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Tenth Quarterly Report  
Regulation of Coal Polymer Degradation by Fungi  
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## Abstract

It has long been known that low rank coal such as leonardite can be solubilized by strong base (>pH 12). Recent discoveries have also shown that leonardite is solubilized by Lewis bases at considerably lower pH values and by fungi that secrete certain Lewis bases (*i.e.*, oxalate ion). During the current reporting period we have studied the ability of a strong base (sodium hydroxide, pH 12), and two fungi, *Phanerochaete chrysosporium* and *Trametes versicolor*, to solubilize Argonne Premium Coals. In general, Argonne Premium Coals were relatively resistant to base mediated solubilization. However, when these coals were preoxidized (150°C for seven days), substantial amounts of several coals were solubilized. Most affected were the Lewiston-Stockton bituminous coal, the Beulah-Zap lignite, the Wyodak-Anderson subbituminous coal and the Blind Canyon bituminous coal. Argonne Premium Coals were previously shown by us to be relatively resistant to solubilization by sodium oxalate. When preoxidized coals were treated with sodium oxalate, only the Beulah-Zap lignite was substantially solubilized. Although very small amounts of the other preoxidized coals were solubilized by treatment with oxalate, the small amount of solubilization that did take place was generally increased relative to that observed for coals that were not preoxidized. None of the Argonne Premium Coals were solubilized by *P. chrysosporium* or *T. versicolor*. Of considerable interest, however, is the observation that *P. chrysosporium* and *T. versicolor* mediated extensive solubilization of Lewiston-Stockton bituminous coal, the Beulah-Zap lignite and the Wyodak-Anderson subbituminous coal.

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## Executive Summary

In a previous quarterly report we noted that, compared to leonardite, Argonne Premium Coals and preoxidized Argonne Premium Coals were resistant to solubilization by sodium oxalate. In the present investigation, we studied the ability of a strong base to solubilize Argonne Premium Coals. We also showed that *Phanerochaete chrysosporium* and *Trametes versicolor* solubilized preoxidized Lewiston-Stockton bituminous coal, Beulah-Zap lignite and Wyodak-Anderson subbituminous coal. Our results suggest that mechanisms other than oxalate mediated solubilization are probably important in the solubilization of these coals.

Our investigations of the decolorization and depolymerization of coal macromolecule in cultures of *P. chrysosporium* were continued. Results demonstrate that decolorization is, indeed, an appropriate surrogate assay for coal macromolecule depolymerization.

We also repeated our studies assessing the role of laccase in leonardite solubilization. Under our conditions, laccase had no role in leonardite solubilization.

The interaction of crude lignin peroxidase with biomimetically solubilized coal macromolecule was also investigated. It has been suggested by others that this enzyme is involved in the depolymerization of soluble coal macromolecule. At low concentrations soluble coal macromolecule, inhibition is reversible and appears to be competitive with the reducing cosubstrate (veratryl alcohol) and the oxidizing cosubstrate (hydrogen peroxide). At high concentrations of coal macromolecule, inhibition appears to be irreversible.

## Introduction

There is considerable interest in the mechanisms by which fungi solubilize coal (1). Results in our laboratory and by others (Reference 1 and references therein) suggest that coal solubilization and subsequent depolymerization of the solubilized coal macromolecule are distinct events. Fungi solubilize leonardite by secreting oxalate ion which chelate metal ions in coal and, in the process, break ionic linkages which bind coal macromolecules together (2,3). Subsequent depolymerization of soluble coal macromolecule appears to occur by another process, possibly involving enzymes of the lignin degrading system. A number of common Lewis bases are also able to solubilize leonardite. Because oxalate is important in leonardite solubilization *in vivo*, the use of oxalic acid salts *in vitro* for this purpose may be considered to be a biomimetic process. It was of interest to determine the role, if any, of oxalate in the solubilization of other coals. In a previous quarterly report we noted that compared to leonardite, Argonne Premium Coals and preoxidized Argonne Premium Coals were resistant to solubilization by sodium oxalate. In the present investigation, we studied the ability of a strong base to solubilize Argonne Premium Coals. We also assessed the ability of *Phanerochaete chrysosporium* and *Trametes versicolor* to solubilize these coals. Our results suggest that, in addition to oxalate-mediated solubilization, there may be other mechanisms that are important in the solubilization of these coals.

We also continued our investigations of the decolorization and depolymerization of coal macromolecule in cultures of *P. chrysosporium*. Our results demonstrate that decolorization is, indeed, an appropriate surrogate assay for coal macromolecule depolymerization.

We also repeated our studies assessing the role of laccase in leonardite solubilization. Under our conditions, laccase had no role in leonardite solubilization.

Lastly, the kinetics and mechanism by which soluble coal macromolecule inhibits lignin peroxidase was studied.

## Results and Discussion

### **Solubilization of Preoxidized and Nonoxidized Argonne Premium Coals by Sodium Hydroxide and by Two Wood Rotting Fungi.**

In general, Argonne Premium Coals were relatively resistant to base mediated solubilization. However, when these coals were preoxidized (150°C for seven days), substantial amounts of several coals were solubilized. Most affected were the

Lewiston-Stockton bituminous coal, the Beulah-Zap lignite and the Wyodak-Anderson subbituminous coal. Argonne Premium Coals that were not preoxidized were also resistant to solubilization by *P. chrysosporium* and *T. versicolor*. However, these fungi were able to solubilize substantially, preoxidized Lewiston-Stockton bituminous coal, Beulah-Zap lignite and Wyodak-Anderson subbituminous coal. In our ninth quarterly report we noted that, relative to leonardite, Argonne Premium Coals and preoxidized Argonne Premium Coals were resistant to sodium oxalate mediated solubilization.

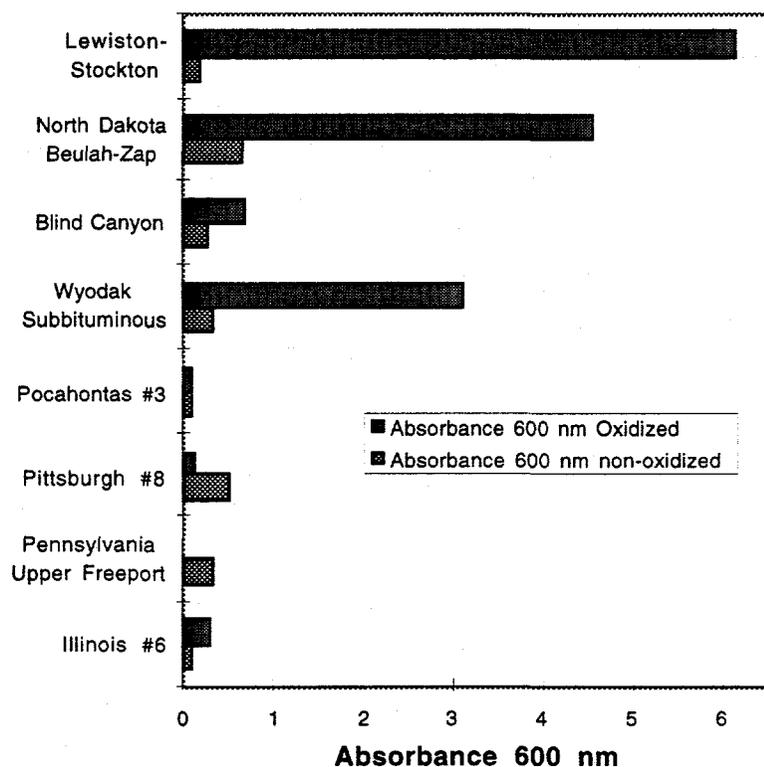


Figure 1. Solubilization of Argonne Premium Coals by Sodium hydroxide (pH 12). For each coal, a 20 mL suspension of 1 M sodium hydroxide and 20 mg of each coal was prepared and mixed for 24 h. An aliquot was then centrifuged and the absorbance of the sample at 600 nm was determined.

After acquiring results of base and biologically mediated solubilization we have reexamined this data and note that, although small amounts were solubilized, preoxidation did appear to have a marginally beneficial effect on oxalate mediated solubilization. Of the preoxidized Argonne Premium Coals tested the one of lowest rank, the Beulah-Zap lignite was most affected by preoxidation and most amenable to oxalate mediated solubilization. Solubilization, however, was considerably less than that observed for leonardite.

Our studies dealing with coal solubilization by fungi are of most interest. That the Beulah-Zap lignite was solubilized can be explained by the fact that of all the Argonne Premium Coals, this coal was the most amenable to oxalate mediated solubilization. The fact that the Lewiston-Stockton bituminous coal and the Wyodak-Anderson subbituminous coal were solubilized by *P. chrysosporium* and *T. versicolor* is important because these coals are clearly not amenable to oxalate mediated solubilization and these results suggest that other factor, possible degradative enzymes, are involved in biodegradation of these higher rank coals. There are only a few studies dealing with the solubilization of subbituminous and bituminous coals by wood rotting fungi. Achi (4) showed that several members of the Basidiomycetes were able to mediate solubilization of a preoxidized Nigerian subbituminous coal and Scott *et al.* (5) reported that *T. versicolor* solubilized trace amounts of a Wyodak subbituminous coal. Stewart *et al.* (6) reported that *P. chrysosporium* was able to mediate solubilization of trace amounts of Pennsylvania Upper Freeport Bituminous Coal, but did not solubilize a similar sample that had been preoxidized. Our observation that *P. chrysosporium* and *T. versicolor* solubilized substantially, a bituminous coal (preoxidized Lewiston-Stockton bituminous coal) is a relatively uncommon observation for a member of the Basidiomycetes. It should be noted, however, that certain Ascomycetes (*Penicillium sp.* and *Cunninghamella sp.*) have been reported to solubilize three bituminous coals (Illinois # 6, Pittsburgh # 8 and Pennsylvania Upper Freeport bituminous coals). Taken together, these reports and our observations suggest that oxalate mediated solubilization is important only for the initial solubilization of highly oxidized low rank coals. For subbituminous and bituminous coals that are amenable to fungal solubilization it appears that some process other than metal chelation and subsequent cleavage of ionic linkages is required as an initial event in solubilization of such coals. Although oxalate is not responsible for initial solubilization of subbituminous and bituminous coals, it is possible that oxalate may still have a role in aiding or increasing solubilization of such coals.

#### **Decolorization of Coal Macromolecule in Liquid Cultures.**

In our ninth quarterly report we demonstrated that extensive decolorization of coal macromolecule occurred in stationary and agitated liquid cultures of *P. chrysosporium* ( $83.3 \pm 2.3\%$  and  $89.6 \pm 1.0\%$ , respectively). During the current reporting period, we repeated this experiment in stationary cultures using a greater initial concentration (820 mg/L) of soluble coal macromolecule. We observed  $46.2 \pm 3.0\%$  (N=9) decolorization. More importantly we observed a considerable decrease in molecular weight of soluble coal macromolecule. During this twelve day incubation MW decreased from ~30,000 to ~18,000 in all nine incubations.

#### **Effect of laccase on Coal Solubilization.**

It has been suggested that the laccase from *Trametes versicolor* may have a role in solubilization of low rank coal (7,8). However, a more recent assessment suggests that laccase has a limited role in solubilization of leonardite (9). Additionally, previous

experiments in our laboratory do not support a role for laccase in this process. Because of conflicting data in the literature, we repeated this investigation during this reporting period. In addition to assessing the role of laccase in coal solubilization we also wished to further develop procedures that would allow us to purify laccase on a scale smaller than the 60 L scale that was published by Fahraeus and Reinhammer (10). In these investigations 250 mL agitated pelleted cultures of *T. versicolor* were grown in 500 mL Erlenmeyer Flasks using procedures that are very similar to those previously published (10). Our procedures differed in that cultures were grown in 500 mL flasks instead of 1 L flasks and the cultures were used as a source of enzyme rather than an inoculum for larger scale production. After two days 2,5-xylydine ( $2 \times 10^{-4}$  M) was added to cultures to induce laccase activity. A second addition of 2,5-xylydine ( $1 \times 10^{-4}$  M) was made after 3 more days along with addition of 1% (w/v) glucose. After 3 more days of incubation when laccase activity was greatest cultures were harvested. Several procedures were used to concentrate the crude enzyme preparation for subsequent chromatography. These were ammonium sulfate precipitation, concentration in Centriprep (Amicon) tubes and lyophilization. Of the three procedures, lyophilization was the most convenient. Following lyophilization, the enzyme preparation was dissolved in a minimal volume of water and two mL were passed over a PD-10 (G-25) column equilibrated in 100 mM potassium phosphate buffer, pH 6.0 as needed. This preparation was used to assess the effect of laccase activity on leonardite solubilization. In these experiments, 20 mg of leonardite was added to a 20 mL scintillation vial. 1.8 mL of 100 mM potassium phosphate buffer, pH 6.0 and 200  $\mu$ L of the laccase preparation were then added with gentle mixing and the mixture was allowed to incubate at room temperature for 48 h. The initial laccase activity of this preparation was approximately four times that which would be present in induced cultures of *T. versicolor*. During this incubation, no coal solubilization occurred as evidenced by the fact that an increase of absorbance at 600 nm did not occur in treated samples.

#### **Inhibition of lignin peroxidase by biomimetically solubilized coal macromolecule.**

Initial experiments concerning inhibition of lignin peroxidase by biomimetically solubilized coal macromolecule were performed by that part of our research group located at the University of Northern Iowa. Continued studies concerning this subject are also being pursued by our research group located at the University of Notre Dame. Figures 2 and 3 confirm our earlier observation that soluble coal macromolecule is a potent inhibitor of lignin peroxidase mediated veratryl alcohol oxidation. Initial observations suggested that soluble coal macromolecule is a good inhibitor of lignin peroxidase because it is a good substrate. It was also observed that in incubation mixtures containing both veratryl alcohol and soluble coal macromolecule, a lag

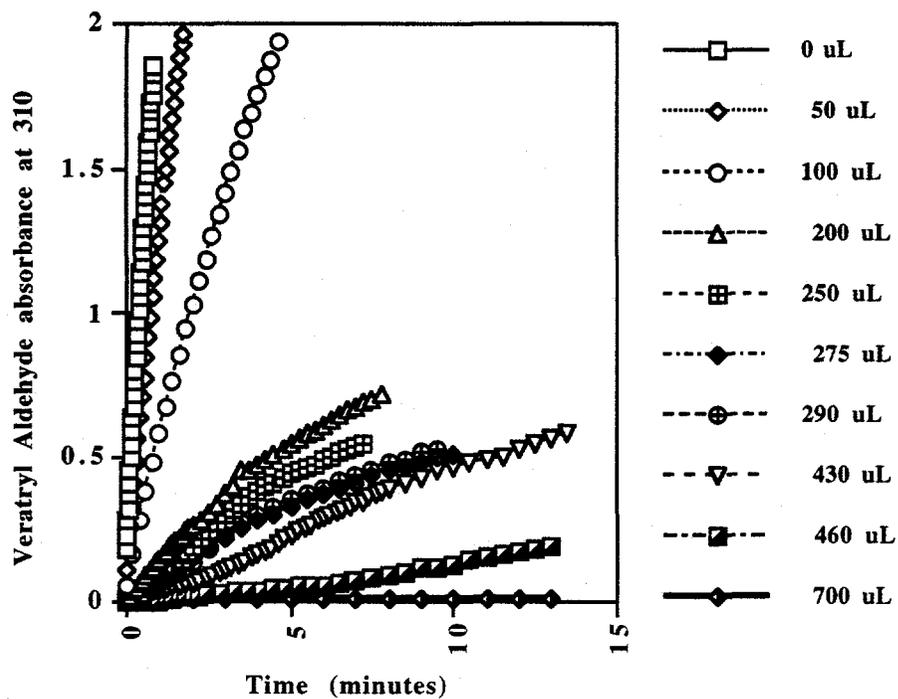


Figure 2: Inhibition of veratryl alcohol oxidase activity in the presence of oxalate solubilized coal.

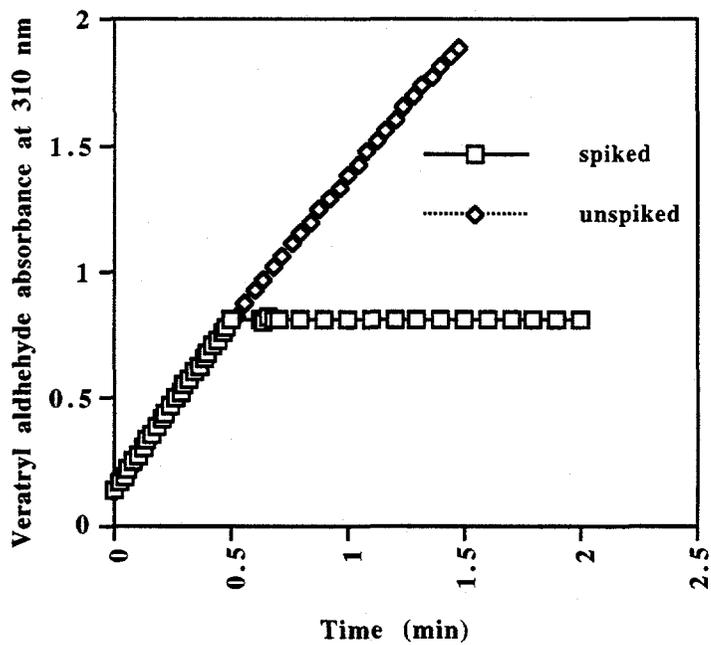


Figure 3: Complete inhibition of veratryl alcohol oxidase activity by 10X concentrated stock of oxalate solubilized coal.

occurred before veratryl alcohol oxidase activity could be observed (measured by monitoring formation of veratryl aldehyde at 310 nm). This suggested that, for lignin peroxidase, coal macromolecule is a much better substrate than veratryl alcohol (its natural reducing cosubstrate). An important new observation is that at very low concentrations of soluble coal macromolecule inhibition of veratryl alcohol oxidase activity still occurs, but the lag does not occur. This is important because it allowed inhibition by Lineweaver-Burke analysis to be assessed (figures 4 and 5). In these experiments, lignin peroxidase activity was assayed by procedures described by Tien and Kirk (11). The concentration of stock coal macromolecule was 185  $\mu\text{g}/1\text{ mL}$ .

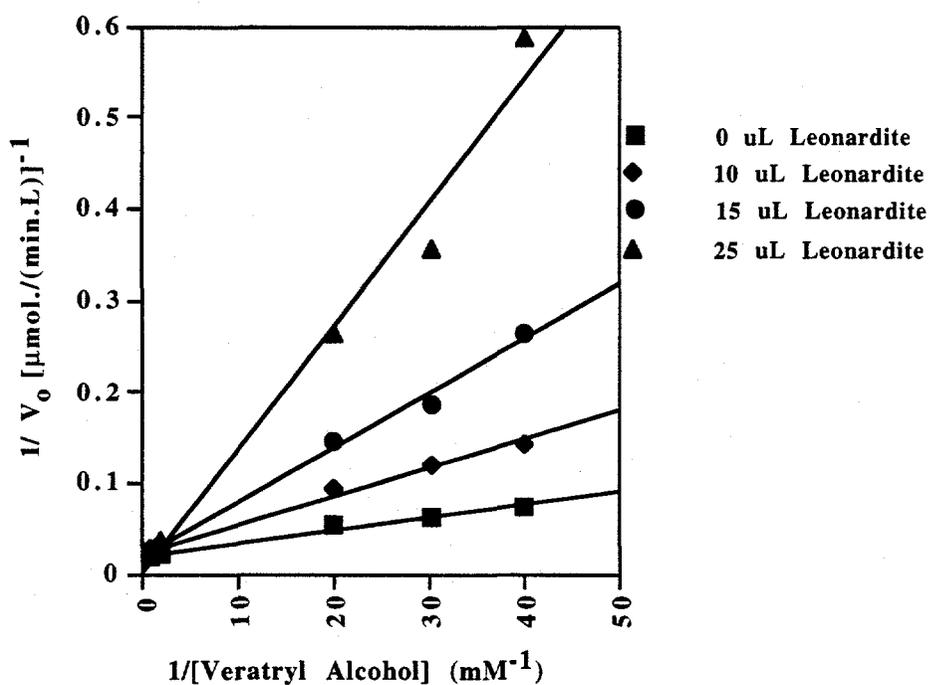


Figure 4: Double Reciprocal Plot for veratryl alcohol oxidase activity with oxalate solubilized leonardite and veratryl alcohol as the varied substrates.

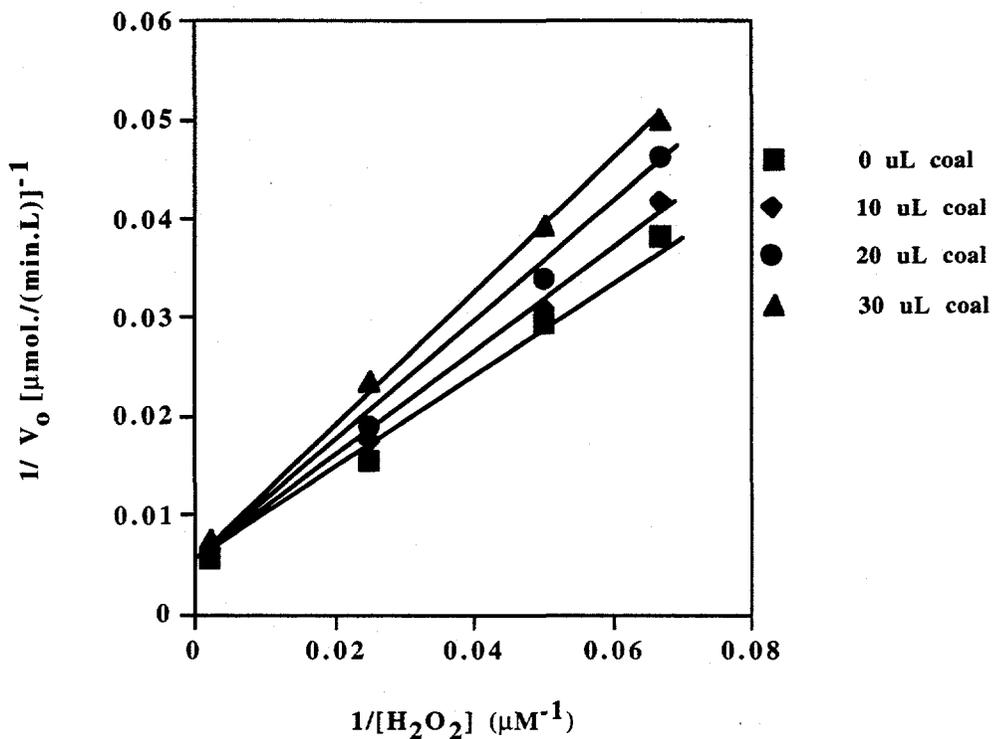


Figure 5: Double reciprocal plot for veratryl alcohol oxidase activity with oxalate solubilized macromolecule and hydrogen peroxide as the varied substrates.

Of considerable interest is the observation that soluble coal macromolecule appears to be a competitive inhibitor for both the oxidizing cosubstrate (hydrogen peroxide) and the reducing cosubstrate (veratryl alcohol). It is unusual for another molecule to be a competitive inhibitor for both cosubstrates in a peroxidase mediated reaction. It is not, however, impossible as this could occur if coal macromolecule serves as a good reducing cosubstrate (*i.e.*, being competitive with veratryl alcohol) and as a reversible competitive inhibitor (but not as a substrate) for the oxidizing cosubstrate (hydrogen peroxide). The inhibition of lignin peroxidase mediated veratryl alcohol oxidation by soluble coal macromolecule is further complicated by the fact that inhibition is irreversible when high concentrations of coal macromolecule are present. Elucidation of the mechanisms by which lignin peroxidases are inhibited and inactivated by soluble coal macromolecule is a topic of continued investigation by our research groups.

## Conclusions

1. *P. chrysosporium* and *T. versicolor* solubilized preoxidized Lewiston-Stockton bituminous coal and Wyodak-Anderson subbituminous coal. This is of interest because these coals are not solubilized appreciably by sodium oxalate. Thus, the mechanism by which these coals are solubilized by *P. chrysosporium* and *T. versicolor* appears to be different than the mechanism by which highly oxidized low rank coals, such as leonardite are solubilized.
2. Decolorization of soluble coal macromolecule in fungal cultures appears to be a good surrogate assay for depolymerization.
3. Laccase does not appear to have a role in solubilization of leonardite.
4. The inhibition of lignin peroxidase by soluble coal macromolecule is complex and occurs by several mechanisms. At low concentrations coal macromolecule is a competitive inhibitor of both veratryl alcohol (the enzyme's natural reducing cosubstrate) and hydrogen peroxide (the enzyme's natural oxidizing cosubstrate). At high concentrations coal macromolecule appears to be an irreversible inactivator of the enzyme.

## References

- 1) Crawford, D.L. (Ed.) Microbial transformations of low rank coal. CRC Press, Boca Raton 223 p.
- 2) 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.
- 3) Frederickson, J.K., Stewart, 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.)
- 4) Achi, O.K. (1993) Studies on the microbial degradation of a Nigerian Preoxidized subbituminous coal. Int. Biodeter. Biodeg. 31:293-303.
- 5) Scott, C.D., G.W. Strandberg and S.N. Lewis (1986) Microbial solubilization of coal. Biotechnol. Prog. 2:131-139.
- 6) Stewart, D.L., B.L. Thomas, R.M. Bean and J.K. Fredrickson (1990) Colonization and degradation of oxidized bituminous and lignite coals by fungi. J. Ind. Microbiol. 6:53-59.
- 7) Pyne, J.W., D.L. Stewart, J. Fredrickson and B.Wilson (1987) Solubilization of leonardite by an extracellular fraction from *Coriolus versicolor*. Appl. Environ. Microbiol. 53:2844-2848.
- 8) Cohen, M.S., W.C. Bowers, H. Aronson and E.T. Gray Jr. (1987) Cell-free solubilization of coal by *Polyporus versicolor*. Appl. Environ. Microbiol. 53:2840-2843.
- 9) Quigley, D.R. (1993) The enzymology and chemistry of coal biosolubilization. In: Microbial transformations of low rank coal. (Ed. D.L. Crawford) CRC Press, Boca Raton 223 p.
- 10) Fahraeus, G. and B. Reinhammar (1967) Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. Acta Chem. Scand. 21:2367-2378.
- 11) Tien, M. and T.K. Kirk (1984) Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H<sub>2</sub>O<sub>2</sub>-requiring oxygenase. Proc. Natl. Acad. Sci. USA 81:2280-2284.