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Renewal Proposal to the Department of Energy
Division of Biological Energy Research
Office of Basic Energy Sciences

Current grant number DE-AS07-80-ER10775, The Metabolism of Aromatic Compounds
by Actinomycetes.

Principal Investigator:

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Moscow, ID 83843

Current Support, Year One: \$67,080, beginning September 1, 1980

INTRODUCTION

By way of this renewal proposal I am requesting continued funding for year two of my research grant DE-AS07-80-ER10775, Metabolism of Aromatic Compounds by Actinomycetes. Below, I have summarized our current research progress. There have been no significant deviations from the original research plan which was funded, and progress is satisfactory. In light of the fact that we are only about 7 months into year one of this 3 year project, we have only recently begun to make substantial progress towards fulfilling the objectives of the research grant. However, as outlined below, I feel that the project is on schedule and significant findings are being made. Research objectives continue to emphasize the study and elucidation of pathways of low molecular weight aromatic lignin fragment metabolism by actinomycetes. Principal lignin degradation fragments released as catabolic intermediates during lignin degradation by actinomycetes are being identified, and the pathways of metabolism of these intermediates by selected actinomycete strains are being elucidated. Groups of genetic mutants blocked at various points in the metabolism of lignin or of specific lignin fragments are being isolated and used in these elucidation studies and in work aimed at determining the potential for utilizing blocked mutants for the bioconversion of lignin to low molecular weight phenolic chemicals. Thus, lignin might serve as a renewable resource for the production of aromatic chemicals now derived from petroleum.

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Also enclosed is a budget for Year Two of funding. The budget request is for \$62,676, the amount previously requested in the initial proposal which was funded.

CURRENT PROGRESS

Personnel.

I have now hired a technician and postdoctoral research associate and both are working on the project. I will probably add a graduate student to the project during the summer or fall of 1981. This student will be funded by a departmental graduate assistantship.

Current Research Accomplishments.

We have just recently begun to accumulate substantial new data concerning lignin fragment metabolism by Streptomyces. We are also just beginning a concentrated mutagenesis-screening procedure to isolate mutants of lignin-degrading Streptomyces which are blocked in the catabolism of low molecular weight lignin degradation intermediates. We are also now in the process of isolating and characterizing new actinomycete strains capable of decomposing a number of known lignin degradation intermediates. In the discussion below, I will summarize our progress in each of these areas.

Catabolism of Lignin Degradation Intermediates by Selected Lignin-decomposing Streptomyces Strains. We are near completion of a study which characterizes the lignin fragment accumulation patterns of low molecular weight aromatic compounds which accumulate as water soluble products during degradation of lignin by Streptomyces viridosporus T7A. In this study, we grew S. viridosporus on a hardwood (maple), softwood (spruce) and two grass (quackgrass, corn) lignocelluloses, and determined the amounts and kinds of single-ring aromatic fragments which were released into the culture medium during growth of the culture on lignin. A paper concerning this work will be presented at an upcoming symposium on Biotechnology in Energy Production and Conservation in Gatlinburg, Tennessee (May 1981). Fragment accumulation patterns with strain T7A are reproducible and specific for each lignocellulose substrate. Numerous aromatic compounds are present in small amounts in culture media, and we have identified

some of the compounds using gas chromatography-mass spectroscopy. Single-ring aromatic lignin-derived chemicals identified thus far include p-hydroxybenzoic acid, vanillic acid (4-hydroxy-3-methoxybenzoic acid), syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid), p-hydroxyphenylacetic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), p-coumaric acid (3-[4-hydroxyphenyl]-2-propenoic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid) and the ketol 1-hydroxy-3-[4-hydroxy-3-methoxyphenyl]-2-propanone. Other as yet unidentified compounds are also routinely detected. p-Hydroxybenzoic acid, vanillic acid and syringic acid are quantitatively important lignin fragments. Many of the other lignin fragments are probably degraded through these three substituted benzoic acids. The quantitatively most important and probably "key intermediate" fragments appear to be vanillic acid, syringic acid and p-hydroxybenzoic acid. The relative importance of each as a key intermediate varies with the lignin type (grass, hardwood, softwood).

We have also made progress in delineating how certain Streptomyces strains further decompose lignin degradation intermediates. We have completed a study on the pathway of vanillic acid catabolism by a lignin-degrading strain of S. setonii (75Vi2). We are well into a study of how S. setonii metabolizes phenylpropanoid aromatics including ferulic, coumaric and cinnamic acids. We are also studying how S. viridosporus (T7A) degrades these same compounds.

S. setonii decomposes vanillic acid via a pathway not previously confirmed for any microorganism. The streptomycete first decarboxylates vanillic acid to guaiacol and then demethylates guaiacol to catechol which is then cleaved by catechol 1,2-dioxygenase. The pathway is unusual in that most organisms degrade vanillic acid through protocatechuic acid by demethylating vanillic acid.

Vanillic acid is quantitatively a significant lignin degradation intermediate detected during lignin degradation by S. viridosporus and S. setonii. Thus, in these organisms a considerable percentage of the carbons of lignin may be metabolized through catechol via vanillic acid. We are now screening for vanillic acid catabolism blocked mutants which accumulate vanillic acid or catechol in substantially increased amounts as compared to the wildtype. The isolation of such mutants will help us to determine more accurately the percentage of lignin carbons catabolized through vanillic acid by these streptomycetes.

The metabolism of vanillic acid by S. viridosporus T7A is of particular interest to us. Under certain cultural conditions (inadequate yeast extract cosubstrate concentration) the culture will not metabolize vanillic acid, although it will oxidize vanillin to vanillic acid. When adequate levels of yeast extract are supplied, the culture will metabolize vanillic acid through catechol. We do not see this pattern with S. setonii, which readily catabolizes vanillic acid under either set of cultural conditions. We have not determined the exact cause of this yeast extract effect with S. viridosporus, but we are continuing to examine the phenomenon. We are also elucidating the pathways by which known lignin-degrading Streptomyces metabolize other lignin degradation fragments and structurally related compounds including the phenylpropanoid aromatics, ferulic acid (4-hydroxy-3-methoxycinnamic acid), p-coumaric acid (3-[4-hydroxyphenyl]-2-propenoic acid) and cinnamic acid (3-phenyl-2-propenoic acid). Most of our current work has been done with S. setonii. We are delineating the pathways by which this strain metabolizes the aromatics by isolating and identifying intermediates present in culture media after growth of the organism on a specific aromatic, and by examining cell-free extracts for specific enzymes induced when the cells are grown on each aromatic. Although not yet complete, the data thus far indicate that these phenylpropanoid aromatics are degraded by S. setonii by previously described pathways. Though none of the pathways are unique, the data increases our knowledge of how lignin fragment degradation proceeds through classical ring cleavage substrates. Streptomyces setonii catabolizes ferulic acid via vanillic acid, guaiacol and catechol. Catechol is cleaved by catechol 1,2-dioxygenase. p-Coumaric acid is degraded via p-hydroxybenzoic acid and protocatechuic acid. Protocatechuic acid is cleaved by protocatechuate-3,4-dioxygenase. Cinnamic acid is degraded via benzoic acid and catechol. The data obtained from these metabolic pathway elucidation studies, when combined with our knowledge of how Streptomyces species alter polymeric lignin, is allowing us to develop a concept of a complete pathway for lignin degradation by streptomycetes.

Genetic Manipulation of Selected Streptomyces strains. A major emphasis of upcoming work will be in using mutagenesis to obtain streptomycete mutants blocked in the metabolism of specific low molecular weight lignin fragments.

We will concentrate on the isolation of vanillic acid, syringic acid and *p*-hydroxybenzoic acid degradation blocked mutants. By growing these mutants on lignin, we hope to determine more accurately the percentage of lignin carbons decomposed through these three aromatic intermediates, based upon the level of increased accumulation of each aromatic when mutants are grown on lignin (as compared to the baseline levels of accumulation when the wildtype is grown on lignin). We have already developed the mutagenesis procedure which is based upon irradiation of spores with ultraviolet light, and we are currently isolating mutant strains.

Screening of new strains capable of degrading lignin-derived aromatic compounds.

We are now screening for and isolating new actinomycete strains which have the ability to degrade low molecular weight lignin fragments and lignin substructure models. We are at the present time concentrating on phenylpropane fragments (ferulic acid, sinapic acid) and dimeric lignin substructure models (dehydrodivanillic acid, guaiacylglycerol- β -guaiacyl ether). Since we are having to synthesize all but ferulic acid, we are just getting into the actual isolations.

Optimal substrates for bioconversion of lignin to useful chemicals using Streptomyces.

By comparing the growth of two *Streptomyces* (*S. viridosporus* T7A and *S. setonii* 75Vi2) on softwood, hardwood and grass lignocellulose, we have recently discovered that these actinomycetes decompose grass lignocelluloses two to three times as well as they decompose hardwood or softwood lignocelluloses. Both carbohydrate and lignin components are degraded more extensively. We have quantified the degradation of each of the lignocellulose types in terms of loss of lignocellulose dry weight, depletion of lignin and depletion of carbohydrate versus time for each streptomycete. In light of these findings, I feel that it would be better to concentrate in the future on development of lignin to chemical bioconversions using grass type lignocelluloses as substrates for our *Streptomyces* strains. The potential for the development of bioconversions giving high yields of specific chemicals from lignin is greatly increased using grasses as opposed to softwoods or hardwoods.

Budget request.

We are continuing our research in each of the areas discussed above. A budget request for year two is enclosed. The current budget for year one is just adequate, especially in terms of day to day costs for materials and supplies.

Renewal Budget Year Two
Grant DE-AS07-80-ER10775

University of Idaho, Department of Bacteriology and Biochemistry
Principal Investigator - Donald L. Crawford, Associate Professor
Beginning, September 1, 1981

	<u>DOE Funded Person Months</u>	<u>Funds Requested by Proposer</u>
P.I. - Donald L. Crawford	3	None
Postdoctoral Associate	12	14,925
Graduate Student (1)	3	4,805
Scientific Aide II	12	14,600
Total Salaries and Wages		34,330
Fringe Benefits (21% staff, 7% student)		6,536
Total Salaries, Wages and Fringe Benefits		40,866
Equipment		None
Materials and Supplies		3,000
Domestic travel		600
Publication costs		150
Total Materials, Travel and Publication costs		3,750
Total Direct Costs		44,616
Indirect Costs (30% of Total Direct Costs, Minus Capital Outlay)		13,385
Total Direct and Indirect Costs		58,001
Total Amount of this Request		58,001

Signatures:

Donald L. Crawford 4-10-81
Principal Investigator
Associate Prof. of Bact.

G. R. Reynolds 4-13-81
University of Idaho
Authorized Administrative Officer