Quarterly Report 2

A Study of Over-Production and Enhanced Secretion of Enzymes

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April 8, 1993

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Significance

This project is concerned with the over-production of ligno-cellulolytic enzymes which are relevant to the paper-pulp industry and agricultural community. Since ligno-cellulosics are components of wood, the project involves the Forest, a renewable energy resource.

Technical Report

During January, February and March, 1993, the following were accomplished:

Over-production of Polyphenol Oxidase - To determine whether the synthesis of polyphenol oxidase (PPO) could be enhanced via substrate induction, 25 ml liquid cultures (24 ml growth medium together with 1 ml fungal inoculum (Moore et al., 1989) were supplied with 1 ml sterile Kirk and Kelman’s (1965) medium containing reagent grade catechol (an o-diphenol) to yield 100 mM catechol per 26 ml culture. In contrast, control cultures were supplied with 1 ml sterile growth medium lacking the diphenol. Both control and catechol-treated hyphae were cultured 7 days in a Labline gyroty r shaker at 150 rpm and 25 ± 2°C when three control cultures and three cultures supplemented with catechol were transferred to catechol-free medium for 7 days. Other cultures supplemented with growth medium either containing or lacking catechol at the time of inoculation with hyphae but not transferred as above were harvested at 14 days by gentle vacuum filtration. Thus, whether transferred or not, the total time of hyphal culture was 14 days. Harvested growth medium was rapidly frozen in liquid N₂, stored at -20°C, thawed (PPO appears to be stable to freezing and thawing), re-packaged and re-frozen for lyophilization. Subsequent to lyophilization, freeze-dried growth medium will be re-suspended in 2 ml 100 mM, pH 5.0 acetate buffer for an overnight dialysis at 4°C against 41 of the buffer. In addition, freeze-dried mycelia will be homogenized with a mortar and pestle on ice for 10-15 min and the homogenate centrifuged at 500×g to ‘pellet’ the hyphal cell wall. The supernatant will be dialyzed as above. Following dialysis, both the mycelial homogenate and the growth medium will be assayed for 280nm absorbing substances and PPO to yield intracellular and extracellular PPO spc. act. It is anticipated that this experiment will be completed by mid-April.

In addition to the above, a co-operative molecular genetics program with Dr. David McMillin of the Clark Atlanta University Biology Department was initiated. Dr. McMillin has begun isolating Coriolus versicolor genomic DNA in an effort to clone a PCR (polymerase chain reaction) amplified, restriction endonuclease generated fragment of genomic DNA. This work is progressing rapidly and should complement that of Dr. A. L. Williams at Howard University.

Establishment of the Route of Polyphenol Oxidase Secretion- During this quarter, a concentrated effort was undertaken to complete the intracellular localization of PPO within hyphae by a TEM substrate method (Czaminski and Catesson, 1974; Eppig, 1974; Martyn et al., 1979; Vaughn and Duke, 1981). To this end, hyphae cultured for 12 days were pre-fixed in 2.5% glutaraldehyde buffered with 0.1M cacodylate, pH 7.4 for 30 min, washed with
cacodylate, treated 18h with 50 mg dihydroxyphenylalanine (DOPA) per 10 ml cacodylate buffer and post-fixed in cacodylate buffered 2% $\text{Os}_4$ for 2h (controls were treated with cacodylate only). Following post-fixation, the hyphae were dehydrated with acetone and embedded in low viscosity Spurr's embedding medium (Spurr, 1969). Examination of DOPA-treated and non-treated hyphae revealed considerable reaction product indicative of PPO within treated hyphae. A thorough analysis of the product's distribution is in progress and will involve examination of numerous hyphae.

Besides the TEM substrate induction method, efforts were continued to localize intracellular PPO by immunoelectron microscopy. A second ‘batch’ of antibody to PPO prepared by immunizing, boosting and bleeding New Zealand rabbits is in the process of being tagged with colloidal gold for the immunoelectron localization of PPO within hyphae liquid cultured over time. Figure 2B presents the achieved immunochromatographic purification of antibody for serum derived from rabbits immunized with Sigma's PPO. The elution profile is similar to that obtained (Figure 2A) for the first ‘batch’ of antibody (Moore et al., 1993 a).

The colloidal gold tagging procedure being employed is depicted in Figure 3. During the current quarter, 0, 3, 6, 9 and 12 day liquid cultured hyphae will be fixed, dehydrated and embedded in Lowicryl K4M (Abrahamson, 1986; Altman et al., 1984) for their utilization in localizing PPO with colloidal gold tagged antibody.

With regard to electron microscopy, the wood-decay literature suggests that there may be a difference in hyphal morphology when mycelia are grown on liquid or solid surfaces. This is extremely important as wood-decay fungi grow upon the inner surface within a wood cell’s lumen. To this end, a comparative fixation analysis of hyphae grown upon agar and in liquid culture is in progress. The hyphae so grown were pre-fixed, washed, post-fixed, dehydrated and embedded for TEM. During this quarter, these hyphae will be sectioned and electron micrographs prepared.

Regulation of PPO Secretion- Experiments (described in previous reports) concerned with controlling secretion through the addition of the respiration inhibitors, NaF and NaAzide as well as the PPO inhibitor, diethylthiocarbamate, will be completed this quarter. Their results will be detailed in the next report.

Purification of Extracellular Oxidase- Attempts continue to separate extracellular Coriolus versicolor PPO cellulase by various chromatographies, e.g., from affinity, hydroxylapatite and hydrophobic interaction. To expedite this, commercially available PPO (Sigma, St. Louis, Mo) and cellulase (Onozuka) are being employed as model enzymes to gain insight into how C. versicolor's PPO and cellulase may elute from the above as well as other resins (Moore et al., 1993b).
REFERENCES


Dialyze antibody (affinity purified) 1 liter against PO₄ buffer 6 h (room temperature)

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Dialyze against 2nd 1 liter PO₄ buffer

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Centrifuge 1:20 dilution 2800 rpm 25 min, 4°C

pellet (discard)       supernatant (10 ml of gold sol) Adjust pH to 7.4

Invert 6 times and let sit for 5 min

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Add fresh/filtered 1% PEG-Carbowax 20 to 10 ml gold

↓

Centrifuge 10K 30 min at 4°C

Aspirate supernatant and discard

pellet

↓

resuspend in 1 ml (20mM Tris-Buffered Sol)

↓

Vortex suspension dilute 1:20 w/dH₂O

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Absorbance 520nm