DOE/CH/10520--T1

Clark Atlanta University
Department of Biological Sciences

Quarterly Report One for S9X021591 DOE

National Renewable Energy Laboratory

by

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12-28-92

MASTER

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OBJECTIVES AND APPROACHES

Wood decay within Forests, a significant renewable photosynthetic energy resource, is caused primarily by Basidiomycetous fungi, e.g., white rot fungi. These organisms possess the ability to degrade lignin, cellulose and hemicellulose, the main organic polymers of wood. In the case of the white-rot fungi, e.g., Coriolus versicolor, the capacity results from the fungus' ability to elaborate extracellular cellulolytic and ligninolytic enzymes. With regard to the latter, at least one of the enzymes, polyphenol oxidase (PPO) appears within the external milieu in a highly time-dependent fashion when C. versicolor is cultured in a defined growth medium. It appears that extracellular PPO arises from intracellular PPO. Fungal PPO seems to convert the putative tree-synthesized disease resistance factors, diphenols to diquinones and oligomerizes syringic acid, a lignin derivative. Because PPO appears to be inducible, it is conceiveable that the C. versicolor culture system could be a model for achieving over-production of enzymes relevant to the paper-pulp industry and the agricultural community. The current project is concerned with the over-production and enhanced secretion of PPO, cellulase and lignin peroxidase. The project is divided into two segments: 1) over-production of lignocellulolytic enzymes by genetic engineering methodologies and hyper-production and enhanced secretion of these enzymes by biochemical/electron microscopical techniques. The former approach employs recombinant DNA procedures, e.g., isolation of C. versicolor genomic DNA, ligation of appropriate nuclease generated DNA fragments into a vector and the subsequent transformation

of Escherichia coli to yield E. coli harboring a C. versicolor DNA insert. This approach is being carried out by Dr. Arthur L. Williams at Howard University. The biochemistry/electron microscopical method involves substrate induction and the time-dependent addition of respiration and PPO inhibitors to elevate C. versicolor's ability to synthesize and secrete lignocellulosic enzymes. In this connection, cell fractionation/kinetic analysis, TEM immunoelectron microscopic localization and TEM substrate localization of PPO are being employed to assess the route of secretion. This approach is being performed by Dr. W.V. Dashek and N.L. Moore at Clark Atlanta University. Both approaches will culminate in the batch culture of either E.coli or C. versicolor, in a fermentor with the subsequent development of rapid isolation and purification procedures to yield elevated quantities of pure lignocellulosic enzymes.

During the past year, research efforts were directed toward determining the route of polyphenol oxidase (PPO) secretion by the wood-decay fungus, Coriolus versicolor. This basidiomycete secretes PPO (an enzyme converting tree-synthesized, disease resistance factors, o-diphenols to o-diquinones) and ligno-cellulolytic enzymes. The latter possess commercial uses in the forest (renewable energy resource) paper-pulp and bioconversion industries as well as the agricultural community.

In addition, research activities were continued to over-produce and to purify PPO as well as define the time-dependent intra- and extra-cellular appearances of C. versicolor ligninases and cellulases. These investigations were performed in an effort to over-produce, stimulate secretion and purify these enzymes from batch-cultured C. versicolor for scientific and commercial applications.

Technical Progress

Overproduction of Lignocellulolytic Enzymes by Substrate Induction

During the first quarter of the current DOE award, Coriolus versicolor hyphae were cultured upon Kirk's medium solidified with agar for subsequent inoculation of liquid culture medium with mycelium for the substrate induction of PPO. The in progress substrate conditions will consist of the following catechol additions (inducer) to 24 ml liquid cultured C. versicolor.

Flask Number	Addendum	Time of Addition (Days)
1	1 ml growth medium	0
2	"	0
3	"	0
4	1 ml growth medium with catechol	0
5	"	0
6	"	0
7	1 ml growth medium	3[a]
8	"	3
9	"	3
10	1 ml growth medium with catechol	3
11	"	3
12	"	3

[a] On-set of PPO synthesis (Moore et al., 1992)

At day 6, mycelia will be harvested and transferred to phenolic-free medium until day 13 of culture. Then, mycelia will be harvested by separation of the growth medium from mycelia through gentle vacuum filtration. Both intracellular and extracellular PPO will be quantified as in Moore et al. (1989) to assess whether the spc. act. of PPO has been enhanced through the time-dependent addition of a phenolic inducer to the growth medium. Subsequent exepriments will involve the time-dependent supplementation of growth medium with syringic and gallic acids, possible PPO inducers other than catechol (Moore et al., 1989).

Route of and Enhanced Secretion of Lignocelluloytic Enzymes

During the last quarter, we concentrated on two approaches to establishing the route of PPO secretion in liquid cultured Coriolus versicolor. These approaches centered about the TEM substrate and the transmission immunoelectron microscopic localization of PPO within liquid cultured hyphae. The former approach involved interposing dihydroxyphenylalanine (DOPA) between glutaraldehyde pre-fixation and osmium tetroxide post-fixation with the subsequent preparation of hyphae for TEM. In this regard, two activities were accomplished. These were ascertaining the purity of the PPO substrate, DOPA, and the sectioning of controls (exposure of hyphae to buffer lacking DOPA) for the above experiment.

The purities of DOPA as well as the related amino acids, phenylalanine and tyrosine were assessed by thin layer chromatography utilizing ethanol, ammonium hydroxide and H_2O as the developing solvent.

In addition to this approach, TEM immunoelectron microscopic efforts have been continued to localize PPO within hyphae cultured over time. To this end, another batch of anitbody was purified by immunochromatography employing Mabtrap G. This antibody will be tagged with colloidal gold for the TEM immunoelectron microscopic localization of PPO as above. During the second quarter, hyphae will be fixed and then embedded in Lowicryl K4M for ultramicrotomy and the subsequent employment of antibody for the localization of PPO in sectioned hyphae.

Purification of Extracellular PPO

An abstract regarding the partial purification of extracellular PPO is being prepared for the Annual Meeting of the American Society of Plant Physiologists. It details the progress that has been made toward purifying extracellular PPO to homogeneity. The abstract will be submitted during Jan, 1993 for review by the NREL and subsequent modification prior to submission to Plant Physiology.

Summary of Accomplishments

An improved PPO substrate induction protocol has been initiated possibly leading toward a statistically significant, quantifiable enhancement in the amounts of both intracellular and extracellular PPO. This will be extended to cullualses and eventually lignin peroxidases,

enzymes of commercial and agricultural significances. With regard to establishing the route of secretion, progress occurred toward localizing PPO via TEM substrate and TEM immunoelectron microscopic procedures. Finally, an abstract detailing further accomplishments beyond previously published information concerning the partial purification of extracellular PPO has been prepared.

Collaborative Efforts

Dashek has been verbally notified that another co-operative agreement with the Forest Product's Laboratory is likely. This agreement involves a non-enzymatic mechanism for the partial degradation of wood cellulose.

Budget

DOE Grant S9XO21591 - W.V. Dashek, 12-24-92

Category	Allocated	Spent	Remaining
Salaries			
W.V. Dashek	$ 5,000.00	University Deduction	
N.L. Moore (Sec)	$ 4,000.00	$ 500.00	$ 3,500.00
Graduate student			
N.L. Moore	$15,000.00	None	$15,000.00 *
EM Technician	$ 2,500.00	None	$ 2,500.00 **
Supplies			
Office	$ 2,500.00	$ 332.85	$ 2,167.15
Scientific	$ 3,500.00	$ 2,884.02	$ 615.78

* Will be converted into DOE approved laboratory technician as Ms. Moore completed a second MS degree.

**EM technician has been contacted and has submitted a quote (see enclosure).

Publications

Moore, N.L., L.A. Brako, C. Clausen, B.R. Jones and W.V. Dashek. 1992. Distribution of polyphenol ocidase in organelles of hyphae of the wood-deteriorating fungus, Coriolus versicolor. Biotoxins, Biodegradation and Biodeterioration Res. 4, Plenum Press (in press).

Moore, N.L. and W.V. Dashek. 1992. Distribution of polyphenol oxidase in cultured hyphae of Coriolus versicolor, a wood-decay fungus. SIM Abstracts (Abstract 6, p. 71).

In preparation

Moore, N.L., L.A. Brako, C. Clausen and W.V. Dashek. Subcellular distribution of polyphenol oxidase in hyphae of the wood-decay fungus, Coriolus versicolor. International Biodeterioration.

Moore, N.l. and W.V. Dashek. 1993. Partial purification of Coriolus versicolor's extracellular polyphenol oxidase (PPO). Plant Physiol. (Abstract).

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