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Cultivation of *Phanerochaete chrysosporium* and Lignin Peroxidase Activity

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Effects of exogenous veratryl alcohol addition on the growth of basidiomycete *Phanerochaete chrysosporium* ME-446 and the induction of lignin peroxidase activity were investigated in this study. The organism was grown in ligninolytic (low-nitrogen) culture conditions in which extracellular enzymes are produced. Analyses showed that a statistically significant decrease of cell growth was associated with the veratryl alcohol addition. The effect of veratryl alcohol addition on LiP activity was nearly instantaneous and this effect diminished with culture aging. The extent of this effect was different depending on the time of addition, which led to a speculation that there might be some other effector species which played a role in regulation of lignin peroxidase activity.

Over the past several years the degradation of a variety of aromatic pollutants by *Phanerochaete chrysosporium* has been reported (6, 7, 24). *P. chrysosporium*, a white rot wood-decaying basidiomycete, produces two unique extracellular heme peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP) under substrate-limiting conditions (9, 13). They are believed to be the major enzymes responsible for the degradation of both lignin and many of the chlorinated pollutants. A number of studies (17, 18, 19) have demonstrated that both *P. chrysosporium* and LiP are promising for application in hazardous waste treatment. Recently the pathways used by this fungus for chlorinated aromatic pollutant degradation has been examined (11), and a mechanism for the degradation of halogenated organics has also been proposed (3).

Dhawale *et al.* (7) observed the compound degradation of phenanthrene by *P. chrysosporium* under ligninolytic (low-nitrogen) as well as nonligninolytic (high-nitrogen) conditions. Degradation of benzene, toluene, and xylene (BTX), however, was favored under nonligninolytic conditions (24). Involvement of both the mycelium and extracellular enzymes in the degradation process by *P. chrysosporium* has been proposed (18, 19). Both mycelium and extracellular enzyme were required for the complete degradation of 2,4,6-trichlorophenol (1). Yadav and Reddy (24), however, provided data that the degradation of BTEX compounds was due to fungal metabolism and not due to sorption to mycelial biomass.

Lignin peroxidase activity has been reported to appear only during secondary metabolism (9). The external factors affecting the activity of *P. chrysosporium* have been identified by many researchers (13, 14, 17). The optimum temperature for growth of the fungus has been established at 39°C, and the optimum pH between 4.0-4.5. Studies have shown that the LiP activity was enhanced by addition of veratryl alcohol (10, 17), which is a secondary metabolite of *P. chrysosporium*, and excess trace elements (15). Faison *et al.* (10) speculated that veratryl alcohol probably functions as an inducer for lignin peroxidase. Others (2, 12) have shown an improved ligninase production in the presence of surfactants such as Tween 80 (sorbitan polyoxyethylene monooleate). Lewandowski *et al.* (17), however, has pointed out that neither Tween 80 nor veratryl alcohol were absolutely essential to the process in view of the their cost in a commercial application.

While there are some disputes on the role of mycelium and enzymes in the degradation process, this study examined the effect of veratryl alcohol addition on the cultivation of *P. chrysosporium* and the lignin peroxidase activity as a preliminary step toward the characterization of role of mycelium and enzymes in the degradation of BTX compounds.

MATERIALS AND METHODS

Organism and Media

A set of experiments in 3 Series was conducted using

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Table 1. Composition of growth medium.

Component	Concentration
Glucose	10.0 g
KH_2PO_4	2.0 g
$CaCl_2$	0.1 g
NH_4Cl	0.12 g
$MgSO_4$	0.5 g
Thiamine	1.0 mg
Water	1.0 liter

P. crysosporium ME-446 (ATCC 34541). The organism was grown for sporulation for 4 days at 39°C on yeast-malt agar plate and maintained at 4°C. A 100 ml volume of liquid YM broth (Difco) in a 250 ml baffled flask sealed with cotton stoppers was inoculated with the spores, and the cultures were grown at 39°C for 14 days under shaking conditions at 150 rpm. The broth contained yeast extract 3 g, malt extract 3 g, peptone 5 g, and dextrose 10 g per liter. The pre-grown cultures were aseptically homogenized, and this homogenized mycelial inoculum was transferred to each of three 250 ml flasks partially filled with growth medium for cultivation. The pre-grown culture was used at the 10% (vol/vol) level and the composition of growth medium is provided in Table 1. No additional mineral salts were added to the medium. The initial pH of the medium was between 4.3 and 4.7, and no further pH adjustment was made.

Three flasks (Series 1, 2, and 3) were used in parallel for cultivation of *P. chrysosporium*, each having a replicate. Flasks in Series 1 did not receive veratryl alcohol. Veratryl alcohol was added approximately 4 days after the inoculation in Series 2, and it was added at the time of inoculation in Series 3. In addition to the effects of veratryl alcohol on the growth of *P. chrysosporium*, the optimal culture period for the production of ligninase peroxidases in a batch mode operation was also studied. For the purpose of deliberately increasing the enzyme activity, 2 mM of veratryl alcohol (Aldrich Chemical Co.) was added to the flasks. Ten mls of homogenized culture fluids were aseptically withdrawn daily from each flask, and they were analyzed for ammonia nitrogen, glucose, LiP activity, and dry biomass.

Analytical Methods

Dry biomass. For determining mycelial dry weight, 10 ml of culture fluid in each flask was vacuum filtered through tared GF/C grade filter and dried to a constant weight in a 103°C dry oven for 12 h, and the cell mass was calculated by difference. The filtered culture fluids were used in determining glucose, ammonia nitrogen, and enzyme activity.

Substrate concentration. Ammonia nitrogen concentrations were determined by Indo phenol method. Glucose concentrations were determined by DNS (dinitrosalicylic acid) method, using D-glucose as standard. Optical density was measured at 575 nm.

Lignin peroxidase assay. Lignin peroxidase activity (veratryl alcohol oxidase activity) was assayed as described by Tien and Kirk (22) by measuring the rate of veratraldehyde production from veratryl alcohol. Veratraldehyde production was followed spectrophotometrically at 310 nm (Kontron, Uvikon 930). Prior to the reading at the spectrophotometer, 350 µl of the solution (220 mM Na tartrate and 3.33 mM veratryl alcohol, adjusted to pH 3.0 by tartaric acid) was added to 500 µl of sample and next 50 µl of 10 mM H_2O_2. The three highest rates of extinction were averaged (extinction coefficient 9.3). Activity was expressed in units per liter (U/l), which means that one unit of ligninase activity converts one micromole of veratryl alcohol to veratraldehyde per minute.

RESULTS AND DISCUSSION

Growth and Substrate Utilization

The ability of *P. chrysosporium* to degrade chlorinated chemicals and polyaromatic hydrocarbons is related to its lignin degrading enzyme system. Some of the most important components of its lignin-degrading enzyme systems are lignin peroxidases, H_2O_2, oxalate, and veratryl alcohol (3, 4, 23). Veratryl(3,4-dimethoxybenzyl) alcohol, which is a secondary metabolite produced in response to nitrogen limitatioin, has been known to stimulate lignin peroxidase synthesis in cultures (9). The optimum concentration of veratryl alcohol for increase of activity was 0.4 mM (15), whereas Leisola *et al.* (16) reported maximal enhancement of lignin peroxidase activity for veratryl alcohol concentrations between 1 and 2 mM. Sang *et al.* (21) also found that adding 2 mM performed better than 0.4 mM for induction of lignin peroxidase. In this study, 2 mM of veratryl alcohol was added to determine the effects of veratryl alcohol addition on fungal growth and subsequent enzyme activity.

Figs. 1 through 3 present the patterns of cell growth and glucose concentrations for each Series in ligninolytic cultures of *P. chrysosporium* ME-446. Mycelial growth during the experimental period was not substantial, which might be due to the use of cultures pre-grown for 2 weeks.

Suppression of cell growth following the addition of exogenous veratryl alcohol in Fig. 4 is apparent. However, in Series 3, the biomass measured on 34 and 84 h of cultivation time were higher than those for Series 2. The probable reason for this would be an inherent experimental error associated with the difficulty of pipetting fungal culture for measuring dry biomass, and this might have caused the two dry weight values higher than they were supposed to be.

Compared to the differences in cell mass between

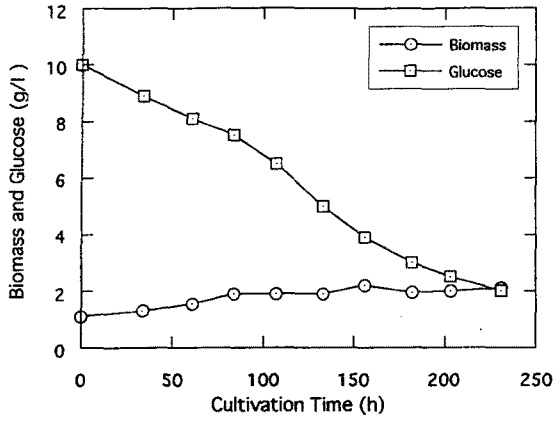

Fig. 1. Cell growth and remaining glucose concentration in Series 1.

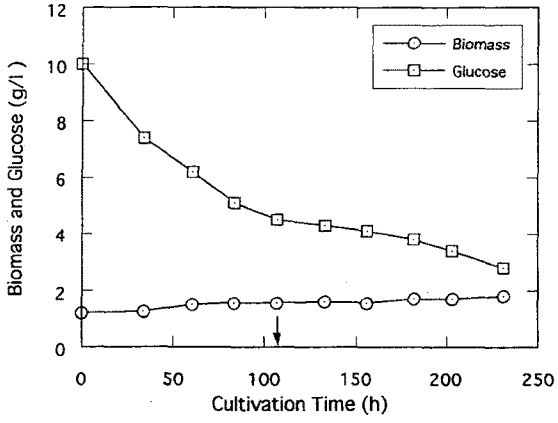

Fig. 2. Cell growth and remaining glucose concentration in Series 2.
Arrow indicates the point of veratryl alcohol addition.

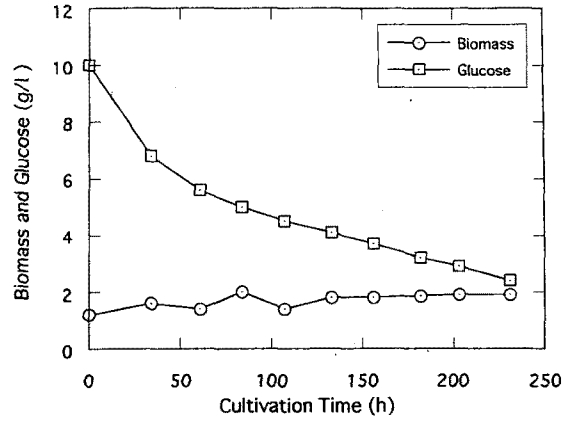

Fig. 3. Cell growth and remaining glucose concentration in Series 3.

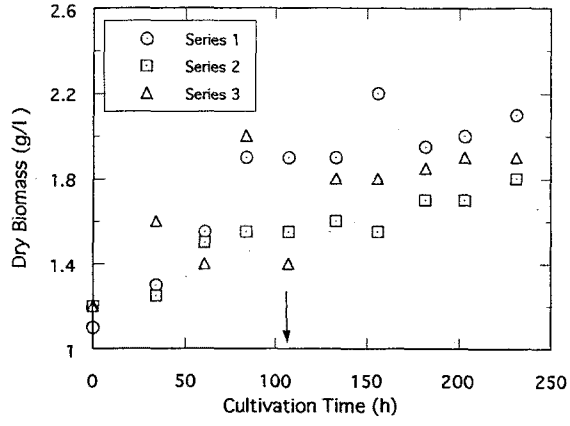

Fig. 4. Comparison of cell growth between 3 experimental Series.
Arrow indicates the point of veratryl alcohol addition for Series 2.

Series 1 and Series 2, the differences between Series 1 and Series 3 appeared to be less significant. In addition to the graphical comparisons, therefore, statistical analysis was conducted according to the procedures in Box *et al.* (5) to test the hypothesis if the exogenous veratryl alcohol addition to the cultures did or did not affect the cell growth. The null hypothesis of H_0 that the difference in cell mass between Series 1 and 3 was merely experimental error was tested using the "paired *t*-test". Data obtained at the start of the experiment were excluded in the test, and calculation of *t* is summarized in Table 2.

Based on the calculation in Table 2, the difference was significant at the 95% confidence level. This led to a conclusion that a statistically significant decrease of cell growth in Series 3 was associated with the veratryl alcohol addition.

Table 2. Calculation summary for a *t* test.

Average of differences between Series 1 and Series 3 = 0.128 g/l
The sample variance = 0.057 g/l
Standard error of the average difference = 0.08 g/l
Degrees of freedom = 8
$t_0 = 1.60$
Pr ($t \geq t_0$, df=8) = 0.078

Lignin Peroxidase Activity

Table 3 provides the LiP activities for approximately 10 days of experimental period for 3 Series. Decrease of ammonia nitrogen concentrations in the cultures (data not shown) did not match the lignin peroxidase production by secondary metabolism. In Series 1 in which veratryl alcohol was not added, lignin peroxidase activity steadily increased along with mycelial growth. Little difference was observed in the activity between Series 1 and Series 2 before veratryl alcohol was added to the Series 2. The LiP activity, however, exhibited over a 3 fold in-

Table 3. Lignin peroxidase (LiP) activity.

Cultivation time (h)	LiP activity (U/l)		
	Series 1	Series 2	Series 3
0	62.3	58.5	609.5
34	69.4	65.3	605.7
61	69.6	69.0	598.5
84	70.1	67.3	504.5
107	71.3	67.4	405.6
133	74.2	236.0	321.5
156	70.8	207.2	240.5
182	68.1	217.1	228.2
203	76.6	203.1	227.4
231	77.2	192.2	197.2

Each measurement was done in duplicate.

crease (236.0 U/l) when measured 26 h after veratryl alcohol addition. The increase in response to veratryl alcohol addition was nearly instantaneous, and this effect progressively diminished with time. Similar to this result, the increase of LiP activity 2 to 4 h after the addition of exogenous veratryl alcohol to ligninolytic cultures was demonstrated (10). Meanwhile, in Series 3, in which the veratryl alcohol was added at time 0, the LiP activity was substantially higher than those for both Series 1 and 2 with an initial value of 609.5 U/l. Reason for the relatively lower LiP activity following the veratryl alcohol addition in Series 2 might be related to the concentration of ligninase in the culture. This result suggested that LiP activity could be regulated by adding veratryl alcohol and/or varying culture condition.

Considering the progressive increase of activity in Series 1, the decrease of LiP synthesis that actually occurred in both Series 2 and 3 might be larger than the measured values. Fig. 5 shows the decline of activity computed by subtracting the LiP activities in Series 1 from those in Series 2 and 3. The differences in the rate of LiP syntheses were assumed negligible in this calculation. As shown in Fig. 5, the decrease in Series 3 was very rapid during the period of 61 to 156 h of cultivation followed by a slow decrease. The decreasing pattern toward the end of cultivation appeared similar for both Series 2 and 3. The decline in LiP activity might be due to 1) degradation of LiP proteins by a protease induced under starvation conditions during secondary metabolism (8) and 2) the loss of extant enzyme in aging cultures (10). According to Michel *et al.* (20), loss of LiP activity corresponded to the depletion of glucose in the culture fluid. They used initial glucose concentration of 10 g/l, and the loss began to appear when the glucose level became below 1 g/l. In this study, however, glucose level was over 2 g/l throughout the experimental period.

In conclusion, results of this study showed that the addition of veratryl alcohol to cultures caused an interference with fungal growth. The effect of veratryl alcohol addition on LiP activity was nearly instantaneous and this effect became retarded as cultivation progressed. The extent of this effect was different depending on the time of addition. This led to a speculation that there might be some other effector species which played a role in regulation of lignin peroxidase activity.

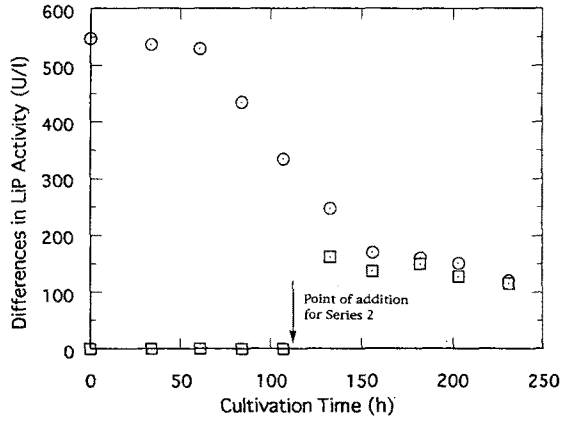

Fig. 5. Decrease of LiP activity in Series 2 and 3 following the addition of veratryl alcohol.
Circle indicates the LiP difference between Series 3 and 1, and square between Series 2 and 1.

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