DE-FG22-94PC94209--14
(6093)

Regulation of Coal Polymer Degradation by Fungi

Quarterly Report
July 1 - September 30, 1997

Work Performed Under Contract No.: DE-FG22-94PC94209

For
U.S. Department of Energy
Office of Fossil Energy
Federal Energy Technology Center
P.O. Box 880
Morgantown, West Virginia 26507-0880

By
University of Notre Dame
Department of Civil Engineering and
Geological Sciences
Notre Dame, Indiana 46556

Disclaimer

Abstract

During this reporting period we continued our investigations of how low rank coals are degraded by wood rotting fungi. Our previous investigations showed that ligninolytic cultures of *P. chrysosporium* could decolorize soluble low rank coal macromolecule. We continue to investigate this phenomenon. Consistent with earlier observations we conclude that soluble coal macromolecule is decolorized in ligninolytic cultures of *P. chrysosporium.* To determine if this fungus can depolymerize coal macromolecule, samples were analyzed by GPC-HPLC. These analyses suggested that when coal macromolecules were incubated with ligninolytic cultures of *P. chrysosporium* a slight decrease in the average peak molecular weight of this mixture had occurred. During this reporting period we also discovered that changes in buffer composition can alter the peak retention times of coal macromolecules during GPC-HPLC probably by causing dissociation and reassociation of individual macromolecules. In other experiments it has been shown that lignin peroxidases that are secreted by ligninolytic cultures of *P. chrysosporium* are responsible, at least in part, for decolorization of coal macromolecules. Taken together, our studies show that the lignin degrading system of *P. chrysosporium* is able to enzymatically attack macromolecules solubilized from low rank coal. The ability of nonacclimated bacteria from sewage sludge to used leonardite and soluble coal macromolecule as a substrate for methanogenesis was also investigated. To date, the bacterial consortium studied was unable to use these substrates for this purpose.

Table of Contents

Page

Executive Summary

During this reporting period our investigations focused on assessing the ability of ligninolytic cultures of *P. chrysosporium* to degrade oxalate solubilized coal macromolecule. Similar to previous findings, we conclude that this fungus does, indeed, mediate extensive decolorization of oxalate solubilized coal macromolecule and that lignin peroxidases that are secreted by this fungus are responsible for this decolorization. It also appears that the mixture of soluble coal macromolecules that had been incubated with ligninolytic cultures of *P. chrysosporium* possess on average a lower molecular weight than nonincubated material. An important observation made during this investigation is that the medium in which soluble coal macromolecule is dissolved affects the peak retention times observed during GPC-HPLC and thus must be considered when interpreting molecular weight data During this reporting period we have also shown that pH adjusted (pH 7) aqueous solutions of sodium oxalate effectively solubilized an air oxidized North Dakota Lignite and a nitric acid oxidized North Dakota Lignite. The ability of nonacclimated bacteria from sewage sludge to used leonardite and soluble coal macromolecule as a substrate for methanogenesis was also investigated. To date, the bacterial consortium studied was unable to use these substrates for this purpose.

Introduction

A major objective of this research is to better understand how wood rotting fungi solubilize low rank coal and how soluble coal macromolecules are depolymerized or otherwise degraded following solubilization. Results from a number of laboratories suggest oxalate ion which is secreted by these fungi has a major role in solubilization of low rank coal (1-3). Oxalate ions are thought to chelate metal ions that hold coal macromolecules together by ionic bridges; forming insoluble complexes. Once these ionic linkages are broken, the relatively polar coal macromolecules become soluble in water. In addition to oxalate, we have shown that a number of other carboxylic acids (Malate, Oxaloacetate, Citrate and Isocitrate) are also able to mediate substantial solubilization of low rank coal. This is of significance because a variety of fungi that are known to secrete such compounds also are known to be able to solubilize low rank coals and probably do so because of their ability to secrete these compounds.

Although enzymes of the lignin degrading system are not required for solubilization of low rank coal, we have shown that the lignin degrading system does appear to be involved in subsequent degradation of the soluble coal macromolecule. We have shown that substantial decolorization of solubilized coal macromolecule occurred in nutrient nitrogen limited malt agar cultures of the lignin degrading fungus *Phanerochaete chrysosporium.* Decolorization of soluble coal macromolecule did not, however, occur in nutrient nitrogen sufficient malt agar cultures of this fungus. This is of significance because the lignin degrading system of this fungus is expressed in nutrient nitrogen limited cultures, but not in nutrient nitrogen sufficient cultures. Thus, these results are indirect evidence that the lignin degrading system has a role in degradation/decolorization of soluble coal macromolecule. Ultimately we hope to use soluble coal macromolecule as a substrate for methanogenesis in anaerobic bacterial fermentations.

Methods and Materials

Many of the procedures used in studies described herein were described in previous quarterly reports. The methods and materials described below refer mainly to experiments involving the use of leonardite or solubilized leonardite as a substrate for methanogenesis.

Oxalate solubilized coal production: Sodium oxalate (1 g) was added to 500 ml of a 1% slurry of leonardite in distilled water and allowed to mix. After 24 hours the mixture was centrifuged for 30 minutes after which the supernatant, containing solubilized coal macromolecule, was separated and dialyzed extensively against distilled water. The dialyzed coal macromolecule was lyophilized and stored at 4^{o}C until used.

Biosolubilized coal production: The white rot fungus *P. chrysosporium* was cultured in shake flasks as outlined by Tien (5). Autoclaved leonardite powder was introduced to the shake flasks during inoculation. The shake flasks were incubated at 35^{o}C for 10 days. Subsequent processing to produce lyophilized coal macromolecule was similar to the protocol used for the oxalate solubilized product.

Anaerobic seed organisms: Anaerobic sludge was collected from an anaerobic digester at the South Bend, Indiana waste water treatment plant. The sludge was thoroughly rinsed in nutrient solution medium prior to use in the anaerobic serum bottle studies. All serum bottles were seeded with the same amount of anaerobic sludge prior to the start of the experiment. Preparation of anaerobic shake flasks was performed in an anaerobic chamber.

Methane and carbon dioxide analysis: Gas analyses were performed on a Varian 3400 gas chromatograph equipped with a 60/80 Carbowax column and TCD detector. Analyses were performed isothermally at 40 ^{o}C. Helium was used as the carrier gas. The retention time was 0.5 and 1.0 minutes for methane and carbon dioxide, respectively.

Chemical Oxygen Demand (COD) : Analyses were performed using a modified Hach (San Francisco, CA) procedure.

Results and Discussion

Biodegradation of Oxalate Solubilized Coal by *Phanerochaete chrysosporium.*

In our previous studies direct visual examination of cultures of *P. chrysosporium* grown in nutrient nitrogen limited liquid medium showed clearly that decolorization of the coal macromolecule had occurred and, when quantified by UV visible absorption spectroscopy it was found that as much as 35-50% decolorization had occurred, depending on the wavelength monitored. During this reporting period we discovered that freezing (-20^{o}C) these samples caused some of the coal macromolecule to precipitate and that this precipitated material did not go back into solution upon vigorous mixing. Addition of 100 uL of 1M dibasic potassium phosphate/ 1 mL of sample did, however, cause the precipitated material to redissolve. GPC-HPLC analysis of these samples monitored at 350 nm is presented in figure 1. The peak retention time for material that had been incubated with *P. chrysosporium* in these experiments appeared to be somewhat greater than that of the control.

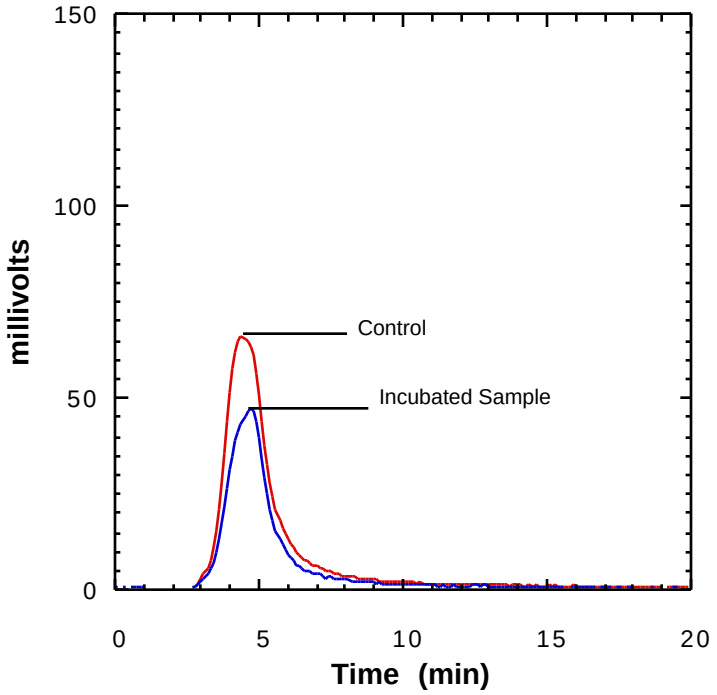

Figure 1. Effect of incubation of soluble coal macromolecule with ligninolytic cultures of *P. chrysosporium* for twelve days. Incubated samples and nonincubated controls were assayed by GPC-HPLC as detailed in previous quarterly reports.

UV-visible absorption spectra of incubated samples and controls are presented in figure 2 and further document that biodegradation of soluble coal macromolecule by *P. chrysosporium* had occurred. Figure 3 is a replot of the spectral data in which the absorbance of incubated samples is expressed as a percentage of the absorbance of nonincubated controls.

Taken together, our results indicate that the lignin degrading system of *P. chrysosporium* does modify the structure of soluble coal macromolecule probably by oxidizing, and thereby, decolorizing chromophoric substructures in this material.

A major goal of our research is to determine if coal depolymerization occurs in this system. Although we observed an increase in the peak retention time of coal macromolecule incubated with *P. chrysosporium*, we did not observe the appearance of distinct fragments indicative of low molecular weight substructures. This could be due to the possibility that low molecular weight material does not accumulate because it is readily metabolized by the fungus.

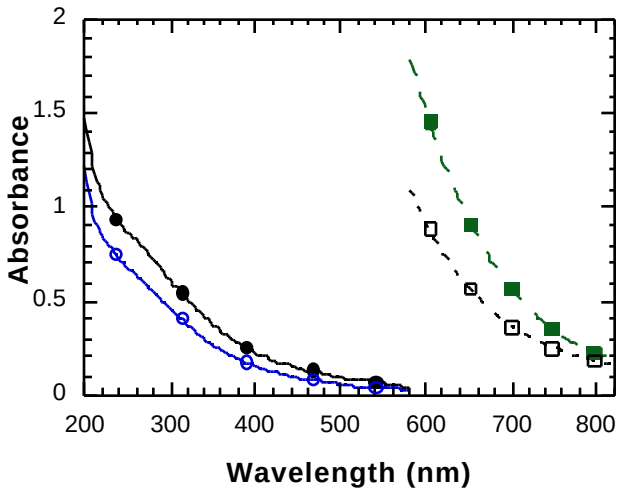

Figure 2. Decolorization of soluble coal macromolecule by a ligninolytic culture of *P. chrysosporium*. The UV-visible spectrum (open symbols) of the extracellular fluid of a ligninolytic culture of *P. chrysosporium* that had been incubated with oxalate solubilized coal macromolecule for twelve days was acquired. This spectrum was then compared with a similar spectrum (closed symbols) of a nonincubated sample that was removed immediately following addition to the culture. The absorption spectra from 200 to 580 nm was acquired using a sample that had been diluted 40:1 with water. The absorption spectra from 580 nm to 820 nm was acquired using the undiluted sample.

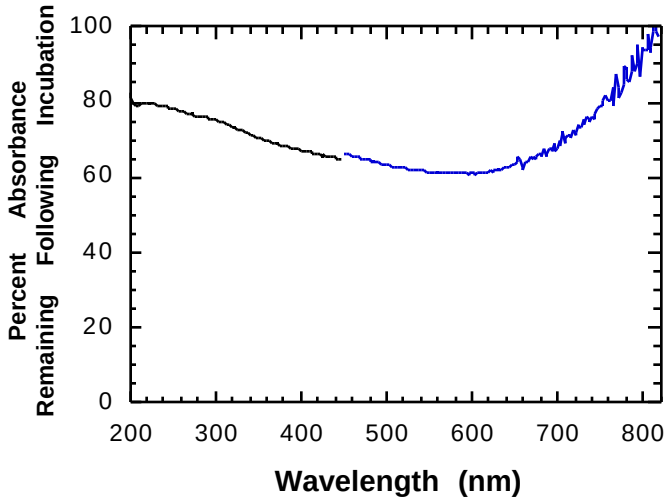

Figure 3. Decolorization of soluble coal macromolecule by a ligninolytic culture of *P.chrysosporium* expressed as a percentage. Absorbance data were acquired on the same sample used to generate figure 2 and expressed as the percent of absorbance due to coal macromolecule remaining after incubation with *P. chrysosporium* for twelve days. The data from 200 to 450 nm was acquired using samples that had been diluted 40:1 whereas the data from 450 nm to 820 nm was acquired using samples that had been diluted 4:1

Interpretation of this data is made more difficult by the fact that the material under study is not homogeneous, but a mixture of macromolecules most of which have molecular weights between 14,000 and 60,000. Furthermore, we found that under certain conditions coal macromolecules appear to undergo associations with each other leading to a decrease in peak retention times (Figure 4). Indeed we discovered that such a phenomenon occurs following the addition of dibasic potassium phosphate to solutions of coal macromolecules. However, because incubated samples and nonincubated controls were treated identically, our conclusion that incubation of soluble coal macromolecules with ligninolytic cultures of *P. chrysosporium* results in a population of coal macromolecules that have somewhat smaller molecular weights is probably correct.

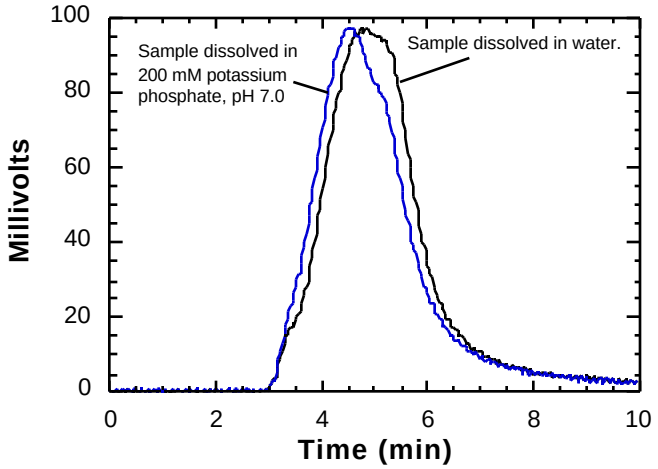

Figure 4. Effect of storage buffer on the elution of soluble coal macromolecule during GPC-HPLC. Oxalate solubilized coal macromolecule that had been extensively dialyzed and subsequently lyophilized was redissolved in water or 200 mM potassium phosphate buffer, pH 7.0 (Final concentration ~5mg/10 mL). Twenty microliter aliquots were assayed by GPC-HPLC.

Interestingly, extracellular lignin peroxidases that are so important in lignin degradation do not appear to have a role in solubilization of low rank coal. It has been reported, however, that lignin peroxidases from *P. chrysosporium* may have a role in mediating subsequent degradation of the soluble coal macromolecule. Wondrack *et al.* (1989) have shown clearly that a soluble coal macromolecule (Fraction III-B) isolated from a nitric acid oxidized North Dakota Lignite is a competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidation. They also reported that lignin peroxidases mediate depolymerization of soluble coal macromolecule. We have reexamined this phenomenon using nitric acid oxidized North Dakota Lignite provided by Dr. Willis Wood. We confirm that solubilized coal macromolecule is a

good competitive inhibitor of lignin peroxidase mediated veratryl alcohol oxidation. We have also developed a colorimetric assay and have demonstrated that lignin peroxidases can mediate decolorization of coal macromolecules and are probably responsible, at least in part, for decolorization of coal macromolecule observed in ligninolytic cultures of *P. chrysosporium.*

The ability of lignin peroxidases to depolymerize soluble coal macromolecule is a topic of ongoing investigation in our laboratory.

Solubilization of Argonne Premium Coals Using Sodium Oxalate.

During this reporting period we further studied the ability of sodium oxalate to solubilize preoxidized Argonne Premium Coals. In a previous quarterly report we noted that sodium oxalate was not very effective in the solubilization of these coals. We have since performed solubilization studies in pH adjusted (pH ~7) aqueous solutions of these coals containing sodium oxalate. The GPC-HPLC of material solubilized from representative Argonne Premium Coals by aqueous solutions of sodium oxalate is illustrated in Figure 5. Of the eight Argonne Premium Coals, only the Beulah-Zap North Dakota Lignite was extensively solubilized by this process.

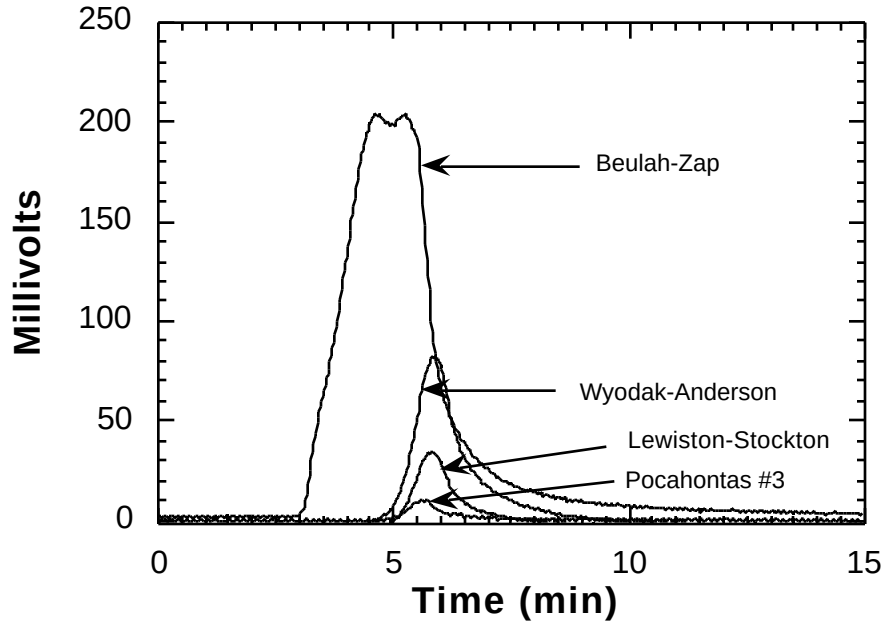

Figure 5. GPC-HPLC of material solubilized from representative Argonne Premium Coals by an aqueous solution of sodium oxalate. Soluble material from the other Argonne Premium Coals had elution profiles very similar to that of soluble material from Pocahontas #3. In these experiments, 50 mg of dry coal was incubated with 10 mL of 75 mM sodium oxalate on a rotary shaker (200 rpm) for 48 h. Samples were then centrifuged and 20 µL aliquots were assayed by GPC-HPLC. The material solubilized from the Beulah-Zap North Dakota lignite was diluted 4:1 prior to analysis.

Solubilization of A Nitric Acid Oxidized North Dakota Lignite Using Sodium Oxalate.

Wondrack *et al.* (1989) prepared for their investigations a soluble fraction (III-B) from a nitric acid oxidized North Dakota Lignite using a procedure that involves solubilization with a strong base. During this reporting period we showed that our procedures using sodium oxalate also were effective in solubilizing this material. One gram of sodium oxalate was added to one gram of nitric acid oxidized North Dakota Lignite in 100 mL of water and stirred. The pH of this material was approximately pH 4.6. Visual examination of this mixture suggested that little or no solubilization was taking place. Sodium hydroxide (1 M) was then added dropwise to the solution until a pH of 6.8 was achieved. The solution then turned dark brown and was stirred overnight. Following centrifugation to remove particulate material, the solution was dialyzed extensively against water and stored. These results are of interest because they demonstrate that like the leonardite used in most of our studies, the nitric acid oxidized North Dakota Lignite used by Wondrack *et al*. (1989) can also be solubilized using less caustic procedures.

Use of Untreated Leonardite and Solubilized Leonardite for Methane Production.

One of the potential uses of solubilized low rank coal is as a raw material in methane production (*i.e.,* generation of a gaseous fuel). Biological methane production is a process that was investigated during this reporting period to assess the feasibility of using solubilized coal macromolecule generated biologically (*e.g.,* using a white rot fungus) and chemically (*e.g.,* using oxalic acid or NaOH).

Methane production in anaerobic shake flasks containing different forms of leonardite.

Figure 6 shows the methane production profiles in anaerobic serum bottles containing different forms of leonardite. Methane production in the control, biosolubilized leonardite and oxalate solubilized leonardite experiments were appreciably similar initially (between 0 and 400 hours). The serum bottles containing biosolubilized leonardite generated slightly more (relative to the control and oxalate solubilized leonardite) methane as the experiment progressed. There was no appreciable difference between the control containing biomass only and that containing oxalate solubilized leonardite. The serum bottles containing untreated leonardite had the least methane production, even lower than the control.

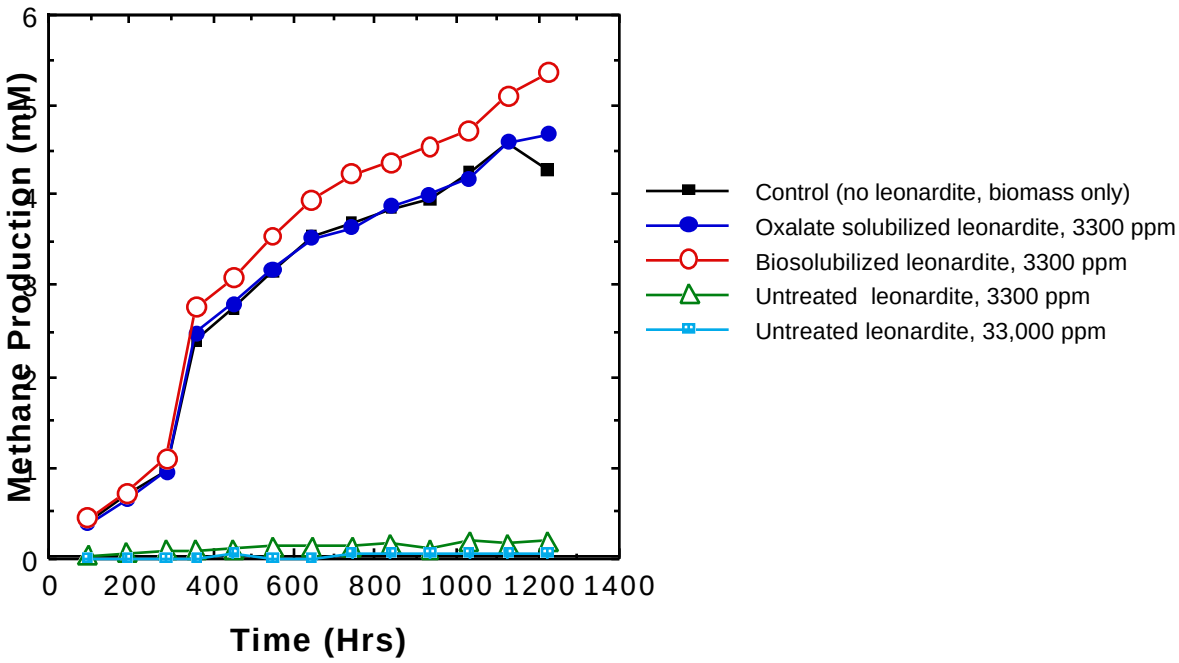

Figure 6:Methane production in anaerobic shake flasks containing different forms of leonardite.

The corresponding carbon dioxide production profiles are presented in figure 7. Anaerobic organisms are known to produce methane from the reduction of carbon dioxide if they have reducing equivalents to drive the reaction (6). Because methane and carbon dioxide production profiles of the serum bottles containing solubilized forms of leonardite and the control are appreciably similar, the reducing equivalents for methane production appear to be from endogenous fermentation (cell lysis) and residual organic debris left over after rinsing the anaerobic sludge and not due to solubilized leonardite. The previously unacclimated seed does not seem to have the metabolic capability to utilize solubilized leonardite as a carbon and/ or energy source.

The carbon dioxide production profiles were greater in the serum bottles containing untreated leonardite than those containing solubilized leonardite or biomass only (figure 7). There is an increase in the carbon dioxide production after 300 hours in incubations containing untreated leonardite. The cause for the rise in carbon dioxide is probably indirectly related to the endogenous metabolism that results in production of

extra cellular fermentation products which react with inorganic forms of carbonates in untreated leonardite to release carbon dioxide into the headspace. The suppression of methane production in the serum bottles that contain untreated leonardite further supports the hypothesis that pathways that produce methane by fermentation are probably inhibited after the fermentation product formation step.

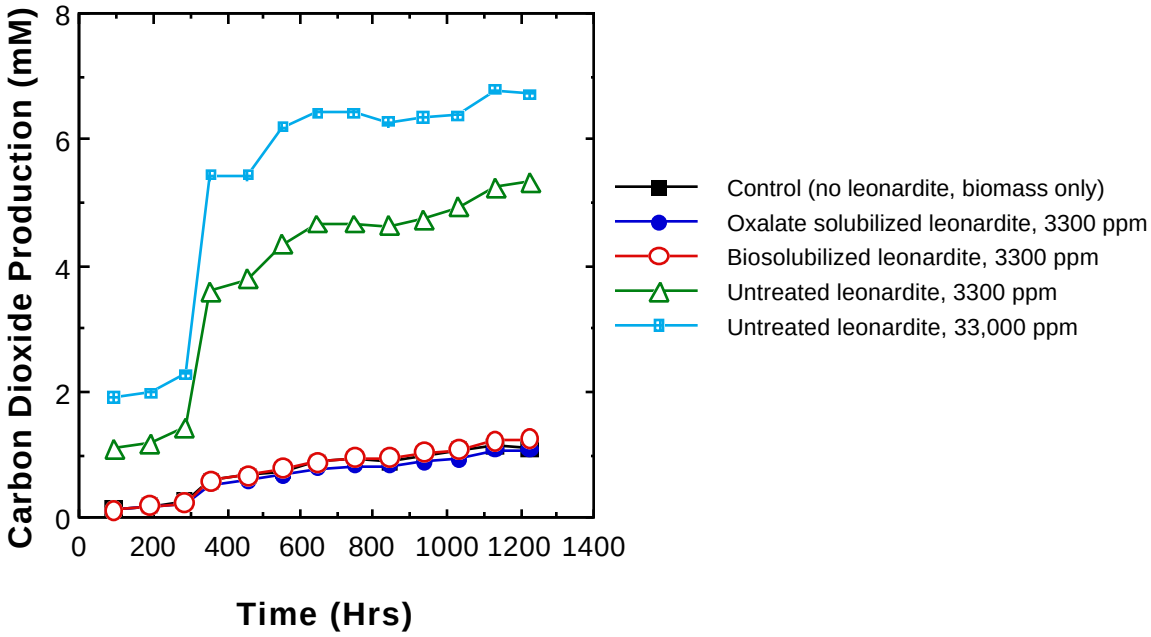

Figure 7: Carbon dioxide production in anaerobic shake flasks containing different forms of leonardite.

Metal ion toxicity is one of the possible causes that could inhibit methanogenesis. Neutron activation analysis of untreated leonardite reported in our seventh DOE quarterly report indicated metal ions (*e.g.,* Cu.) that are known to inhibit methanogenesis are present. Their presence in the untreated leonardite possibly inhibits methanogenesis. Solubilization of the solid untreated leonardite is also a rate limiting step in the anaerobic experiments in which it was used. The pH typically decreases during fermentation which is unfavorable for leonardite solubilization as has been shown in previous progress reports. Solubilized leonardite produced by chelation mechanisms such as that produced using oxalate or other secondary metabolites produced by fungi release chelated metal ions into solution. However, these ions are removed with other lower molecular weight compounds during dialysis. Hence there is no apparent inhibition when solubilized coal is used.

Thus, initial studies indicate that unacclimated seed does not metabolize leonardite in either the soluble or untreated insoluble form. The untreated leonardite appears to be inhibitory to methanogenesis in a unacclimated seed. The next phase of experiments will be performed using anaerobic sludge that has been acclimated to coal model

compounds such as methoxy- phenols, phenol, etc. after which solubilized leonardite will be provided to determine if metabolic pathways can be induced or if organisms can be enriched which have the ability to transform solubilized leonardite into fermentation products and/ or methane. A metal leaching protocol has been developed to remove metal ions from solubilized leonardite. These procedures utilize concentrated hydrochloric acid to decrease the coal macromolecule solutions to pH 2 This precipitates solubilized coal macromolecule while leaving metal ions in solution. The soluble metal ions are removed from the supernatant when it is decanted. The concentrated precipitate is then solubilized with 1N NaOH, reprecipitated and redissolved. This repetitive precipitation procedure removes most of the metals associated with solubilized macromolecule produced from leonardite Experiments using an acclimated seed with metal free solubilized leonardite macromolecule could result in increased methanogenesis and will be a focus of study for the next reporting period.

References

1) Cohen, M.S., Feldman, K.A., Brown, C.S. and Grey, Jr., E.T. (1990) Isolation and identification of the coal-solubilizing agent produced by *Trametes versicolor*. Appl. Environ. Microbiol. **56**:3285-3291.

2) Frederickson, J.K., Steward, D.L., Campbell, J.A., Powell, M.A., McMulloch, M. Pyne, J.W. and Bean, R.M. (1990) Biosolubilization of low-rank coal by a *Trametes versicolor* siderophore-like product and other complexing agents. J. Ind. Microbiol. 5:401-406.

3) Faison, B.D. (1993) The chemistry of low rank coal and its relationship to the biochemical mechanisms of coal solubilization. In: *Microbial transformations of low rank coal*. (Ed. D.L. Crawford) CRC Press, Boca Raton, 223p.

4) Wondrack, L. Szanto, M. and W.A. Wood (1989) Depolymerization of water soluble coal polymers from subbituminous coal and lignite by lignin peroxidase. Appl. Biochem. Biotechnol. **20/21**:765-780.

5) Tien, M., Properties of Ligninases from *P. chrysosporium* and their possible applications, CRC Critical Reviews in Microbiology, 15, 2, p. 141168., 1987

6) Wiegel, J., Acetate and the Potential of Homoacetogenic Bacteria for
Industrial Applications, In Acetogenesis, Ed. Drake, H. L., 1994, Chapman & Hall Inc., New York, NY, 1994.