

FINAL REPORT

DOE/CH/10557--7/

(ANNUAL TECHNICAL SUMMARY REPORT)

**OVER PRODUCTION OF LIGNOCELLULOSIC ENZYMES OF
CORIOLUS VERSICOLOR BY GENETIC ENGINEERING
METHODOLOGY**

Subcontract ³⁶ DE-FG02-93CH10557

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Project Description:

The project seeks to understand the biological and chemical processes involved in the secretion of the enzyme polyphenol oxidase (PPO) by the hyphae, the basic unit of the filamentous fungus *Coriolus versicolor*. These studies are made to determine rational strategies for enhanced secretion of PPO, both with the use of recombinant DNA techniques (Howard University), and without (Clark-Atlanta). This effort is done in concert with work at Clark-Atlanta University but focuses on recombinant DNA techniques to enhance enzyme production.

The major thrust of this project was two-fold: (i) to mass produce *C. versicolor* tyrosinase (polyphenol oxidase) by genetic engineering as well as cultural manipulations; (ii) to utilize polyphenol oxidase (PPO) as a biocatalyst in the processing of lignocellulose as a renewable energy resource. Wood-decay within forests, a significant renewable photosynthetic energy resource, is caused primarily by Basidiomycetous fungi, i.e., white-rot fungi. In the case of the white-rot fungus, *C. versicolor*, its degradative capacity results from the ability to elaborate extracellular cellulolytic and ligninolytic enzymes. With regard to the latter, at least one of the enzymes, PPO, appears within the external milieu in a highly time-dependent fashion when *C. versicolor* is cultured in a defined growth medium.

In this study, the assessment of genomic and cDNA recombinant clones with regards to the overproduction of PPO continued. During antibody (anti-tyrosinase) screening of genetic clones, enhanced production of polyphenol oxidase in several variants was observed. Further, immunoprecipitation and ELISA protocols were employed to detect and quantify tyrosinase (PPO) during cultural manipulations. Catechol and tyrosine appeared to stimulate the production of PPO in bulk cultures. On the other hand, diethylthiocarbamate (DETC) exhibited an inhibitory effect on PPO Activity. In the attempt to over produce PPO, it was noticed that the activity of catechol oxidase (CTO) was higher than PPO. Summarily, owing to the fact that the tyrosinase complex possesses multiple activities, e.g., monophenol oxidase, PPO, CTO, the employment of it as a biocatalyst to process lignocellulose appears feasible.

Further, immunocytochemical techniques were employed to assess the mechanism(s) involved in the secretion of PPO by the hyphae. That is, immunogold was used to label (i.e. tag) native PPO during certain growth conditions. Data from the immunocytochemical analysis suggested a putative route of secretion of PPO in *Coriolus versicolor*. Also, factors influencing PPO secretion were examined.

Background

Wood-Decay in the Forest - Renewable Energy Resource:

Wood-decay within Forests, a renewable photosynthetic energy source, is caused primarily by Basidiomycetous fungi, e.g., white-rot fungi which possess the ability to degrade lignin, cellulose and hemicellulose, the main polymers of wood. In the case of at least one of these fungi, *C.versicolor*, this ability results from the fungus' capacity to elaborate both extra-cellular cellulytic and ligninolytic enzymes, synthesis and secretion of these enzymes can occur *in situ* and *in vitro*. With regard to the latter, at least one of these enzymes, PPO makes its appearance within a culture medium in a highly time-dependent fashion. Presumable, extracellular PPO originates via secretion of intracellular PPO. However, this presumption requires verification. Because PPO, and enzyme capable of converting diphenols to diquinones and oligomerizing the lignin derivative, syringic acid, appears to be inducible, it is conceivable that the *C. versicolor* culture system could be a model for achieving over-production of enzymes, a stated mission of the DOE-ACID Project. In addition, the system, which can be scaled-up to industrial levels of enzyme production, seems to be the one in which the regulation of synthesis and/or secretion of both cellulolytic

and ligninolytic enzymes can be investigated separately; i.e., experiments can be designed whose results may lead to the independent control of synthesis and/or secretion.

Lignocellulose - A Forest By-Product:

Besides its presence within wood, lignocellulose often constitutes an unwanted component in the paper-pulp industry and also renders certain agricultural commodities less digestible to ruminants. Thus, an available supply of lignocellulolytic enzymes could be marked industrial value. To maintain an adequate supply, substrate induction coupled to hyphal 'culture' and/or genetic engineering techniques could be employed to over-produce these enzymes.

The cellulases and ligninases that are secreted by *C. versicolor* can degrade cellulose and lignin, the main organic polymers of wood. *C. versicolor* can be grown in 'batch culture', which makes the over-production and the enhanced secretion of interest, commercially and biologically.

Objectives

1. Detect and quantify the expression of PPO in recombinant clones of *E. coli*.
2. Detect and quantify the over-production of PPO during cultural manipulations.
3. Mutagenize cloned DNA inserts harboring the PPO gene.
4. Assess the expression of recombinant clones carrying mutagenized DNA inserts for their abilities to over-produce PPO.
5. Perfect the PPO system as a model for producing large scale quantities of industrial enzymes.
6. Characterize the catalytic properties and activities of the PPO complex.
7. Utilize PPO as a biocatalyst in wood bioprocessing, e.g., lignocellulose utilization.
8. Initiate the development of a course curriculum impacting upon the environment with heavy emphasis pertaining to biotechnology, manufacturing, and bioprocessing.

Methodology

Part I. Genetic Engineering of the PPO Gene

1. Construction of PPO recombinant clones
2. Screening of recombinant clones of PPO
3. Trafficking of PPO in *E. coli* cells

Part II Biological Processing of the PPO Gene

1. Enzymatic catalysis of PPO.
2. Enzymatic assay of PPO.
3. Bioprocessing of PPO.

1995 Accomplishments:

- Generated several PPO recombinant clones of *C. versicolor* by means of cDNA approach.
- Assessed the expression of the PPO gene in bacterial cells as well as a unique yeast variant.
- Quantified PPO production via antibody probing.
- Enhanced PPO production via cultural manipulations.
- Detected other enzymatic activities associated with the PPO complex.
- Initiated the utilization of PPO as a biocatalyst in the processing of lignocellulose.

1996 Planned Activities:

- Market the mass production of PPO via genetic engineering and cultural manipulations
- Resolve other enzymatic activities associated with the PPO complex.
- Utilize multiple activities of PPO complex in the bioprocessing of lignocellulose.
- Demonstrate other industrial applications of PPO.
- Generate a patent of PPO proteins.

Annual Technical Summary Report:

Results

During this period (8/17/95 - 8/16/96), the assessment of genomic and cDNA recombinant clones with regards to over production of PPO has continued. (Fig. 1).

During antibody, anti-tyrosinase, screening of genetic clones, enhanced production of PPO in several variants was observed. That is, immunoprecipitation and ELISA protocols were employed to detect and quantify tyrosinase, i.e., PPO, during cultural manipulations. Cultural filtrates and tissue extracts were concentrated whereby standardized aliquots were tested in the ELISA protocol. On the other hand, corresponding aliquots were subjected to SDS-polyacrylamide gel electrophoresis subsequent to immunoprecipitation. Based upon these results, one major protein band was detected from the tissue extracts while no visible band was displayed from the filtrates during the 15 day growth period.

Other recombinant clones were generated by means of the cDNA approach (Fig.2). Specifically, two methods were employed to separate poly (A⁺) mRNA from bulk RNA and poly A Tract System proved more efficient (Fig. 3). An aliquot of the poly (A⁺) sample was subjected to formaldehyde-agarose gel electrophoresis to confirm its presence. In order to determine whether the poly (A⁺) mRNA was functional, samples were subjected to the BRL in vitro translation system and two protein bands, approximately 40-60 kDA were visible. These protein bands were transferred to nitrocellulose paper, using the Sartobolt apparatus and probed with fluorescein-PPO antibodies.

Additionally, the enzymatic profile of activities associated with the tyrosinase complex was determined. Catechol and tyrosine appeared to stimulate the production of PPO. The activity of CTO was even higher than PPO. Summarily owing to the fact that tyrosinase complex possesses multiple activities, e.g. monophenol, PPO, and CTO, its employment as a biocatalyst to process lignocellulose appears feasible from this study. This project has afforded now substantial understanding the PPO enzymatic complex.

Electron Microscopy: Immunocytochemical Analysis

Polyphenol Oxidase (PPO) activity and production were studied by means of immunocytochemical methods. For electron microscopic immunocytochemistry, the mycelial mass was fixed in 3% glutaraldehyde and embedded in LR White medium. Subcellular localization of PPO with immunogold revealed that the 6th day mycelia showed maximum metabolic activity which was evidenced by the presence of (a) nuclei, (b) euchromatic material (c) rough endoplasmic reticulum and (d) mitochondria. Vacuoles in the 6th day mycelial cells of *C. versicolor* were small in size

and contained the enzyme polyphenol oxidase which was evidenced by the presence of immunogold particle in the vacuoles. There was an abundance of immunogold particles on the cell-wall and in the cytosolic area of the mycelial hyphae. The 9th day mycelia tissue displayed the maximum level of secretion of the enzyme PPO. This immunocytochemical study of PPO showed the intracellular storage of PPO and the distribution of PPO which was observed as the localization of immunogold particles conjugated to the secondary antibody (goat anti-rabbit IgG) which is specific to the primary antibody rabbit-antityrosinase in the vacuoles, secretory vesicles cell-walls and the extracellular area. The overall putative route of secretion of PPO appears from (1) E.R. → (2) Vacuoles → (3) secretory vesicles (adjacent to the interior of the cell wall) → (4) cell wall → (5) outside of cell wall. (see Figs. 4, 5, 6).

Significance of Biological Processing of PPO

The white-rot basidiomycete, *C. versicolor*, secretes PPO as well as other lignocellulolytic enzymes *in situ* and *in vitro*. The enzymes degrade cellulose and lignin, the principal polymers of wood. Wood is a component for trees which constitutes a renewable forest energy resource. Also, these enzymes may play a role in the recycling of waste from lignocellulose degradation.

To date, environmental biotechnology has a direct and fundamental role in bioremediation, biorestitution, waste treatment, environmental diagnostics and analysis, the preservation of biodiversity, and the development of environmental risk assessment technologies. Some examples of recent developments in toxic organic chemicals and others that emit visible light signals when they encounter specific chemicals in the environment. Applications of biotechnology to energy production and use are closely related to bioprocessing and are likely to be an important part of our economic future by promoting production of fuels from renewable resources.

Output of Project

Data collected during this budget are being compiled into three (3) manuscripts which will be submitted for publication in the very near future.

Also, this subcontract project provided support for another minority student to receive the M.S. degree in Biology (Student - Chandana Bandyopadhyay).

To date the following minority students have received funds from this project to support their graduate training:

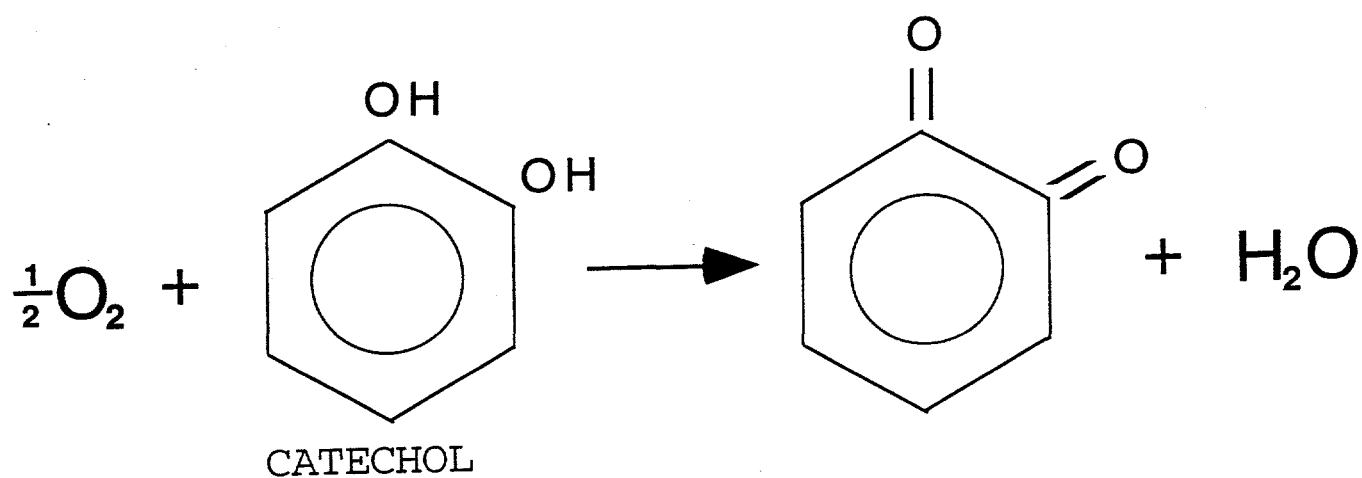
School	Name	Degree	Current Status
Clark Atlanta University	Deneke Mariam	M.S. degree (1989)	N/A
Clark Atlanta University	Nina Moore	M.S. degree (1991)	Research Fellow, Emory University
Howard University	Felicia Goins	M.S. degree (1994)	Instructor/Norfolk State University
Howard University	Augustus Billy	M.S. degree (1995)	Ph.D. Program
Howard University	Chandana Bandyopadhyay	M.S. degree (1997)	Employed at Laser Chemistry Lab.
Howard University	Gay Leslie Brown	Ph.D. degree (pending)	Graduate Student
Howard University	Randolph Taylor	Ph.D. degree (pending)	Graduate Student

Additionally, two minority faculty members at Howard University were supported during the summer from this subcontract.

APPENDIX

(SELECTED DATA OF PPO)

1. PPO enzymatic reaction
2. Molecular Cloning Protocol
3. Oligo - dT fractionation of poly (A⁺) and poly (A⁻) RNA
4. Sixth-day old tissue showing immunogold particles in young hyphae of C. versicolor.
5. Sixth-day old tissue showing immunogold particles in vacuoles and outside of the cells.
6. Ninth - day old tissue showing immunogold particles along cells, vacuoles and secretory vesicles.
7. Protocol for Tyrosinase Assay (i.e., multiple activities).



Conversion of O - diphenols to O - diquinones.

Figure 1: Conversion of O - diphenols to O - diquinones.

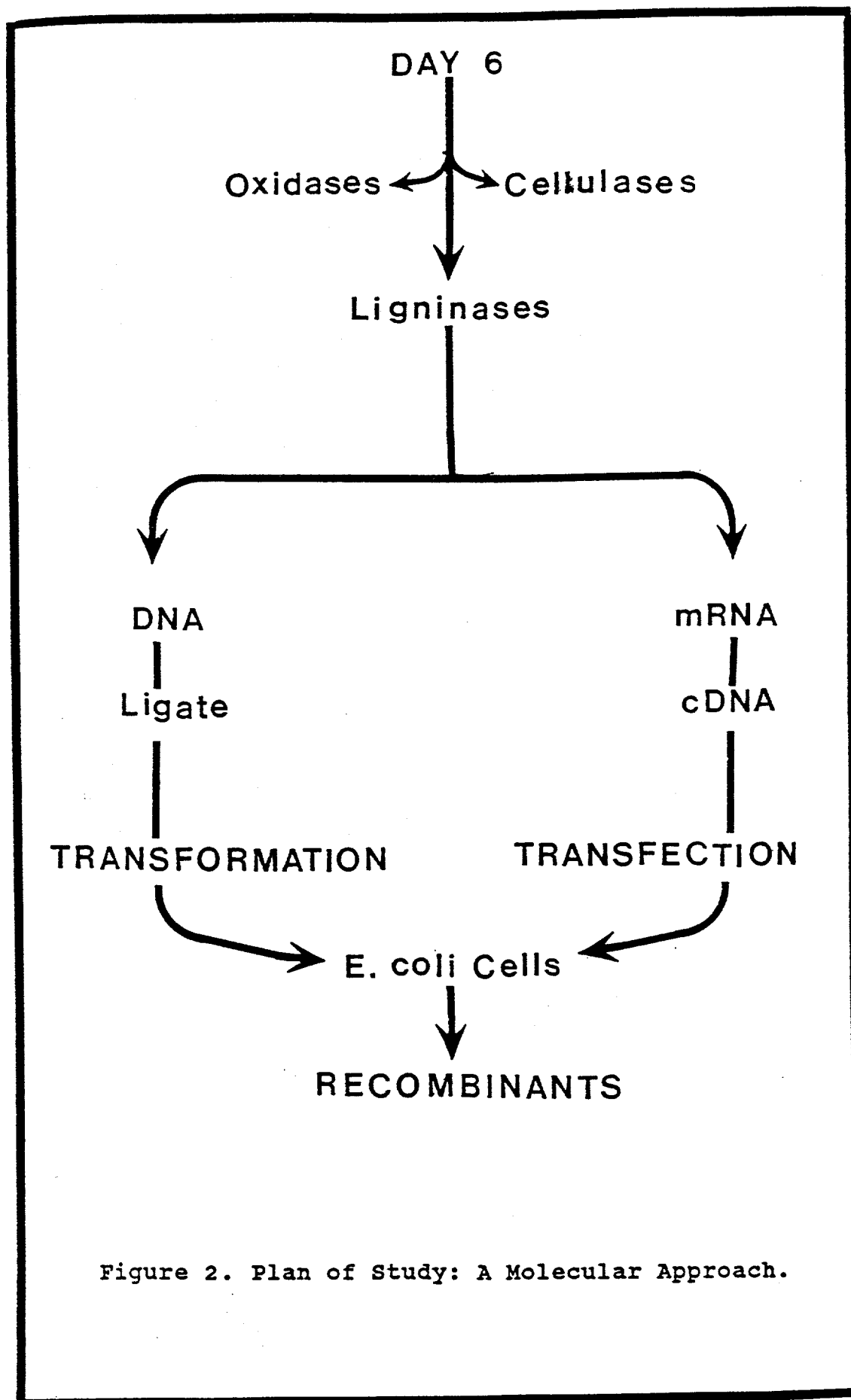


Figure 2. Plan of Study: A Molecular Approach.

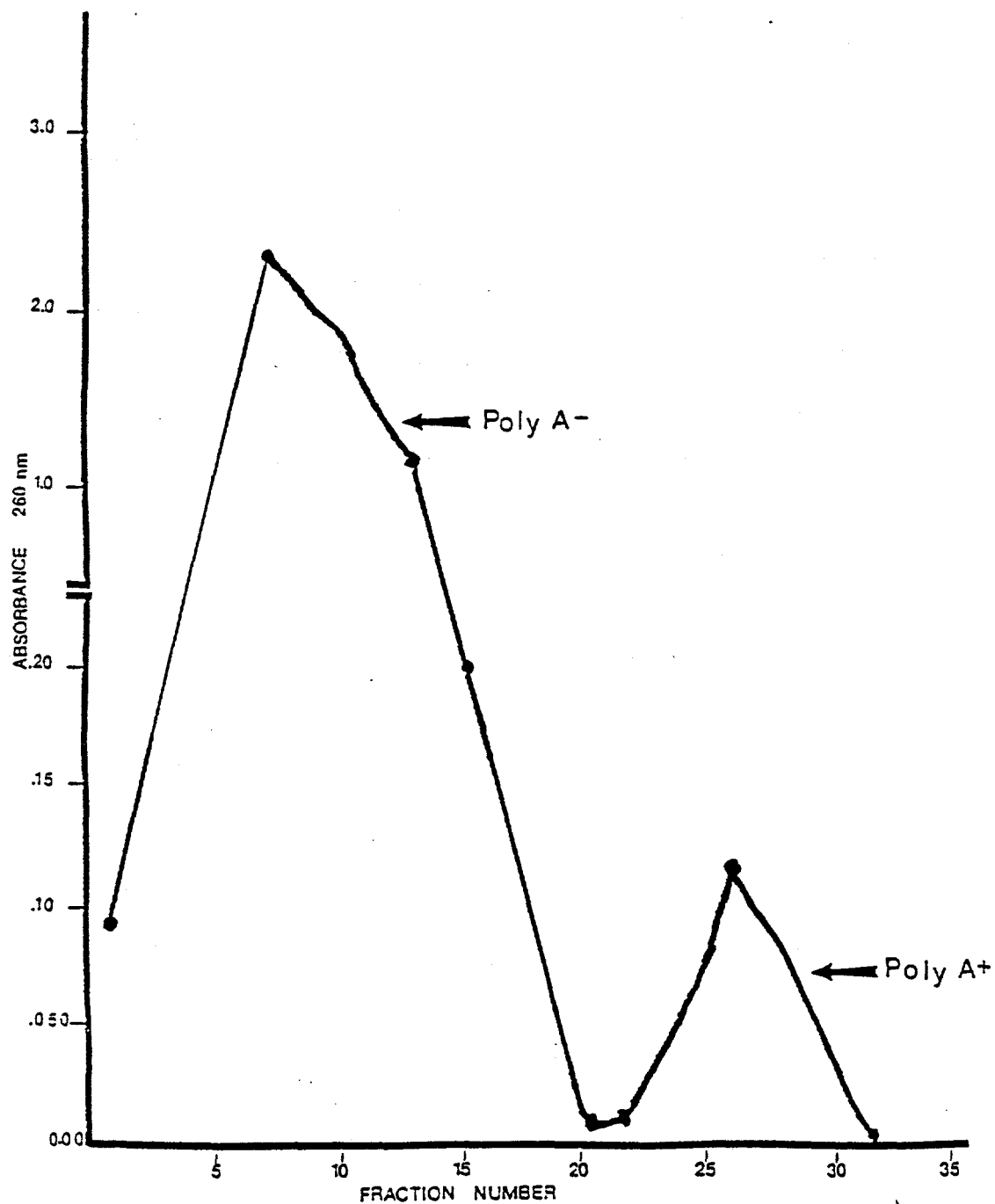


Figure 3. Fractionation of Purified RNA via Oligo (dT)-Cellulose Chromatography.



FIGURE 4

Sixth- day cell showing the presence of a large amount of immunogold particles all along the outside border of the cell-wall (CW) of a young hyphal cell of *Coriolus versicolor* from the sixth day. A large mieochodrion (M) is well-defined. A dense mass of rough endoplasmic reticulum (RER) traverses the entire cytoplasm. Presence of scattered immunogold particles (arrows) is well noted in the extracellular area. ($\times 45,000$)



FIGURE 5

Sixth day hyphal cell (experimental set) showing the abundance of immunogold particles of the cell wall (CW) area. A large amount of rough endoplasmic reticulum (RER) is scattered hroughout the cytoplasm of the cell. A large amount of immunogold particles (arrow) are present in vacuoles and outside the cell. Presence of a nucleus is well-defined. ($\times 33,000$).



FIGURE 6

Hyphal cell of *C. versicolor* (4th day mycelial cell experimental set) showing the presence of a dense mass of rough endoplasmic reticulum (RER). Note the deposition of immunogold particles (arrow-heads) along the cell-wall (CW), the vacuoles (V) and in the secretory vesicle (SV) ($\times 27,000$).

Tyrosinase Assay

Tyrosinase was assayed by the dopachrome method.¹⁶ Ten millimolar L-3,4-dihydroxyphenylalanine (L-DOPA) solution in 0.1 M sodium phosphate (pH 6.0) was prepared. After adding 25 to 100 μ l of the tyrosinase sample to 1.5 ml of L-DOPA solution containing 0.05 g/l of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, absorbance at 475 nm was monitored for 4 minutes using a spectrophotometer. One unit is defined as the amount of enzyme catalyzing the formation of 1.0 μ mole of dopachrome per minute. The molar absorption coefficient of the reaction is 3600 l/(mole \cdot cm). Cell samples for the tyrosinase assay were prepared as follows: Samples from the derepressed cultured broth were diluted to 1.5 OD at 600 nm, and 1-ml aliquots were centrifuged, the cell pellets washed and resuspended in 100 μ l of 50 mM sodium phosphate buffer (pH 6.0). 10 μ l of toluene-ethanol (1:4) mixture¹⁷ was added, and the mixture was mixed vigorously for 30 sec. Twenty-five microliters was then taken and mixed with 1.5 ml of an L-DOPA solution for tyrosinase assay. SDS-PAGE analysis was also carried out to monitor the change of intracellular tyrosinase during the batch fermentation.

HAN *et al.*: TYROSINASE PRODUCTION

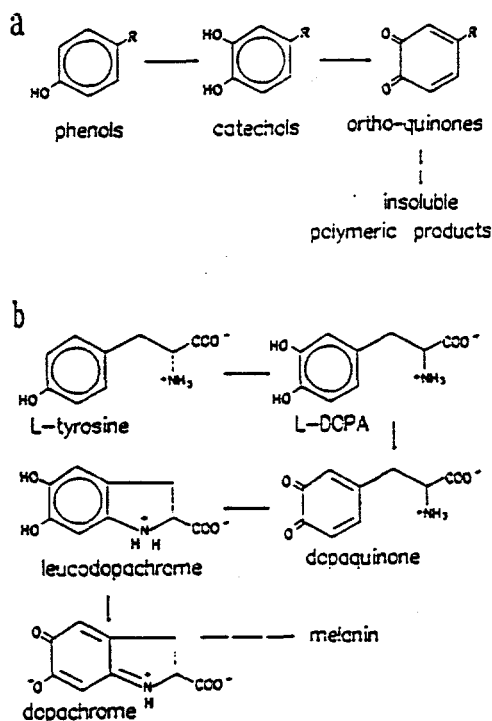


FIGURE 1. (a) The *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones catalyzed by tyrosinase. Because most *o*-quinones are unstable, nonenzymatic polymerization follows, and finally insoluble polymer pigments are formed. (b) An example of the reaction scheme described by (a) for the conversion of L-tyrosine to melanin.

R and D Effort: Chemical Vision 2020 Impact

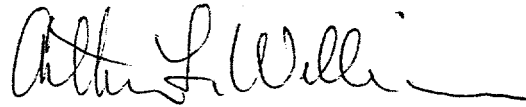
Biotechnology ---- is a rapidly growing field which impacts heavily across many disciplines. To date, environmental biotechnology has a direct and fundamental role in bioremediation, bio restoration, waste treatment, environmental diagnostics and analysis, the preservation of biodiversity and the development of environmental risk assessment technologies. Some examples of recent developments are in toxic organic chemicals and others that emit visible light signals when they encounter specific chemicals in the environment. Applications of biotechnology to energy production and use are closely related to bioprocessing and are likely to be an important part of our economic future by promoting production of facts from renewable resources.

In this Project, we seek to understand the biological and chemical processes involved in the secretion of the enzyme polyphenol oxidase (PPO) by the hyphae, the basic unit of the filamentous fungus, Coriolus versicolor. These studies are made to determine rational strategies for enhanced secretion of PPO, both with the use of recombinant DNA techniques and without. The major thrust of this project was two-fold: (1) to mass produce C. versicolor tyrosinase (polyphenol oxidase) by genetic engineering as well as cultural manipulations; and (2) to utilize polyphenol oxidase (PPO) as a biocatalyst in the processing of lignocellulose as a renewable energy resource. Wood-decay within forests, a significant renewable photosynthetic energy resource, is caused primarily by Basidiomycetous fungi, i.e., white-rot fungi. Specifically, the white-rot basidiomycete, C. versicolor, secretes PPO as well as other lignocellulolytic enzymes in situ and in vitro. These enzymes degrade cellulose and lignin, the principal polymers of wood. Wood is a component of trees which constitutes a renewable forest energy resource. Also, these enzymes may play a role in the recycling of waste from lignocellulose degradation.

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Clearly, this project is linked directly to the Technology Area 1: New Chemical Science and Engineering Technology. That is, this study impacts heavily upon the Biotechnology field from an industrial viewpoint. Finally, this investigator is of the opinion that Industry should scale up its involvement in defining and implementing R & D to optimize this technology world-wide.

Respectfully submitted:

A handwritten signature in cursive script, appearing to read "Arthur L. Williams", followed by a horizontal line.

Arthur L. Williams
Principal Investigator
DOE-BCRT Group