

Fourth Quarterly Report
Regulation of Coal Polymer Degradation by Fungi

July 24, 1995

Robert L. Irvine
Department of Civil Engineering and Geological Sciences
University of Notre Dame
Notre Dame, Indiana 46556

Specific objectives:

1) To test the hypothesis that coal (leonardite) Solubilization and the subsequent depolymerization of the solubilized coal macromolecules are distinct events in lignin degrading fungi. In addition to *T. versicolor*, *Phanerochaete chrysosporium*, another lignin degrading fungus that also has the ability to solubilize coal, will be studied.

2) To test the hypothesis that the processes of coal (leonardite) solubilization and coal macro molecule depolymerization in lignin degrading fungi can be regulated by altering the nutritional status of the microorganism. Coal solubilization is expected to occur in nutrient rich media whereas depolymerization of solubilized coal macromolecules is expected to occur in nutrient limited media.

3) To determine the role of extracellular enzymes (laccases, lignin peroxidases and Mn peroxidases) that are secreted by lignin degrading fungi during coal solubilization or coal macro molecule depolymerization.

4) To assess the role of enzymatically generated oxygen radicals, non-radical active oxygen species, veratryl alcohol radicals and Mn^{+++} complexes in coal macro molecule depolymerization.

5) To characterize products of coal solubilization and coal macro molecule depolymerization that are formed by *T. versicolor* and *P. chrysosporium* and their respective extracellular enzymes. Solubilization products formed using oxalic acid and other metal chelators will also be characterized and compared.

Methods

Molecular weight determinations. During this reporting period Dr. Bumpus' group at the University of Northern Iowa took delivery of a Perkin Elmer high performance liquid chromatograph (Purchased with funds provided by UNI). This instrument, when equipped with a Synchropak GPC 300 column (4.5 x 250 mm) allows the molecular weight of soluble coal macromolecules to be determined by gel permeation chromatography. The retention times of several protein standards of known molecular weight were determined and a calibration curve was constructed.

The apparent average molecular weight of solubilized coal macromolecules was estimated by comparing their retention times with the calibration curve. Elution of protein standards was monitored at 280 nm whereas elution of coal macromolecule was monitored at 350 nm. The flow rate was 0.5 mL/min. The elution solvent was 20 mM potassium phosphate, pH 7.2, containing 100 mM sodium chloride. Initial studies demonstrated that inclusion of the 100 mM sodium chloride was necessary to prevent ionic interactions between protein standards and unreacted silanols on the GPC column. Almost all protein standards were affected. Pepsin with an isoelectric point of <1 migrated with an inordinately rapid retention time for a protein having a molecular weight of only 34,700 and cytochrome c, having a pI of 10.1, appeared to have an inordinate affinity for the column causing it to binding tightly to the column. These problems were circumvented by supplementing the elution buffer with 100 mM sodium chloride. In general, this procedure caused all of the other protein standards (most of which had pI's of between 4 and 6) to have slightly longer (0.3- 0.4 min) retention times. The only protein standard to remain completely unaffected was horseradish peroxidase (pI = 7.2). Apparently, pepsin because of its very low pI undergoes ionic exclusion from column packing material while cytochrome c, because of its very high pI, undergoes ionic adsorption. The protein standards having pI's between 4 and 6 appear to be only minimally affected by ionic interations whereas horseradish peroxidase, which has a pI near the pH of the buffer, is virtually unaffected.

Results

Experiments focusing on Coal solubilization.

During this reporting period, much of our efforts focused on the effect selected growth media have on the solubilization of leonardite by *P. chrysosporium* and *T.versicolor.* Results show that *P. chrysosporium* is able to mediate extensive solubilization of leonardite when grown on Sabouraud agar, Potato dextrose/yeast extract agar and Yeast malt agar. Leonardite solubilization also occurred when the fungus was grown on malt agar. However, solubilization was somewhat less extensive and was not as rapid as that which occurred when the fungus was grown on the other media. *T. versicolor* also mediated extensive solubilization of leonardite when grown on Sabouraud agar and Potato dextrose/yeast extract agar. No solubilization of leonardite occurred when the fungus was grown on malt agar or on a modified

Sabouraud medium in which the ratio of peptone to dextrose was decreased 10 fold. *T. versicolor* growth occurred on all agars tested. These results are important for two reasons. First of all, they demonstrate that solubilization of leonardite by fungi is dependent upon the nutrient medium selected. Thus, those investigators who survey microorganisms for their ability to solubilize coal should do so using several different media. Secondly, the fact that leonardite solubilization by *T. versicolor* occurs on Sabouraud agar, but not on modified Sabouraud agar provides provides a mechanism by which one can study the metabolism of the fungus under conditions which promote or suppress coal solubilization. This observation also partially confirms one of the hypotheses stated in specific objective number two in which we suggested that coal solubilization and depolymerization are under nutritional control.

In addition to solid agar cultures, *T. versicolor* was grown in Sabouraud broth and a modified Sabouraud broth similar in composition to the modified Sabouraud agar medium described above. In stationary cultures, approximately 70% of the leonardite added was solubilized in 13 days of incubation. In the modified Sabouraud broth little coal solubilization occurred. These results are consistent with those observed in solid agar cultures. We have also studied leonardite solubilization by *T. versicolor* in agitated 100 mL Sabouraud broth cultures. Curiously, very little solubilization occurred under these conditions.

Experiments focusing on Coal macromolecule depolymerization.

We have initiated investigations into the solubilization of coal macromolecule by *P. chrysosporium* and *T. versicolor* in nutrient nitrogen limited and nutrient nitrogen sufficient liquid cultures. These studies are in progress.

Biomimetic Solubilization of Coal.

It is known that wood rotting fungi solubilize low rank coal by secreting oxalate which chelates metal ions. This disrupts salt bridges between individual coal macromolecules which, in the absence of such linkages, are relatively soluble in water. It is, therefore, of interest to understand how oxalate solubilizes leonardite. Our results to date demonstrate that in the presence of sodium oxalate, solubilization of leonardite is a relatively slow process, requiring up to 24 h. The ratio of oxalate to

leonardite is also important. At high ratios of oxalate to leonardite, solubilization is less than optimal at high concentrations of leonardite. Interestingly, at high ratios of oxalate to leonardite solubilization is favored at low leonardite concentrations. We have also shown that solubilization of leonardite is favored at pH values greater than the pK_{a2} of oxalic acid (i.e., pH 4.28) and maximal at pH values between pH 7 to 10. This is of importance to the present investigation as many wood rotting fungi grow best at acidic pH. For example, *P. chrysosporium* grows best in a pH range between pH 4 and 5.

The ability of other metal chelators and selected detergents to solubilize leonardite was also studied. Results demonstrated that of the substances tested, only sodium oxalate and sodium citrate possessed substantial ability to solubilize leonardite under the experimental conditions used in this investigation; 71.5% and 45% solubilization, respectively. The metal chelators DTPA, EDTA and NTA solubilized 4.73%, 12% and 7.4%, of the leonardite, respectively, whereas the detergents Triton X-100 and SDS solubilized 9 and 15%, respectively.

Characterization of coal macromolecules solubilized biologically by *P. chrysosporium* and *T.versicolor* and biomimetically by sodium oxalate

Spectral characterization. UV-visible spectra of all coal sample were acquired. Little difference was noted between any of the samples. Characterization of samples by IR spectroscopy is in progress.

Molecular weight determinations. The molecular weights of coal macromolecules solubilized biologically by *P. chrysosporium* and *T. versicolor* and biomimetically using sodium oxalate were determined using high performance liquid chromatography/gel permeation chromatography (HPLC/GPC). Coal macromolecules solubilized had apparent average molecular weights of 17,000, 22,000 and 29,000, respectively.

Personnel: Ms. Kimberly Sturm of Thiel College joined Dr. Bumpus' laboratory at the University of Northern Iowa for the Summer 1995. Ms. Sturm studied the solubilization of leonardite by *T. versicolor.*

Planned Activities

During the next reporting period we plan conduct experiments documenting the depolymerization of coal macromolecule by *P. chrysosporium* and the effect of nutrient limitation on depolymerization. We will also plan to determine levels of oxalic acid produced by this fungus in nutrient limited and nutrient sufficient cultures. Study of the biomimetic solubilization of leonardite by sodium oxalate has been informative in that results are good predictors of the conditions for leonardite solubilization that are likely to be optimal *in vivo.* These investigations will be continued.