Third Quarterly Report
Regulation of Coal Degradation by Fungi

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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 macromolecule 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 macromolecule depolymerization.

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

5) To characterize products of coal solubilization and coal macromolecule 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 and Materials

Coal macromolecule solubilization. The soluble coal macromolecule used in these investigations was solubilized from an aqueous suspension of leonardite by addition of sodium oxalate. Since oxalate ion is responsible for coal macromolecule solubilization in vivo, this method of solubilization represents a biomimetic process. The solubilized coal macromolecule was dialyzed (14,000 MWCO) extensively against distilled deionized water. A ten mL aliquot of the resulting dark brown solution was then placed in a tared glass vial and the water was evaporated overnight in a 105°C oven. The mass of the dried residue was then determined gravimetrically. Depolymerization of coal
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macromolecule was monitored indirectly by observing the decrease (decolorization) of absorbance that occurs in this highly colored macromolecule at 600 nm.

**Equipment.** During the next reporting period Dr. Bumpus' group at the University of Northern Iowa will accept delivery of a high performance liquid chromatograph (purchased with funds provided by UNI). This instrument will allow depolymerization experiments to be monitored by gel exclusion column chromatography rather than indirectly by decolorization of coal macromolecule.

**Microorganisms.** In addition to *P. chrysosporium*, *Trametes versicolor* 12697 is being used in these investigations. This microorganism was acquired from the American Type Culture Collection in January 1995 and has been subcultured. Stock cultures are maintained on potato dextrose agar containing 0.5% yeast extract as recommended by ATCC. Initial observations demonstrate that this fungus grows more slowly than *P. chrysosporium*. Thus, it may be necessary to use longer incubation times in experiments in which this fungus is used.

**Results**

**Experiments focusing on coal solubilization.**

In previous quarterly reports we showed that considerable amounts of leonardite were solubilized by *P. chrysosporium* grown on Sabouraud agar. We also showed that a smaller amount was solubilized when this fungus was cultured on malt agar. During the present reporting period, we showed little or no leonardite was solubilized in malt extract broth cultures of *P. chrysosporium*. In still other experiments conducted during this reporting period we have shown that substantial amounts of leonardite are solubilized by *P. chrysosporium* that was grown on potato dextrose yeast extract agar (These experiments are still in progress. Thus the exact percent of coal solubilized in not yet available. We know that considerable coal is being solubilized as soluble coal macromolecule is diffusing throughout the agar). It is apparent that coal macromolecule solubilization is dependent on the composition of the nutrient medium used. We believe that this is because the composition of certain media is conducive to oxalic acid production while others are not. Oxalic acid, of course, is the agent responsible for solubilization of low rank coal *in vivo*.

Oxalic acid is synthesized in a number of reactions that occur during the metabolism of amino acids. For example during the oxidative cleavage of phenylpyruvate, benzaldehyde and oxalic acid are formed as products (This reaction occurs during the conversion of phenylalanine to hippuric acid). Similarly some organisms convert excess glycine to glyoxalic acid which is further oxidized to oxalic acid. It has also been demonstrated that certain fungi, when grown in the presence of excess succinic acid, produce high concentrations of oxalic acid. Because the glyoxalic acid cycle and the Krebs cycle may operate simultaneously in plants and some microorganisms, high concentrations of a Krebs cycle intermediate could, theoretically, lead to formation of increased amounts of isocitrate which may be converted by isocitrate lyase to reform
succinate with the concomitant production of increased of glyoxallic acid which is then oxidized to oxalic acid.

That media containing high concentrations of amino acids or a nitrogen source promotes solubilization of coal by P. chrysosporium is consistent with the idea that oxalic acid formation from carbon atoms derived from amino acid metabolism is favored in such cultures. Similarly, the fact that minimal amounts of coal solubilization occurs in nutrient nitrogen limited cultures is consistent with the idea that carbon atoms participating in amino acid metabolism in such cultures would be used for protein synthesis—not oxalic acid formation. It is also interesting to note that high concentrations of succinic acid promotes increased production of oxalic acid in some fungi. It will be very important to determine if this occurs in P. chrysosporium and other wood rotting fungi. As noted in our specific objectives, it is our hypothesis that coal solubilization and depolymerization are distinct events and are under nutritional control. Thus one would expect that in cultures having high concentrations of amino acids, coal solubilization should readily occur. However, the lignin degrading system would not be expected to be expressed in nutrient nitrogen rich cultures and depolymerization of the solubilized macromolecule would not be expected to occur. If succinic acid amended cultures promote oxalic acid production in nutrient nitrogen limited cultures of P. chrysosporium, it would be expected that coal solubilization and subsequent depolymerization of solubilized coal macromolecule would both occur. Thus control of solubilization and depolymerization could be achieved simply by selecting the appropriate culture medium.

We have also initiated experiments using T. versicolor. Experiments that are still in progress suggest that, like P. chrysosporium, T. versicolor, when grown on potato dextrose agar containing 0.5% yeast extract, is able to solubilize the low rank coal used in these investigations. Results of these experiments will be summarized in our next quarterly report.

Experiments focusing on depolymerization of solubilized coal macromolecule.

As noted several times in this report, it is our hypothesis that low rank coal (leonardite) solubilization and subsequent depolymerization are distinct events in lignin degrading fungi and that these processes are under nutritional control. To demonstrate that depolymerization of soluble coal macromolecule is under nutritional control, we prepared two sets of malt agar petri plates which contained 1,000 mg/mL each of solubilized coal macromolecule. One set contained no supplemental nutrient nitrogen while the other set was amended with supplemental nutrient nitrogen (12 mM ammonium tartrate). Cultures were inoculated by placing a small piece of agar from a stock malt agar culture of P. chrysosporium in the middle of the Petri plates. Cultures were then allowed to incubate at room temperature in a humidified growth chamber for three weeks. During this time P. chrysosporium grew to the edge of inoculated Petri plates. Although those cultures that received supplemental amounts of nutrient nitrogen appeared to exhibit more luxurient growth, substantial decolorization (depolymerization) occurred only in those cultures which did not receive supplemental nutrient nitrogen.
This is of interest because it is known that the lignin degrading system of *P. chrysosporium* is suppressed in cultures containing supplemental nutrient nitrogen whereas expression of the lignin degrading system in this microorganism is promoted in nutrient nitrogen limited cultures. These results are also consistent with the hypothesis that it is the lignin degrading system of this fungus that is responsible for coal macromolecule depolymerization. It should be noted that unamended malt extract has a carbon to nitrogen ratio of ~50:1. Thus, this growth medium may be considered to be nitrogen limited.

In other experiments, we have shown that in malt extract broth cultures, coal macromolecule is decolorized (i.e., depolymerized). However, this process is slow, taking several weeks for complete decolorization to occur.

**Personnel:** No personnel changes have been made during this reporting period.

**Planned Activities**

During the next reporting period, we plan to continue investigations aimed at addressing specific objectives 1, 2 and 3. Specifically, we plan to conduct experiments that will determine if, as strongly indicated, oxalic acid production (and therefore coal solubilization) is favored in cultures containing high concentrations of amino acids, nutrient nitrogen and succinic acid. In these experiments, several standard media which contain high concentrations of amino acids or peptone will be used. Additionally, a defined nutrient nitrogen limited medium will be used. It is not expected that this medium will promote coal solubilization. However, we intend to also conduct experiments in which this medium is supplemented with selected amino acids, peptone and other nitrogen sources to show unequivocally whether or not oxalic acid and coal solubilization is, indeed, dependent on these nitrogen sources. Experiments will be conducted in agar and broth cultures and oxalic acid will be assayed by the spectrophotometric method described by Allan et al. (1986).

**Literature cited**


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