Eleventh Quarterly Report
Regulation of Coal Polymer Degradation by Fungi
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Disclaimer

Abstract

Previous studies in our laboratory used a spectrophotometric assay to study biomimetic solubilization of leonardite by sodium oxalate. It was found, however, that in extended incubations of several days, this assay resulted in overestimation of the percent of leonardite that was solubilized. This problem did not appear to be significant for short term incubations (*i.e.*, up to ~24 h) and was circumvented in long term incubations by using a gravimetric assay to assay for solubilization. In other studies during this reporting period we examined oxalate production by *P. chrysosporium* and *T. versicolor* grown in Fahreus-Reinhammar medium in agitated pelleted culture. It was found that in this system concentrations of oxalate are produced that are much lower than those that would be optimal for leonardite solubilization.

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Executive Summary

In data presented in previous quarterly reports we assessed leonardite solubilization by a spectrophotometric assay in which the absorbance at 600 nm of solubilized leonardite was used to calculate the amount of this low rank coal that was solubilized. Although spectrophotometric assays can be used, gravimetric procedures are more direct and precise. We had not previously used gravimetric procedures because filters that allowed sufficient flow also allowed very fine particulate material to flow through the filter while filters that retained this very fine particulate material became quickly plugged. This problem was resolved by using 0.45 µm Nylon filters and by redesigning experimental protocols such that smaller masses of leonardite and smaller volumes of aqueous leonardite suspensions are used.

It is known that oxalate ion has a critical role in leonardite solubilization by fungi. Thus during this reporting period we studied oxalate ion production in agitated pelleted cultures of *Phanerochaete chrysosporium* and *Trametes versicolor.* Of interest is the observation that oxalate production by these fungi when grown in Fahreus-Reinhammer medium was substantially less than that reported by others.

The ability of *P. chrysosporium* and *T. versicolor* to solubilize leonardite was reinvestigated using several solid media. *P. chrysosporium* mediated extensive solubilization of leonardite when the fungus was grown on Sabouraud agar and nitrogen limited Sabouraud agar. Less extensive solubilization was observed when the fungus was grown on Yeast malt agar and Potato dextrose agar. *T. versicolor* mediated extensive solubilization of leonardite when the fungus was grown on yeast malt agar, Sabouraud agar and nitrogen limited Sabouraud agar. Solubilization was less extensive when the fungus was grown on potato dextrose agar. Little or no solubilization of leonardite occurred when *P. chrysosporium* or *T. versicolor* were grown on malt agar.

Introduction

Our research focuses on the mechanisms by which wood rotting fungi solubilize, leonardite, a low rank coal. In experiments summarized in previous quarterly reports, we assessed leonardite solubilization by a spectrophotometric assay. In this assay, the absorbance at 600 nm of a solution of soluble coal macromolecule is acquired and a conversion factor is used to convert absorbance to mg soluble coal macromolecule/mL. Although this is an easy and straight forward assay, there are a few drawbacks to its use. For example, the absorbance of soluble coal macromolecule tends to increase slightly with pH. Furthermore, it appears that, in some investigations, long term incubation of leonardite with sodium oxalate appears to result in preparations of soluble coal macromolecules having greater extinction than that found for preparations of coal macromolecules that were used to standardize this assay. This appears to have resulted in overestimation of the amount of coal solubilized in long term incubations.

During this reporting period we also measured the amount of oxalate produced by agitated pelleted cultures of *P. chrysosporium* and *T. versicolor.* The ability of *P. chrysosporium* and *T. versicolor* to solubilize leonardite as a function of the composition of solid growth media was also investigated.

Results and Discussion

Development of gravimetric assays to assess biomimetic solubilization of leonardite.

During several experiments during which our spectrophotometric assay was used to study the effect of incubation time on the biomimetic solubilization of leonardite, we noted that after several days a rather dramatic increase in absorption at 600 nm (the wavelength used to monitor leonardite solubilization) occurred. This initially suggested to us that during extended incubation, a process occurs that results in increased solubilization of leonardite. More recent experiments (Table 1) suggest that this is not the case. In these experiments, in which leonardite solubilization was assessed gravimetrically, 1 gm of leonardite was incubated, with stirring, with 100 mL of 74.5 mM sodium oxalate for 10, 20 and 26 days. Experiments were performed in triplicate. Following the prescribed incubation period incubation mixtures were centrifuged at ~13,000 x g for 30 min. This was necessary to ensure that very fine particulate material was pelleted. The supernatant was carefully removed and the pellet was dried at 105^{o}C overnight. The dried pellets were then scrapped from the centrifuge tubes and their masses were determined using an analytical balance.
In Table 1 the gravimetric assay demonstrates that extended incubation in the

presence of sodium oxalate results in a maximum leonardite solubilization of about 48%. Table 1 also demonstrates that during extended incubations when percent leonardite solubilization is calculated spectrophotometrically, the percent of leonardite solubilized is overestimated substantially. It should be noted that in previous experiments, the time of appearance of increased absorbance that occurred upon extended incubation was not predictable. In some experiments it appeared after two to three days while in others it occurred after five to six days. In Table 1, this phenomenon was apparent only in the experiments that were incubated for twenty six days. Taken together, these results indicate that solubility assays based on visible absorbance of coal macromolecules tend to overestimate the degree of coal solubilization that occurs during extended incubations.

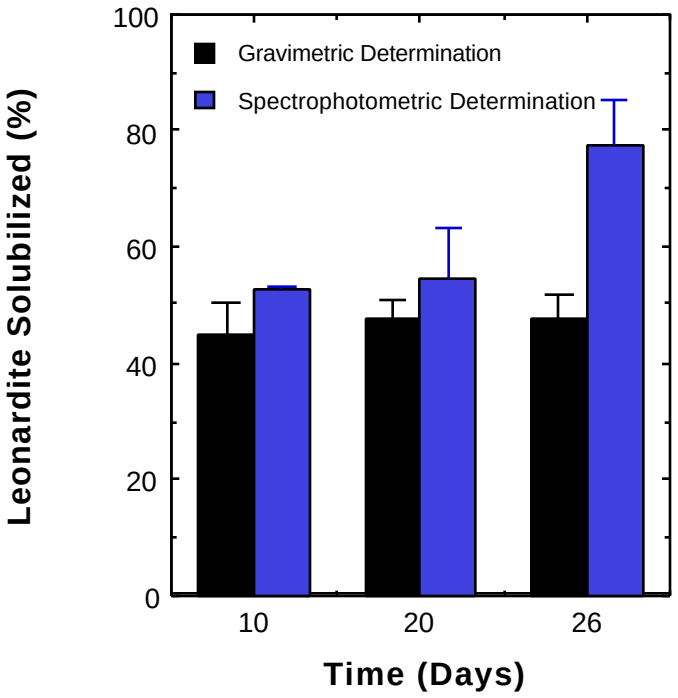

Table 1. Biomimetic solubilization of leonardite in sodium oxalate solution (Centrifugation gravimetric assay). In these experiments, 1 gm of leonardite was stirred with 100 mL of 74.5 mM sodium oxalate in water for the times indicated. Reactions mixtures were centrifuged at 13,000 x g for 30 min. The supernatants were then carefully removed. The pellets were then dried and their masses were determined. The percent of leonardite that was solubilized was calculated from this data. The absorbance at 600 nm of the supernatants of each sample was also determined and the percent solubilization was calculated using the relationship A_{600} 1.71 = 1 mg/mL .

We have also developed another gravimetric procedure to assess leonardite solubilization. A straight forward procedure to assess leonardite solubilization would entail determining the initial dry mass of leonardite, treating it with a solubilizing agent, in aqueous solution, filtering the solution and collecting the particulate material that

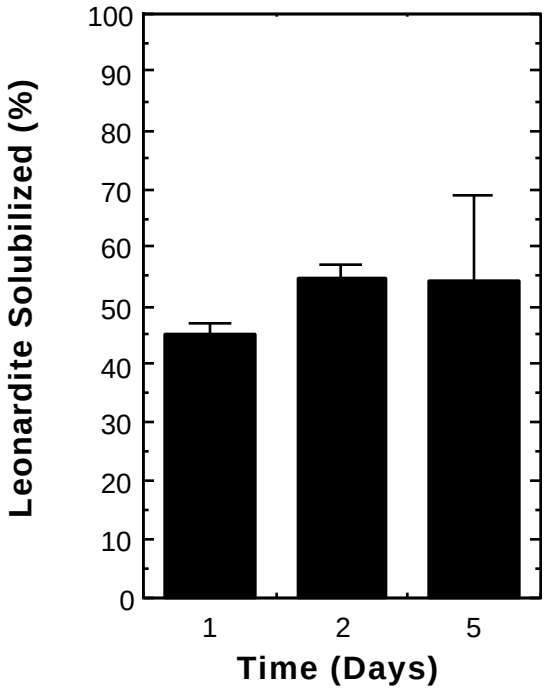

Table 2. Biomimetic solubilization of leonardite in sodium oxalate solution (Filtration gravimetric assay). In these experiments, 20 mg of leonardite was stirred with 10 mL of a 74.5 mM solution of sodium oxalate in water for the times indicated. Samples were then filtered through a tared 0.45 µm nylon filter. The filter containing nonsolubilized material was dried and its mass was determined. The percent of leonardite that was solubilized was calculated from this data.

was not solubilized on a tared filter paper. The mass of the nonsolubilized particulate material could then be determined and the amount or percent of leonardite solubilized could be calculated. In practice, this approach is complicated by two things: 1) nonsoluble particulate material is extremely fine and is not retained by many types of filter paper used in the laboratory and 2) Filter paper that is able to retain this fine material is easily plugged. After considerable trial and error, we developed a workable procedure. In our most recent experiments (Table 2), we have reduced the volume to be filtered in our experiments to 10 mL. Similarly we have reduced the amount of leonardite used in these experiments to 20 mg. The reduced mass and volume allows us to collect insoluble particulate material on 0.45 µm nylon filters and to calculate the percent of leonardite solubilized as outlined above. Results in Table 2 are generally consistent with gravimetric results presented in Table 1 in which the percent of

leonardite solubilized was calculated following centrifugation, drying and determination of the mass of the pellet.

Determination of oxalate concentration.

It appears that oxalate has an important role in solubilization by fungi of low rank coals such as leonardite. It is therefore useful to be able to measure oxalate concentration in fungal cultures. There are several techniques available to measure oxalate in aqueous solution. We used the procedure described by Dutton *et al* . (1991). In this procedure oxalate concentration is measured by high performance liquid chromatography. Production of oxalate in pelleted agitated cultures of *P. chrysosporium* and *T. versicolor* grown in Fahraeus Reinhammer medium (2) are presented in Figures 1 and 2.

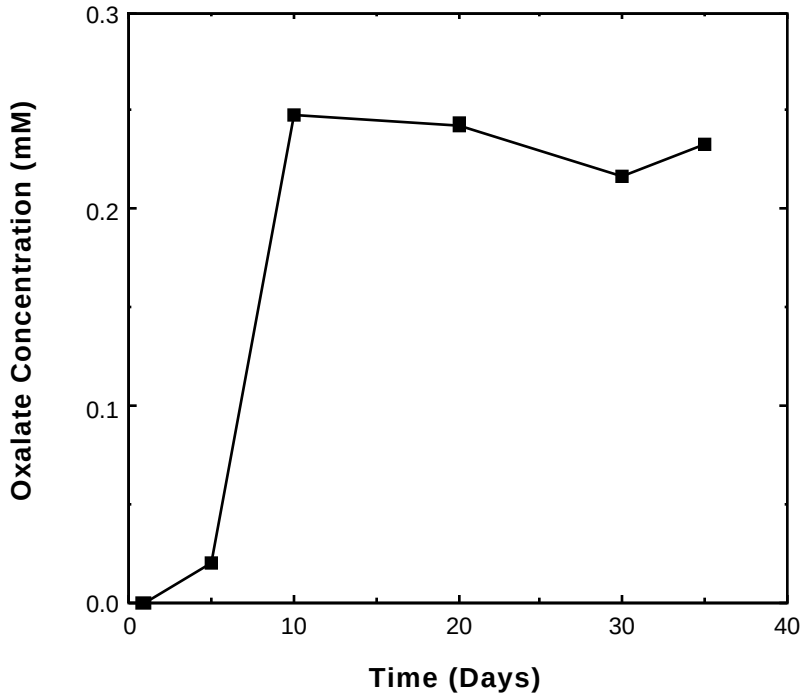

Figure 1. Oxalate production in agitated pelleted cultures of *P. chrysosporium.* Fungal cultures (100 mL) were grown on Fahreus-Reinhammar media.

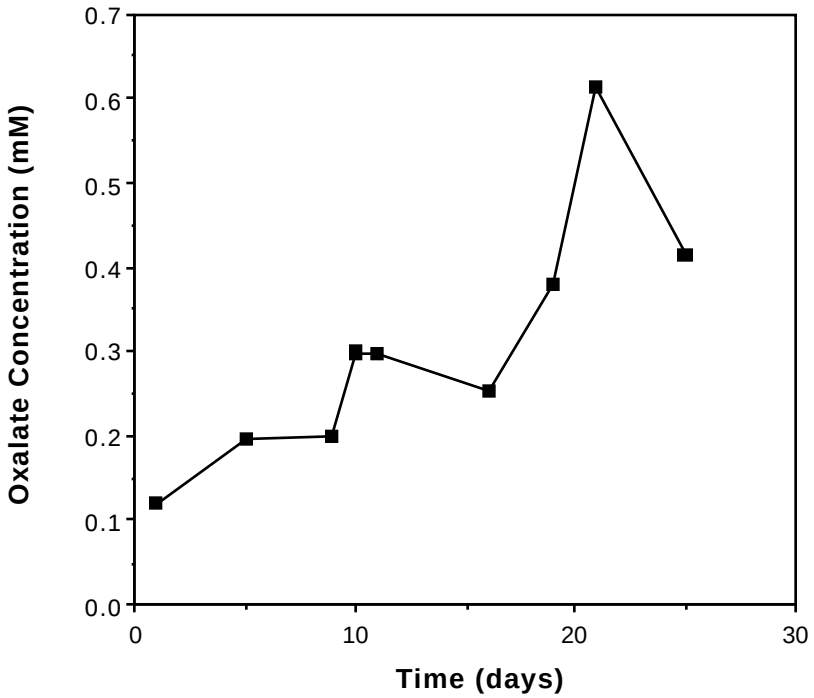

Figure 2. Oxalate production in agitated pelleted cultures of *T. versicolor.* Fungal cultures (100 mL) were grown on Fahreus-Reinhammar media.

The highest concentrations (~0.25 and 0.6 mM, respectively) of oxalate produced were found to be substantially less that known to be optimal (*i.e.,* ~75 mM oxalate) for leonardite solubilization.

Conclusions

1) During extended incubations in which biomimetic solubilization of leonardite by sodium oxalate is studied using a spectrophotometric assay, the potential exists for overestimating the amount of leonardite that is solubilized. This can be avoided in such investigations by using gravimetric assays.

2) An assay based on one described by Dutton *et al.* (1991) was used to measure concentrations of oxalate produced in pelleted agitated cultures of *P. chrysosporium* and *T. versicolor* grown on Fahraeus Reinhammar medium. It appears that concentrations of oxalate are substantially lower than those that are optimal for leonardite solubilization.

References

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