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DOE/ER/13586--T2

DE93 011726

TO: Cathy Waldrop, USDOE

FAX #: 615-576-2865

FROM: Sandy Watson for Don Crawford, Professor

DATE: April 20, 1993

# of pages (including cover) 16

MESSAGE: I am FAXing a copy of the final technical report and cover letter that was sent to Dalles Hoeffer today. Call me if you have any questions.

We are transmitting this fax to you by means of our Omnifax G36D.

Please call Sandy Watson at (208) 885-7966 if you do not receive all of the pages.

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Bacteriology
Biochemistry

April 19, 1993

Mr. Dalles Hoeffer
Contract Management Division
Financial Assistance Branch
USDOE
785 DOE Place, 1221 Mail Stop
Idaho Falls, ID 83401-1562

RE: Expired Grant DE-FG07-86ER-13586

Dear Mr. Hoeffer:

I received a call from Linda Janson, accountant with the UI Grants and Contracts office, last week. She had received a call from you asking for a final technical report for the above referenced grant. Once again I am submitting this report, for the *third* time. This report was initially submitted October 21, 1992. A call was received in February 1993 asking for this same report as it had not yet been received. I mailed another copy to you at that time. This is not the first time this has happened to me. Your apparent lack of coordinated effort or ability to properly reference information has me rather upset and concerned. I trust this copy will "cure" the deficiency in your records.

According to Ms. Janson, Cathy Waldrop has contacted you regarding the need for the final report. I have FAXed a copy of this letter and report to her also. Please make sure this report is filed and properly referenced so another request is not received from you. Thank you.

Sincerely,

Don L. Crawford

Don L. Crawford, Professor
Department of Bacteriology and Biochemistry

/sfw

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Bacteriology
Biochemistry

October 21, 1992

Dr. Robert Rabson
Director, Division of Energy Biosciences
Office of Basic Energy Sciences, ER-17
U.S. Department of Energy
Washington, D.C. 20585

Dear Dr. Rabson:

Enclosed is the **Final Technical Report** for my recently expired grant DE-FG07-86ER-13586, Biochemistry and Genetics of Lignin Biodegradation by *Streptomyces*. I have also sent a copy to the Idaho Operations Office of the Department of Energy. Let me know if you need anything additional.

I appreciate the funding that your program provided me between 1980 and today. That funding was extremely important to the success of my lignin biodegradation research program. While disappointed that you chose not to continue my grant for another round, I understand that you have limited funds to support research in this particular area. It is a pity that research on lignin biodegradation has now come to be essentially totally dominated by *Phanaerochaete* researchers who tend to have very narrow perspectives. Their dominance and self-centeredness has in turn affected the grant peer review process to a point where they are now pretty much able to be self-perpetuating. In the meantime, my research group has moved on. We are now using the biochemical, genetic, and enzymological knowledge base accumulated over the past decade to develop a new generation of biodegradable synthetic polymers and chemicals. We are also doing molecular ecology research that will ultimately determine the relative importance of different ligninolytic microorganisms in the process of lignin turnover in soil. We are also developing selected bioremediation processes that are much more effective and practical than the use of *Phanerochaete*. Our funding base has begun to shift to the U.S. EPA, U.S. DOD, and private industry.

I look forward to possibly working with you in the future.

Sincerely,

Don L. Crawford
Professor of Bacteriology

CC. Idaho Operations Office of DOE

DOE/ER/13586--T2

FINAL TECHNICAL REPORT

DOE Grant DE-FG07-86ER-13586

Genetics and Chemistry of Lignin Degradation by *Streptomyces*

Principal Investigator:

Donald L. Crawford
Professor of Bacteriology
Department of Bacteriology and Biochemistry
University of Idaho, Moscow, ID 83843

Grant Period: September 1, 1989 to August 31, 1992
Grant Amount: $246,000

Contracting Officer :

Dr. Robert Rabson, Director
Division of Energy Biosciences
Office of Basic Energy Research, ER-17
Department of Energy
Washington, D.C. 20585

Contracting Office:

Idaho Operations Office
Department of Energy
Idaho Falls, Idaho

Table of Contents

Summary of Research Accomplished

Our research goal was to define the involvement of lignin peroxidases and other extracellular enzymes in lignin degradation by *Streptomyces*. We examined the biochemistry and genetics of lignin degrading enzyme production by several strains of *Streptomyces*. The lignin peroxidase ALiP-P3 of *S. viridosporus* was characterized kinetically and its activity optimized for oxidation of 2,4-dichlorophenol and vanillyl-acetone. Sensitive spectrophotometric assays were developed for monitoring oxidation of these substrates. ALiP-P3 reaction chemistry was examined using both spectrophotometric assays and gas chromatography/mass spectroscopy. Results showed that the enzyme oxidizes phenolic lignin substructure models in strong preference to nonphenolic ones. The peroxidase was also shown to depolymerize native lignin. We also cloned the ALiP-P3 gene in *S. lividans* in plasmid vector pIJ702. The cloned gene was partially sequenced. We also immunologically characterized the lignin peroxidase of *S. viridosporus* T7A and showed it to be structurally related to peroxidases produced by other lignin-solubilizing *Streptomyces*, but not to the H8 lignin peroxidase of *P. chrysosporium*. Studies with peroxidase deficient mutants of strain T7A showed that lignin peroxidases of *S. viridosporus* are directly involved in the solubilization of lignin. Additional research showed that other enzymes are also probably involved in lignin solubilization, possibly including extracellular esterases. Overall the results expanded our knowledge of the biochemistry and genetics of actinomycete lignin degradation and helped define the role of peroxidases in lignin solubilization. The ultimate aim of the research is to utilize ligninolytic *Streptomyces*, or recombinant strains derived from them, and/or to utilize their enzymes, for 1) the bioconversion of lignin to useful chemicals, 2) the biological pulping or bio-bleaching of pulp, 3) the enzymatic activation of lignin polymers, and/or 4) the degradation of toxic aromatics in pulp mill waste effluents.

Background and Recent Research Progress

Chemistry, Enzymology and Genetics of Lignocellulose Degradation by Streptomyces and Other Actinomycetes. Lignin-degrading white rot fungi such as *Phanerochaete chrysosporium*, and lignin-solubilizing bacteria such as *Streptomyces* species, produce extracellular peroxidases that are involved in the initial oxidative depolymerization of lignin (1-4). In *P. chrysosporium*, these lignin peroxidases utilize H_2O_2 to nonselectively and nonstereospecifically oxidize chemical bond structures within lignin by withdrawing a single electron from a susceptible linkage. This in turn results in the formation of a cation radical within the molecule, which generates unstable structures that react with H_2O or O_2 and cause the lignin macromolecule to decompose (3). Because lignin peroxidases are nonspecific, they oxidize a variety of linkages within lignin and will catalyze the oxidation of numerous other compounds (3). Once the initial oxidative depolymerization of lignin occurs, the resulting products become substrates for other catabolic enzymes. The lignin peroxidases, thus, serve the important function of oxidizing and depolymerizing lignin to generate lower molecular weight catabolic intermediates. While more is known concerning lignin peroxidases of *P. chrysosporium*, it appears that the peroxidases of this fungus (2,3) and of lignin-solubilizing *Streptomyces* (1,2,4; also see **Recent Results**) function similarly.

As discussed below, lignin peroxidases of *P. chrysosporium* oxidize numerous chemical structures. Thus, the fungus decomposes a variety of xenobiotics (5-7). In fact, *P. chrysosporium* is the most versatile xenobiotic-decomposing microorganisms thus far described. *Streptomyces* are also noted for their abilities to decompose aromatic compounds (2), and some of these compounds are metabolized via pathways involving peroxidases (4).

Streptomyces are filamentous, aerobic bacteria (8) that participate in the decomposition of plant residues and organic polymers in soil, particularly polysaccharides such as chitin, cellulose, hemicellulose, and starch (8,9). They decompose lignocellulose in a process involving the oxidative depolymerization and solubilization of lignin (1,10) concomitant with the hydrolysis and assimilation of cellulosic and hemicellulosic polysaccharides (1). Thus, these actinomycetes are important to the long term maintenance of soil fertility. They participate in humification via the degradation of lignin into water soluble polymeric fragments that become incorporated into soil humus fractions (11-13).

Streptomyces and other actinomycetes thus far characterized have lesser abilities to decompose lignin than do white-rot fungi (2). They typically oxidize and decompose lignin to water soluble polymeric products, called acid precipitable polymeric lignins (APPL's) (2,10,11,14,15). Though most *Streptomyces* incompletely mineralize lignin, they catalyze its oxidative depolymerization via the excretion of lignin-oxidizing peroxidases that are involved in lignin degradation via the generation of APPLs (2,4,15-17). Peroxidase deficient mutants of *S. viridosporus* are also deficient in APPL-producing ability, but when such mutants express the recombinant plasmid-encoded ALiP-P3 lignin peroxidase gene of *S. viridosporus* (18,19), they are complimented back to APPL-producing ability (17,18). As with *P. chrysosporium* lignin peroxidases (2,3), the *Streptomyces* peroxidases carry out C-alpha/C-beta cleavage of dimeric lignin substructure models (4,20). The actinomycete enzymes, however, prefer phenolic substrates as opposed to nonphenolic ones preferred by *P. chrysosporium* lignin peroxidase (20). Thus, it appears that the initial oxidation of lignin by *Streptomyces* involves a peroxidase-based mechanism akin to that of white-rot

fungi. The inability of the *Streptomyces* to extensively mineralize lignin must be the result of their having lesser enzymatic capabilities to further degrade lignin fragments, and/or less powerful lignin peroxidases than those produced by fungi.

Low molecular weight aromatic substrates derived from lignin are readily degraded by actinomycetes. *Streptomyces* have been shown to degrade single ring aromatic compounds via classic aromatic catabolism pathways (21-26). Individual strains, however, vary greatly in their versatility. Some lignin-solubilizers such as *S. viridosporus* T7A degrade a small number of aromatic compounds (22,27), while others such as *S. setonii* 75Vi2 degrade a wide range (24-28). The ability of actinomycetes to degrade single ring aromatics is not always correlated with the ability to solubilize lignin (Sinden, D.L. 1979. M.S. Thesis, Univ. Idaho). While little research has been devoted to determining how actinomycetes catabolize multi-ring aromatic compounds, some strains do metabolize multi-ring substrates (21). The extracellular peroxidases of lignin-solubilizing *Streptomyces* such as *S. viridosporus* also catalyze reactions that oxidatively split dimeric lignin substructure model compounds into single-ring products (4,20). Therefore, it is likely that multi-ring aromatics are degraded by a variety of actinomycetes.

While it is apparent that peroxidases play an important role in lignin solubilization by *Streptomyces*, other enzymes appear to also be involved. Work in our laboratory has shown that extracellular esterases produced by *S. viridosporus* may be important in the cleavage of aromatic acids from lignocellulose (29). Recent results (see below) indicate that esterases may be important to the lignin solubilization process, possibly by acting to hydrolyze lignin polysaccharide bonds during lignocellulose degradation. Extracellular oxidases are also produced by *S. viridosporus*, including oxidases that oxidize unsaturated aromatic aldehydes (30) and those that oxidize short chain aliphatic aldehydes (18) to the corresponding acids. These enzymes also generate H_2O_2 as a byproduct of the oxidation. Such oxidases might act during lignin solubilization by oxidizing lignin's aromatic aldehyde groups to the corresponding acids, and they may also be important in generating extracellular H_2O_2 for use by the organism's peroxidases.

Confirmatory Research of Others. My research group has, until fairly recently, been pretty much alone in studying lignin degradation by actinomycetes. However, other researchers have recently become interested in our findings and have either obtained our *Streptomyces* strains (*S. viridosporus* T7A and *S. badius* 252), and/or have used their own strains of lignin-solubilizing actinomycetes to carry out research on lignin biodegradation (2). As their findings have been published, they have confirmed and extended our work. As a result, the reticence of some to believe that actinomycetes might possess lignin-oxidizing enzymes should now have been overcome. There is substantial accumulated literature on lignin metabolism by actinomycetes, from multiple laboratories. Other laboratories now actively doing research with ligninolytic *Streptomyces* include those of W. Zimmermann (Zurich), A. McCarthy (Liverpool), H. Giroux (Canada), R. Korus (Idaho), R. Vicuna (Chile), and R. Blondeau (France). Among the key confirmatory observations reported from these laboratories are:

1) the oxidative, enzymatic solubilization of lignin by actinomycetes (31-36),

2) the excretion of extracellular peroxidases by *Streptomyces* during growth on lignocellulose and during lignin solubilization (35,36),

3) the oxidation of veratryl alcohol by ligninolytic *Streptomyces* and their extracellular enzymes (36,37), and

4) the production of other extracellular enzymes relevant to lignin degradation, including esterases and oxidases (1, 31-37).

In particular, McCarthy's group (34-36) has found that extracellular peroxidases and veratryl alcohol oxidase activities became tightly bound to solubilized lignins present in culture supernatants when cultures of six different actinomycetes were grown on lignocellulose. Giroux's work (32,33) has shown that extracellular enzyme preparations from cultures of both *S. viridosporus* and *S. badius* will solubilize and depolymerize Kraft lignins. Blondeau's work (38, and unpublished data) shows that *S. viridosporus* produces extracellular peroxidases that appear to be involved in its degradation of humic acids.

Thus, the literature on lignin degradation by *Streptomyces* and other actinomycetes is now accumulating at a more rapid pace, and publications from other laboratories are confirming and extending our previous findings. Future research with a variety of actinomycetes will, of course, more clearly define the roles of extracellular peroxidases, oxidases, esterases, and polysaccharides in the lignin solubilization process.

Research Findings and Accomplishments

Our most recent research with lignin peroxidase deficient mutants of *S. viridosporus* has further increased our understanding of the importance of peroxidases and possibly esterases in lignin solubilization by *Streptomyces* (17). Peroxidases are required for efficient solubilization of lignin by this actinomycete. Peroxidase deficient mutants are significantly impaired in APPL production ability. These mutants also tend to be altered in the production of certain extracellular esterases.

Using polyclonal antibodies, we have also examined the immunological relatedness of the lignin peroxidases produced by several lignin-solubilizing *Streptomyces*, to one another and to the H8 lignin peroxidase of *P. chrysosporium* (16). Polyclonal antibodies prepared against purified ALiP-P3 protein bind to the peroxidases of other lignin-solubilizing *Streptomyces*. However, they do no bind the H8 ligninase, nor does an anti-H8 antibody bind the ALiP-P3 enzyme. Thus, the actinomycete peroxidases appear to be structurally similar, but distinct in structure from the fungal enzyme, at least at the immunological level.

We have also further characterized the reaction chemistry of the *S. viridosporus* ALiP-P3 lignin peroxidase using phenolic and nonphenolic lignin substructure model compounds and native lignins as substrates (20,39). This work shows that the enzyme preferentially oxidizes phenolic substrates as opposed to nonphenolic ones. Very importantly, the ALiP-P3 peroxidase depolymerizes native lignin when reactions are run using the procedure of Hammel (40). This finding confirms a lignin catabolism function of this actinomycete enzyme.

Finally, we have recently used our knowledge of lignin degradative enzyme chemistry to synthesize a group of modified azo dyes that are still excellent dyes, but are much more readily biodegraded by ligninolytic actinomycetes and fungi than are current commercial azo dyes (41). By introducing lignin-like substitution patterns into

aromatic ring components of these dyes, they were made to be susceptible to oxidation and complete degradation by the ligninolytic systems of these microorganisms.

Significance

Our results and that of others shows that there is potential for developing lignin bioconversion processes that can address this nation's long term needs for chemical feedstocks, energy in the form of alternate liquid fuels, and a cleaner environment. The enzymes and genetic systems used by microorganisms to degrade lignin are now being elucidated rapidly. Results of studies with extracellular peroxidases indicate that these lignin oxidizing enzymes, or chemical processes that mimic them, might replace the chlorine-based technologies currently used to delignify and bleach pulp. If developed, optimized biological or biomimetic delignification processes would be less energy intensive and more environmentally benign than currently used chlorine-based processes. Lignin-oxidizing enzymes may also prove useful for the controlled modification of lignin to produce useful, more chemically reactive polymers. A possibly soon to be realized use of ligninolytic microorganisms will be for the bioremediation of toxic chemicals (5-7,39). The peroxidases of ligninolytic fungi (5-7,41) and of actinomycetes (20,39) nonspecifically attack a variety of chemical bond structures within a variety of nitroaromatics, chloroaromatics, azo dyes, and other xenobiotics. As a result, lignin degrading microbes such as *P. chrysosporium* are being actively studied for their bioremediation potential. When used in concert with cellulolytic and/or hemicellulolytic microorganisms (or isolated cellulases or hemicellulases), lignin-oxidizing enzymes may also prove important to the development of bioconversion processes for production of liquid or gaseous fuels (e.g., ethanol, methane) or chemical feedstocks from lignocellulose. For such bioconversions to proceed, the "lignin barrier" must be removed or opened to allows cellulases to penetrate and access polysaccharide components. Ligninases might serve this function in saccharification processes. One can also envision using recombinant *Streptomyces*, amplified in the production of lignin peroxidases, and/or other ligninases, to directly bioconvert lignin into low molecular weight chemicals.

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Publications, Theses, and Presentations Supported by This Grant

Published Papers (1988-1992)

1) Ramachandra, M., D.L. Crawford, and G. Hertel. 1988. Characterization of an extracellular lignin peroxidase of the lignocellulolytic actinomycete *Streptomyces viridosporus* T7A. Appl. Environ. Microbiol. **54**: 3057-3063.

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*This research was partially supported by USDA. **This research was partially supported by EPA and NSF.

Theses

1) Jennifer K. Spiker, M.S. Bacteriology. University of Idaho, Spring 1991. Substrate Specificity and Reactions Catalyzed by the Lignin Peroxidase of *S. viridosporus*.

2) Timothy S. Magnuson, M.S. Bacteriology. University of Idaho, Spring 1991. Enzymology and Immunochemistry of Extracellular Ligninases from Lignin-Solubilizing *Streptomyces* strains.

Presentations

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