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Effects of Mercury on the White Rot Fungus *Phanerochaete chrysosporium*

S. S. Dhawale,[1] A. C. Lane,[1] S. W. Dhawale[2]

[1]Department of Biology, Indiana University Purdue University at Fort Wayne, Fort Wayne, Indiana 46805, USA

[2]Department of Natural Sciences, Indiana University East, Richmond, Indiana 47374, USA

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Mercury, one of the most toxic heavy metals, is a major environmental pollutant and has caused major outbreaks of heavy metal poisoning in Japan, Iraq and North America (Fergusson 1990). Mercury finds its way into the environment through effluents from industries and agriculture, through the burning of fossil fuels and from natural sources. The toxicity of mercury mostly stems from its high affinity for thiol groups in amino acid residues and other cellular molecules. By binding to a macromolecule, mercury can inactivate it, since thiol groups are often involved in maintaining the structure or function of macromolecules. Binding of mercury can alter membrane permeability (Cooper et al. 1984), stimulate proteolysis, decrease soluble and structural protein contents and cause progressive increase in accumulation of free amino acids (Suresh et al. 1991).

Fungi are capable of accumulating high concentrations of non-nutrient metals such as cadmium and mercury (Kojo and Lodenius 1989). Hence, the use of filamentous fungi as biosorbents for heavy metals in waste water management has been proposed (Siegel et al. 1990). The use of *P. chrysosporium*, a filamentous basidiomycete, in the removal of environmental pollutants is currently being explored by many laboratories around the world (Boominathan and Reddy 1992). A considerable amount of research has focused on the degradation of organic compounds by this organism (Aust 1990; Bumpus and Aust 1987). However, studies on the removal of toxic inorganic compounds from the environment by *P. chrysosporium* are limited (Shah 1991).

In one of our biodegradation experiments we observed that *P. chrysosporium* cultures remained viable in 100 ppm of mercury. This apparent resistance to mercury suggested that this fungus may be useful in removing mercury from contaminated soil and water. We felt that efficient use of this fungus in mercury bioremediation would be facilitated by examining the effect of mercury on *P. chrysosporium* and by

Correspondence to: S. S. Dhawale

determining whether this fungus shows accumulation of mercury. Hence, we studied the effect of various levels of mercury on the mycelial dry mass, germination, soluble mycelial proteins, extracellular proteins and morphology. We also report the results of our investigation on bioaccumulation of mercury by *P. chrysosporium.*

MATERIALS AND METHODS

Stationary liquid cultures of *P. chrysosporium* strain BKM-F1767 (ATCC 24725) were grown at 37° C using high nitrogen medium, conidial inoculum and culture conditions described by Tien and Kirk (1988). This synthetic medium provides sufficient nutrients for the experimental period used in our studies. The cultures were flushed with oxygen immediately after inoculation (day zero) and again on day 2. Filter-sterilized 0.1 M $HgCl_2$ was used as a stock. Triplicate cultures were used for each treatment in all experiments. Dry mass was determined by harvesting the mycelial mats on preweighed Whatman #1 filters. The filters containing mycelia were dried at 60° C until constant wight was obtained (1 week). Statistical analysis was performed using a two-tailed independent t-test ($p < 0.05$).

For protein estimation mercury was added on day 1 at concentrations of 0, 10 and 2.5 ppm. One set of triplicate flasks from each concentration was harvested on days 1 through 7. Mycelia were separated from the extracellular fluid. The mycelia were suspended in 5 ml of homogenization buffer (0.06 M Na_2HPO_4, 0.04 M $NaH_2P)_4$, 0.05 M 2-mercaptoethanol, pH 7.4,0.8 % NP40 and 1% Tween 80) and blended. The homogenized suspension was centrifuged at 2500 x g for 15 minutes and the supernatant was collected. Proteins were estimated as described by Bradford (1976). To determine protein profiles on polyacrylamide gels, 4 sets of 40 cultures (10 ml per culture) were grown for 6 days. These sets received either 0, 10 or 25 ppm of mercury on day 1, or 50 ppm of mercury on day 2. After harvesting, the extracellular fluid from each set of 40 flasks was pooled and concentrated using an Amicon PM10 membrane. The final volumes of all concentrated samples were adjusted to 2 ml. The mycelia from each set of 40 cultures were also pooled, homogenized in 34 ml of homogenization buffer and centrifuged at 2500 x g for 15 min. The supernatant was concentrated to 2 ml using an Amicon PM10 membrane. Protein profiles were obtained on 6.5% SDS-Polyacrylamide gels stained with Coomassie blue (Laemmli 1970). Qualitative estimation of free amino acids was performed by paper chromatography on Whatman #3 as described by Boschman and Wells (1990).

Recovery of mercury was determined by AAS (atomic absorption spectrophotometry using Perkin Elmer model# 2280). $HgCl_2$ standards were used for calibration. Detection limit for mercury was 4.2 ppm. Cultures received either 0, 10 or 25 ppm of mercury on day 1, or 50 ppm of mercury on day 2. Cultures which did not receive mercury were used to get a baseline. Control flasks containing appropriate medium and mercury, but no conidial inoculation, were also oxygenated and incubated along with other samples. In our initial experiments, the method of recovering mercury associated with mycelial fraction involved overnight digestion of mycelia at 4 °C in 5 ml of 6 M HNO_3. Each digested sample was then filtered through a 0.45

µm filter and the filtrate used for AAS analysis. The extracellular fluid samples were simply filtered through 0.45 µm filter and analyzed by AAS. However, the method was later modified to improve recovery of mercury. In the modified method the mycelial mats were removed from the culture flasks using a glass rod and rinsed in 5 ml of H_2O. The extracellular culture fluid was transferred to a centrifuge tube and the culture flasks were rinsed with 3 ml of H_2O. All rinses were added to the extracellular fluid, which was then filtered through a 0.45-µm syringe filter. The mycelial mats were individually homogenized in 3 ml of TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.9). The homogenizer was rinsed 2 times with 3 ml of TE-buffer. The rinses and homogenate for each sample were pooled, and to digest the cell wall, 5 mg/ml novozyme were added. The suspension was incubated for 2 hr at 37°C and then 3 ml of a concentrated H_2SO_4/HNO_3(1:1) mixture were added. The homogenate was shaken and incubated overnight at 60°C. Flasks were tightly closed using a rubber stopper during the incubation and inverted several times at the end of the incubation period to dissolve any elemental mercury that might have been formed. The homogenate was centrifuged at 2500 x g for 15 min, and the supernatant was filtered through a 0.45 µm filter. Both extracellular and mycelial samples were then analyzed by AAS.

Mycelia and spores were stained with DAPI (4,6- Diamidino-2 phenylindole, Sigma catalog # D1388) as described by Dhawale and Kessler (1993).

RESULTS AND DISCUSSION

Dry masses of cultures treated with 0, 10 ,25 or 50 ppm of mercury and harvested on consecutive days are shown in Figure 1A. Control cultures attained highest dry mass on day 3 after which they entered the biomass reduction phase. In *P. chrysosporium*, the biomass reduction phase represents a stringent response phase which is characterized by weight loss, synthesis of brown pigment, production of secondary metabolites and production of idiophasic proteins (Boominathan and Reddy 1992; Broda et al. 1989). Hence, we continued monitoring dry weights through day 7. All mercury-treated cultures showed arrest in dry mass gain. The 10 ppm mercury-treated cultures started recovering from growth inhibition in approximately 24 hr but the maximum dry weight attained by the 10 ppm mercury-treated cultures was significantly lower than controls on day 4. Interestingly, from day 4 the difference between the dry masses of control and 10 ppm mercury-treated cultures were insignificant. The cultures treated with 25 ppm of mercury required a longer time to begin recovery, while 50 ppm mercury-treated cultures showed no recovery by the end of treatment time. Experiments were not continued for a longer period because control cultures after this time were in a starvation phase due to nutrient depletion.

Figure 1B shows the results of experiments in which mercury was added on day 2 and dry masses of cultures harvested on consecutive days were determined. On days 3, 4 and 5, dry masses of controls were significantly higher than those of mercury-treated samples. However, on days 6 and 7, the 10 ppm mercury-treated cultures did

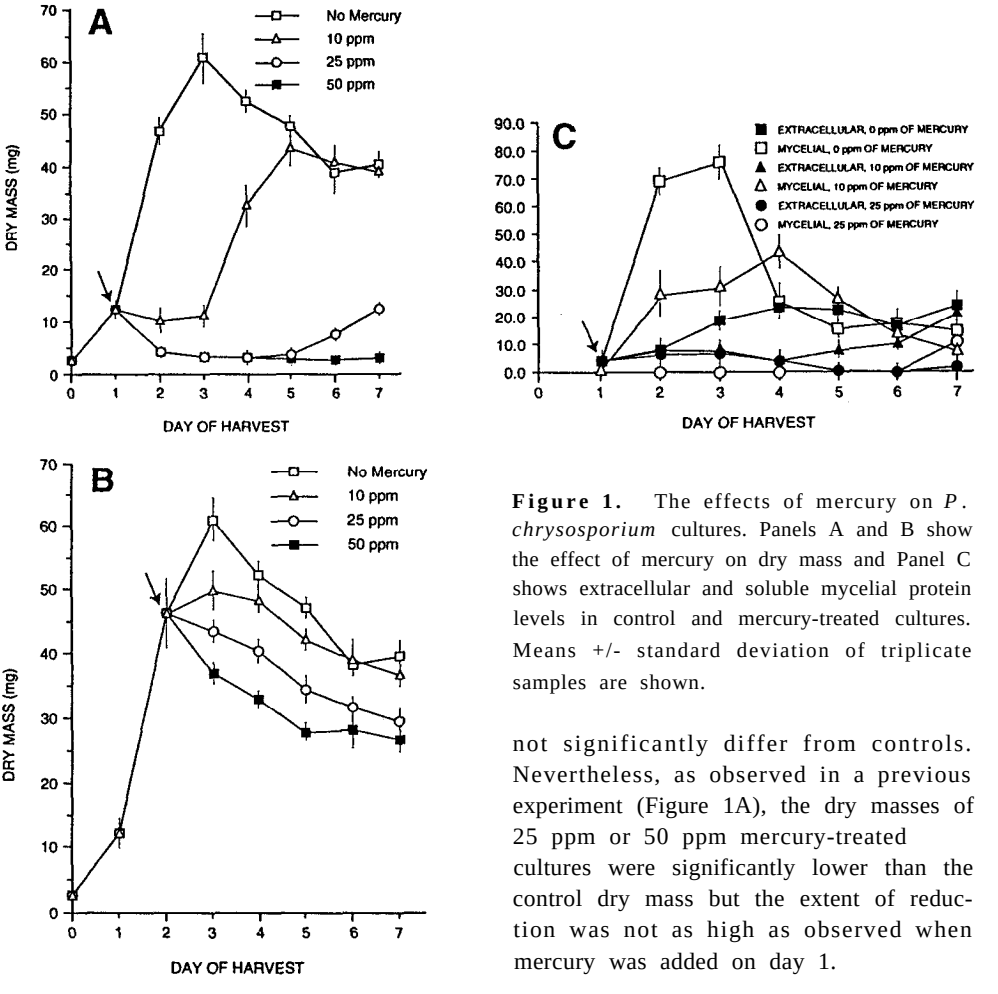

Figure 1. The effects of mercury on *P. chrysosporium* cultures. Panels A and B show the effect of mercury on dry mass and Panel C shows extracellular and soluble mycelial protein levels in control and mercury-treated cultures. Means +/- standard deviation of triplicate samples are shown.

not significantly differ from controls. Nevertheless, as observed in a previous experiment (Figure 1A), the dry masses of 25 ppm or 50 ppm mercury-treated cultures were significantly lower than the control dry mass but the extent of reduction was not as high as observed when mercury was added on day 1.

Sensitivity of ungerminated conidia to mercury was determined by adding low levels of mercury to the cultures at the time of inoculation. Mercury concentrations as low as 10 ppm completely inhibited germination. Therefore, much lower concentrations of mercury (0.5, 1.0 and 2.0 ppm and 5 ppm) were used. Dry masses of 0.5, 1.0 and 2.0 ppm mercury-treated cultures showed no reduction when compared to untreated controls shown in Fig 1A. However, 5 ppm of mercury caused nearly 50% reduction in dry mass (data not shown). Thus, germination appears to be the most sensitive stage.

To determine whether there was a correlation between amounts of soluble mycelial proteins, extracellular proteins and dry masses, a time course study was conducted. In this study 0, 10 or 25 ppm of mercury were added to cultures on day 1 and the

extracellular fluid and mycelia were processed on day 7 as previously described in Materials and Methods section. Figure 1C shows the amounts of extracellular and soluble mycelial protein contents for mercury-treated and untreated cultures. When these data were compared to the dry mass data shown in Figure 1A, it could be seen that in control cultures the levels of soluble mycelial proteins correlated directly with dry mass changes. In these cultures the maximum amount of soluble mycelial protein was found on day 3 while the maximum amount of extracellular proteins was found on day 4. The cultures that received 10 ppm of mercury contained significantly less soluble mycelial proteins on days 2 and 3. However, on day 4 mycelial protein levels of 10 ppm mercury-treated cultures were significantly higher than the control. Unlike controls, these cultures did not enter the biomass reduction phase until day 5. The extracellular proteins in 10 ppm mercury-treated cultures were significantly lower than controls on days 2, 3, 4, and 5. Nonetheless, on days 6 and 7 the differences in protein levels between controls and 10 ppm mercury-treated cultures were not significant. For the entire experimental period soluble mycelial protein levels as well as extracellular protein levels were significantly lower than controls in 25 ppm mercury-treated cultures. However, the recovery appears to begin on day 7 for these cultures. Thus, a direct correlation between dry masses and soluble protein levels is apparent.

To determine if the decrease in protein levels might indicate a decrease in specific proteins, SDS PAGE protein profiles of cultures that received 0, 10 or 25 ppm of mercury on day 1 were obtained. Extracellular fluid and mycelia from large scale cultures harvested on day 7 were pooled, processed and concentrated. The extracellular protein patterns for 10 and 25 ppm mercury-treated cultures were identical to those of untreated controls but 25 ppm mercury-treated cultures showed reduced amounts in all extracellular protein bands (data not shown). The mycelial protein patterns, however, did display alterations. Figure 2 shows that the high molecular weight band which is present in the control (lane 1) is absent from 10 ppm and 25 ppm mercury treated samples (lanes 2 and 3, respectively). Strikingly, the 25 ppm mercury-treated cultures displayed a prominent but diffuse low molecular weight band which was not observed in the controls or in 10 ppm mercury-treated samples. When the 25 ppm sample was subjected to electrophoresis on 10 % SDS- polyacrylamide gel, the low molecular weight band yielded a smear indicating that the diffused band contained proteolytic products.

Since the addition of mercury on day 2 appeared to have less toxicity (based on dry mass data shown in Figure 1A), we compared untreated controls to 50 ppm mercury-treated (added on day 2) cultures. Extracellular and soluble mycelial protein patterns for 0 and 50 ppm mercury-treated samples were similar to each other, but the amount of protein in numerous bands was lower in mercury-treated cultures. However, the low molecular weight smear seen in Figure 2, was not observed in the 50 ppm mercury-treated mycelial samples. Qualitative estimation of free amino acids by paper chromatography showed that in comparison to control samples, 50 ppm mercury-treated cultures contained high amounts of free amino-acids in extracellular fluids (Figure 2).

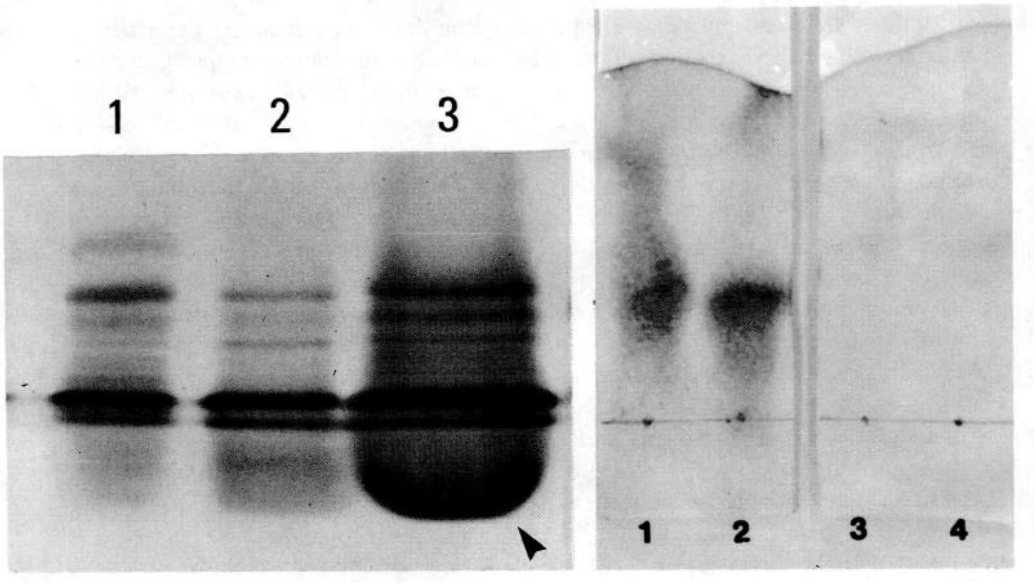

Figure 2. Effect of mercury on mycelial proteins and the amount of free amino acids in the extracellular fluid of *P. chrysosporirium* cultures. Left panel shows SDS-PAGE gels displaying mycelial proteins from cultures grown in presence of 0 ppm (lane 1), 10 ppm (lane 2) or 25 ppm (lane 3) mercury. Right panel shows qualitative estimation of free amino acids. Equal volumes of extracellular fluid from 50 ppm mercury-treated (lanes 1 and 2) and from untreated control cultures (lanes 3 and 4) were analyzed by paper chromatography.

Cultures that received 50 ppm of mercury on day 2 were also analyzed for their mercury content. Mycelia and extracellular fluids were harvested day 7 and the medium containing 50 ppm of mercury and no inoculum served as a control. Even though rubber stoppers were used to prevent loss of mercury in the form of mercury vapors, only 27.1 ppm of mercury was detected in the medium from control flasks after 8 days of incubation. Since this appeared to be the maximum amount that could be recovered from controls at the end of experiment, this amount was arbitrarily set at 100% for making comparisons. In the initial experiments where the mycelia were digested with nitric acid alone, the total mercury recovered was only 52%. However, the modified method of processing mycelia where lysozyme was used to digest the cell wall, the total mercury recovery was 106 %, with 14% found in the extracellular fluid and 92 % in the mycelial fraction. These data suggest that the organism accumulated mercury in its mycelial fraction. The observation that digestion of cell wall from mycelia yields much higher amounts compared to simple acid treatment of mycelia suggests that mercury is associated with the cell wall. This is similar to the biosorption observed using other fungi (Seigel et al. 1990). Adsorption to the cell wall may be a mechanism which leads to the observed high tolerance for mercury.

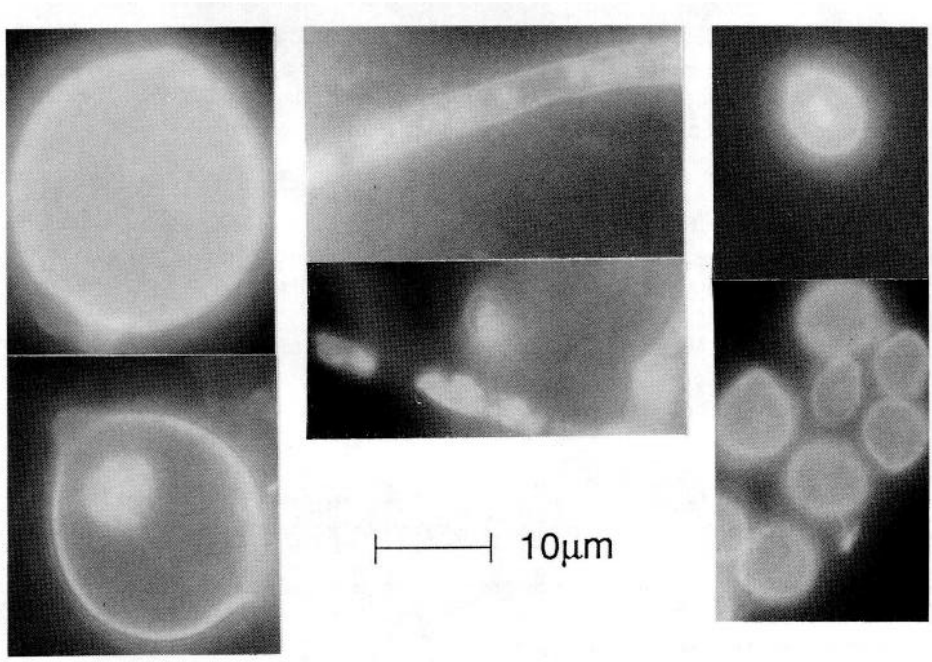

Figure 3. DAPI-stained chlamydospores (left), hyphae (center), and conidiospores (right). Top panel depicts untreated sample while the bottom panel shows mercury-treated samples.

To study the effect of mercury on morphology, teased mycelial mats from untreated controls and 50 ppm mercury-treated cultures (mercury added on day 3) were stained with DAPI on day 7. The average width of 25 randomly chosen hyphal segments from untreated cultures was 3.717 ± 0.408 µm, while that of 50 ppm mercury-treated cultures was 2.692 ± 0.448 µm. Thus, the hyphae from mercury-treated cultures were significantly thinner. The conidia, chlamydospores and hyphae grown in the presence of 50 ppm mercury exhibited abnormal morphology (Figure 3). In methylene blue stained preparation the cytoplasmic membrane appeared to be pulling away from the cell walls (data not shown). This observation, taken together with increased extracellular amino acid levels and altered protein patterns suggest alteration in membrane permeability.

Mercury is toxic to all forms of life but the tolerance levels vary between organisms as well as between tissues of an organism. For example, in humans 1 ppm mercury in blood is toxic and 4 ppm of mercury (4 mg of mercury/ kg. of body weight) is lethal. However, the 96-hr LC_{50} value of mercury for freshwater fish *Cyprinus carpio is* 0.5 ppm (Suresh et al. 1991). In comparison, *P. chrysosporium* appeared to possess tolerance to high levels of mercury. Ungerminated cultures were most sensitive to mercury, but even these cultures were not affected by up to 2 ppm of mercury and total inhibition of germination was observed only at 10 ppm of mercury.

Growing cultures appeared to tolerate even higher concentrations of mercury. It appeared that culture age plays a role in determining the levels of mercury tolerance. When mercury was added on the second day, 10 ppm of mercury had no apparent effect on dry mass or on morphology and 25 ppm of mercury could be tolerated.

In summary, the results of this study show that *P. chrysosporium* is tolerant to high levels of mercury and it appears to accumulate mercury. Hence, this fungus has a potential for use as a metal biosorbant in emerging bioengineering technologies for treating industrial effluents and contaminated waters and soils.

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