Biodegradation 7: 175–182, 1996.

Degradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium* grown in ammonium lignosulphonate media

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Accepted 7 November 1995

Key words: dehalogenation, fungi, lignosulphonates, pentachlorophenol, *Phanerochaete chrysosporium*

Abstract

Removal and degradation of pentachlorophenol (PCP) by *Phanerochaete chrysosporium* in static flask cultures was studied using ammonium lignosulphonates (LS), a waste product of the papermill industry, as a carbon and nitrogen source. After 3 days, cultures of *P. chrysosporium* grown in either a 2% LS (nitrogen-sufficient) medium or a 0.23% LS and 2% glucose (nitrogen-deficient) medium removed 72 to 75% of PCP, slightly less than the 95% removal seen using nitrogen-deficient glucose and ammonia medium. PCP dehalogenation occurred despite the fact that extracellular enzyme (LiP) activity, measured by a veratryl alcohol oxidation assay and by PCP disappearance in cell-free extracts, was inhibited by LS. This inactivation of LiP likely contributed to the lower percent of PCP dehalogenation observed using the LS media. In order to better understand the relationship between PCP disappearance and dehalogenation, we measured the fate of the chlorine in PCP. After 13 days, only 1.8% of the initial PCP added was recoverable as PCP. The remainder of the PCP was either mineralized or transformed to breakdown intermediates collectively identified as organic halides. The largest fraction of the original chlorine (58%) was recovered as organic (non-PCP) halide, most of which (73%) was associated with the cell mass. Of the remaining chlorine, 40% was released as chloride ion, indicating a level of dehalogenation in agreement with previously reported values.

Introduction

The white rot fungus *Phanerochaete chrysosporium* has been widely studied for its ability to degrade lignin (Jeffries et al. 1981; Kirk et al. 1978) and a variety of environmental soil pollutants, including pentachlorophenol (PCP) (Mileski et al. 1988; Lamar et al. 1990; Alleman et al. 1995). When *P. chrysosporium* is grown in defined medium (glucose, nitrogen and mineral salts) in the laboratory in static flask cultures, 85 to 97% of PCP is transformed with 12–60% of [^{14}C]PCP recovered as $^{14}CO_2$(Mileski et al. 1988). If white rot fungi are to be used for large scale bioremediation of PCP-contaminated soils and wastewaters, however, glucose media will need to be replaced by other, less expensive, carbon sources. Bioremediation field tests have been conducted using different strains of white rot fungi grown on fortified grain-sawdust mixtures (Lamar et al. 1993). Growth of *P. chrysosporium* on this media in field tests resulted in only 67 to 72% of PCP removal from soils with the amount of PCP degraded assumed to be identical to PCP removed.

Since PCP is a major component of creosote, a wood preservative and frequent contaminant at wood preserving facilities, we have been considering as potential growth substrates for white rot fungi other products or byproducts available to the paper industry. In this project we report the results of our work on examining the use of lignosulfonates (LS) as a fungal growth medium. LS are a waste product of the papermill industry generated during the bleaching and pulping process. These solutions are highly colored (dark brown) due to their high lignin content and are therefore usually disposed of by incineration. However, LS contain large concentrations of wood sugars (~25%, Table 1), nitrogen (17.6 g/l as NH_3^+-N), and

other trace minerals needed for growth of fungi making LS a logical choice as an alternative growth substrate.

The presence of large concentrations of lignin in LS was considered an asset for their use since lignin-peroxidases (LiP) produced by fungi to degrade lignin have been implicated in the degradation of many toxic chemicals, and lignin has been shown to enhance LiP activity (Jeffries et al. 1981). However, the specific role of LiP in PCP degradation is unclear. Studies have shown that LiP isolated from cultures of *P. chrysosporium* can partially degrade aromatic compounds (Mileski et al. 1988; Lin et al. 1990) as evidenced by evolution of $^{14}CO_2$ from radiolabeled PCP. Although the PCP degradation rate is increased in the presence of LiP (Boominathan et al. 1990), PCP is degraded under both lignolytic and non-lignolytic conditions (Armenante et al. 1994). Structurally similar compounds, such as 2,4,6-trichlorophenol, are also partially degraded in the absence of LiP, but complete degradation does not occur unless both mycelia and enzymes are present (Armenante et al. 1994). The sole importance of the specific enzymes classified as LiP even in lignin degradation is questionable since partial lignin degradation has been observed with mutant cultures unable to excrete LiP (Dorosetz et al. 1990).

The persistence of the dark color of LS could pose a problem for its use in soil bioremediation or wastewater treatment processes. However, *P. chrysosporium* can de-color sulphonated azo dyes (Paszczynski et al. 1992), compounds which are structurally similar to the substructures of LS, and chlorolignins in paper-mill wastestreams (Pellinen et al. 1988). We therefore speculated that LS might also be sufficiently de-colored during the growth of white rot fungi.

In order to determine the usefulness of LS for bioremediation processes involving PCP, we measured the disappearance of PCP in static flask cultures for fungi grown in liquid media using LS as either the sole carbon and nitrogen sources (2% LS) or as the sole nitrogen source (0.23% LS with 2% glucose). To show that the disappearance of PCP resulted in mineralization, the concentrations of total organic halide and inorganic halide (both in the liquid and solid phases) were measured during PCP degradation by fungi grown in defined (glucose and nitrogen) medium. We assumed the pattern of degradation seen in the defined medium was the same for LS media since we could not accurately measure organic halides due to the high chloride concentration of LS media. Because of the important role of LiP in PCP degradation (Armenante et al. 1994), we also monitored the effect of LS on LiP activity in cell-free suspensions.

Methods

Cultures

Phanerochaete chrysosporium (BMK-F-1767) was obtained from the University of Arizona Mycological Collection, Department of Plant Pathology, University of Arizona, Tucson. Cultures were maintained on 2% (wt./vol.) malt agar slants for long term storage (greater than two months). All inoculations were made using fungi grown for at least 5 days in a static culture flask. Fungi and media were poured into an autoclaved stainless steel blender and mixed for 1 min. A 100 μl aliquot of this suspension was aseptically transferred to a sterile 250 ml wide-mouth flask containing 20 ml of media. Flasks were capped with a #8 stopper that had two 2.5 mm holes bored through it. These holes were lightly stuffed with cotton stopper gauze. The flasks were incubated for 3 days at 37°C, moved to a 30°C incubator and the headspace purged with sterile O_2. After 1 min of purging at 600 ml/min, both holes were sealed with 500 μl culture tubes.

A nitrogen-deficient 2%-glucose liquid medium used in some experiments was prepared according to Alleman et al. (1995). LS used in two other media were added on a weight/volume basis (0.23% and 2%). A nitrogen-deficient medium using LS as the sole nitrogen source was prepared by substituting 0.23% of LS for the 0.178 g/l of NH_4NO_3 in the nitrogen-deficient glucose medium. This 0.23% LS medium therefore contained the same concentration of ammonia as a standard nitrogen-deficient medium. No glucose or NH_4NO_3 were used for the 2% LS medium (a nitrogen-sufficient medium defined as medium containing >2 g/l of NH_3) since ammonia and sugars already in the LS solution served as the nitrogen and carbon sources.

Fungal growth

In order to determine the effect of LS on the growth of *P. chrysosporium* in static flask cultures, fungi were incubated isothermally at 37°C under an air atmosphere. After 7 days cultures were filtered onto glass-fiber filters (Whatman GF/C 2.5 cm) at low vacuum (<5 mmHg), and rinsed with ultra-pure water (MQ, Millipore Corp.). Filters were dried overnight in a 105°C oven and weighed. The mean and mode dry weight of

a GF/C filter was determined to be 0.092 g (±0.002 g; range of 0.088–0.094 g; n=14) by filtering sterile media onto a filter by the same method. This filter weight was assumed for all cell mass determinations. Since dry cell masses were in the range of 0.02 g, an error of ≤10% was produced using average filter weights.

Chloride mass balance

The extent of PCP degradation in static flask cultures was evaluated based on the disappearance of PCP and the production of organic and inorganic halides. PCP was added by injecting 10 μl of a 25 mg-PCP/ml-ethanol solution into quadruplicate flasks 5 days after inoculation with fungi. On day 8 the contents of all four flasks, including mycelium, were combined and poured into a stainless steel blender. To remove mycelium and PCP that was attached to the sides of the flask, each flask was rinsed with 3 ml MQ, 1 ml 95% ethanol, and 3 ml MQ. In order to ensure both the liquid and mycelia samples were homogeneous, this mixture was blended on high speed for 2 min. This suspension was then centrifuged (15,000 × g) at 4°C for 30 min to separate mycelium from the liquid medium.

The total organic halide balance was measured in terms of six quantities in liquid (l) and solid (s) phases in terms of inorganic halide in the liquid and solid phase (IX_l, IX_s); non-PCP organic halide (OX_l, OX_s); and PCP that was not degraded (PCP_l, PCP_s). IX_l was measured directly by injecting 100μl of the fluid into the titration cell of a Dohrmann DX-20 organic halide analyzer. PCP_l was measured directly by injecting 20 μl into a high performance liquid chromatograph (HPLC, Beckman), equipped with a C-18 reverse phase column (Rainin microsorb-mv). The mobile phase was a 75:25:0.125 mixture of acetonitrile:water:acetic acid. PCP was monitored at 238 nm and peak counts compared to a standard calibration curve. PCP_s was extracted from the mycelia into hexane by adding 1 ml of NaCl saturated water followed by 10-ml of hexane. The solution was vigorously shaken and the hexane removed. This procedure was repeated, and the two samples from the replicate flasks combined into a single sample. The hexane sample was evaporated with argon and the precipitate resuspended in mobile phase for HPLC analysis.

Because OX was not directly measurable, it was calculated from TOX (total organic halide, including PCP) using:

$$OX = TOX - PCP \qquad (1)$$

TOX_s was measured by removing a small amount of centrifuged wet cells followed by pyrolysis/combustion (Dohrmann DX-20) of a known mass. TOX_l was measured by sorption of a known volume of culture fluid onto powdered activated carbon (PAC) columns followed by pyrolysis/combustion.

The percent recovery of chloride from the initial PCP added (PCP_i) for all experiments was calculated from total organic and inorganic halide:

$$\%Recovery = \frac{TOX_l+TOX_s+IX_l+IX_s}{PCP_i} \times 100 \qquad (2)$$

Since there was no measurable chloride associated with the mycelium in control cultures with 50 μg Cl- (no PCP) added, we assumed IX_s=0. Percent recovery of Cl was 90–110% for all samples.

PCP disappearance

Fungal cultures were grown in replicate 250-ml flasks for 5 days and dosed with PCP as described above. After incubation for 3 to 21 days all PCP was extracted from the culture (containing both the mycelia and the growth medium) using the hexane extraction protocol described above.

Enzyme activities

Extracellular enzyme (LiP) activity was measured by the veratryl alcohol oxidase activity assay (Linko & Haapla 1993). Enzyme activity was also measured by direct oxidation of PCP as described by Mileski et al. (1988) except that DMSO (1%) was substituted for N,N-dimethyl-formamide, and the concentration of PCP was reduced by a factor of 100 to accommodate low enzyme activity (U=10). One unit of enzyme activity (U) oxidizes 1 μmol of veratryl alcohol per minute.

Analysis of lignosulphonates

Decoloration of LS was characterized by UV-visible spectral scans taken at 300–700 nm (Shimadzu UV-160A). The concentration of ammonia in LS was determined using an ion selective electrode (Orion).

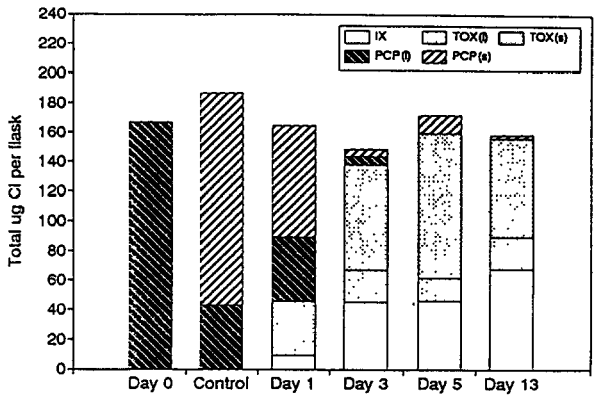

Figure 1. Chlorine mass balance in static cultures of *P. chrysosporium* grown in a 1% glucose nitrogen-limited medium spiked with 250 μg-PCP. PCP was added after five days of growth. Control was a killed culture harvested two days after inoculation. Figure legend indicates distribution of chloride into five different fractions: total organic halide in liquid, TOX(l), and solid, TOX(s), phases; PCP associated with solid, PCP(s), and liquid PCP(l), phases; and inorganic halide, IX, in the liquid phase indicating the concentration of chloride ion produced from dehalogenation of PCP.

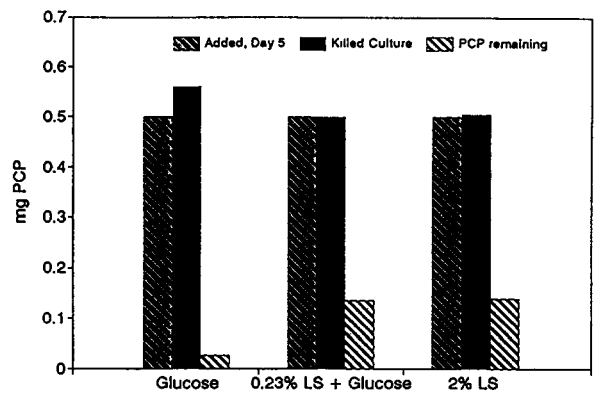

Figure 2. PCP remaining in cultures of *P. chrysosporium* after three days of incubation in nitrogen-limited glucose and 0.23% LS media. PCP was added after five days of growth.

Results

PCP removal and degradation

P. chrysosporium grown in static flasks in a nitrogen-limited medium degraded, but did not completely dehalogenate, PCP (Figure 1). After 13 days, however, only 1.8% of the original PCP was recoverable as PCP. Most of the PCP (75%) became immediately cell bound, and was transformed to intermediate compounds within the mycelium. By day 5 these breakdown intermediates, defined as non-PCP organic halide, accounted for 70% of initial PCP (as chlorine). Once these intermediates appeared, further degradation proceeded slowly, decreasing to only 58% by day 13. Complete dehalogenation, measured by inorganic chloride (IX), showed a steady increase through day 13, with a final dehalogenation of 40%.

Effect of LS media on PCP removal

We compared the ability of fungi grown in a LS medium to degrade PCP with fungi grown in a nitrogen-limited 2% glucose medium by measuring the disappearance of PCP from solution. Cultures of *P. chrysosporium* grown on nitrogen-limited glucose-ammonia medium removed 95% of the initial PCP after 3 days. When LS was used as the nitrogen source, PCP removal was 75%. Similarly, when LS was used as a carbon source, PCP removal was 72% (Figure 2). Recovery of PCP from heat killed control cultures appeared to be unaffected by LS but was slightly more than 100% (100–112%), likely due to some volatilization of the hexane used for extraction.

Effect of LS on LiP activity

Since LiP enzymes have been shown to be important in the breakdown of some chemicals, we examined the effect of LS on LiP activity. In all cases the addition of $\geq$0.23% LS to culture media inhibited LiP activity. Cell free suspensions grown in static flasks had an LiP enzyme activity of U=13 (veratryl alcohol oxidase assay) but decreased to U=0 (no measurable activity) after the addition of 0.23% LS (data not shown). The extent of inhibition was further examined by adding smaller concentrations of LS to cell-free extracts and measuring the resulting activity. Enzyme activity was partially inactivated by a 0.0001% LS solution, with no detectable activity remaining after 0.001% LS (Figure 3). Because this LS concentration is 100x less than that used in this study it is unlikely that there was any LiP enzyme activity in any of our static flask cultures containing LS. In shake flask cultures containing $\geq$0.23% LS (and 0.1% Tween 80) we similarly did not observe any enzyme activity although others have observed high enzyme activities (U<250; Linko 1992) in the absence of LS under these conditions.

To be certain that the assay (veratryl alcohol oxidase) was not selectively inhibited, LiP activity was also measured by incubating extracellular broth with

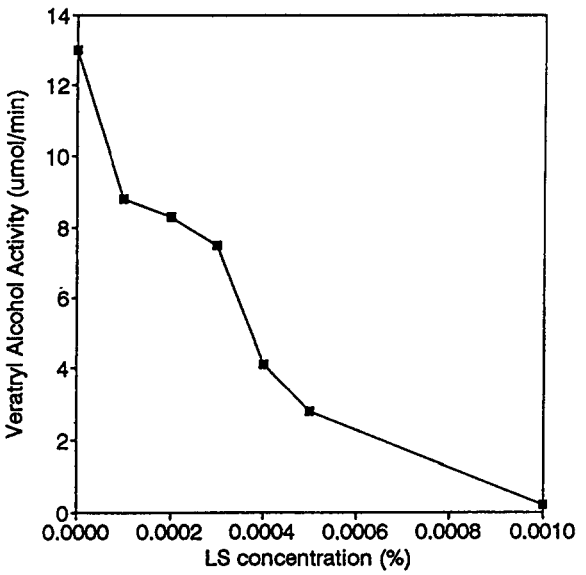

Figure 3. Inhibition of LiP activity by LS as measured by the veratryl alcohol oxidation assay.

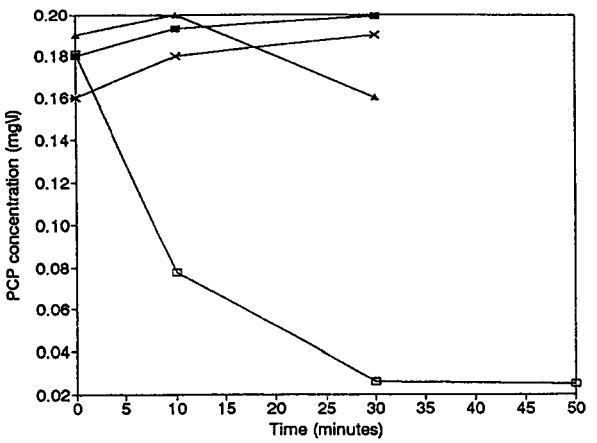

Figure 4. Disappearance of PCP with extracellular broth taken from five day old cultures of *P. chrysosporium* (nitrogen-limited glucose medium (□), autoclaved (enzyme denatured) nitrogen-limited glucose medium (■), 0.23%LS medium (▲), 50:50 mixture of nitrogen-limited glucose medium and autoclaved 0.23% LS medium (×)).

PCP. Figure 4 shows that LiP activity is easily measurable in cultures grown in a nitrogen-limited glucose medium, with PCP decreasing from 0.18 μM to 0.025 μM within 30 min. The same suspension could be denatured by autoclaving, giving no activity. Extracellular broth from cultures grown in a 0.23% LS medium showed no decrease in PCP concentration. We confirmed that LS inhibited enzyme activity, and did not simply repress the expression of LiP enzymes, by incubating the nitrogen-limited glucose medium used above in a 50/50 mixture with denatured 0.23% LS medium. The previously active enzymes showed no removal of PCP after 30 min.

In addition to checking for enzyme activity by the veratryl alcohol oxidase assay and the direct PCP oxidation method, we also used the azure B assay (Archibald 1992) and attempted to precipitate enzymes with acetone described previously (Kuwahara et al. 1984); these methods also showed no LiP activity.

Decoloration of LS amended media

LS are highly colored because of the lignin content and, if used as a growth substrate for a liquid phase treatment system, would need to be de-colored to meet effluent discharge regulations in the US. A small amount of decolorization was indicated by a UV-visible spectral scan (300–700 nm) of the 2% LS medium before inoculation and after two weeks of growth. The range with the greatest color decrease, 450–550 nm, had only a 30% decrease in absorbance, and there was minimal color removal outside this range. Unfortunately, this small absorbance change was not detectable by eye.

Biomass yield on LS amended media

Increasing the biomass yield enhances the degradative capacity of a culture and limits PCP toxicity to fungi (Alleman et al. 1992). The addition of LS to media improved the biomass yield of *P. chrysosporium* in static cultures (Table 2). Based on a 1% glucose medium, fungal dry cell weights from a medium including LS were as much as 95% higher than nitrogen-limited cultures (0% LS) and 60% higher than nitrogen-sufficient cultures (2 g/l ammonia), perhaps due to the sugars in the LS. Although a separate experiment showed a 2% LS solution could support fungal growth with no other additions to the medium (data not shown), we used LS to replace only carbon and/or nitrogen so the comparison of PCP removal with a standard nitrogen-limited glucose medium reflected only one variable.

Discussion

PCP was successfully degraded by cultures of *P. chrysosporium* grown in media containing LS, although to a lesser extent than cultures grown in a nitrogen-limited glucose medium. In the nitrogen-

Table 1. Analysis of ammonium lignosulphonates[a].

Component	Percent[b]
Total solids	50.86
Sodium	0.28
Potassium	0.14
Calcium	0.09
Magnesium	0.04
Sulphur (as S)	6.78
Sulphate (as S)	1.23
Ash	1.63
Galactose	1.2
Glucose	2.3
Mannose	9.8
Arabinose	3.4
Total free sugars	20.4
Hydrolyzed galactose	4.2
Hydrolyzed glucose	5.2
Hydrolyzed mannose	12.6
Hydrolyzed arabinose	1.2
Hydrolyzed xylose	2.1
Total hydrolyzed sugars	25.3
Ammonium lignosulphonates	69.5
Manganese (2% LS solution)	2.7 mg/l[c]

[a] Data provided by ITT Rayonier, percentages on (wt./wt.) basis.
[b] Percentages are based on Total solids.
[c] Measured by atomic absorption spectophotometry (flame ionization).

limited glucose medium 95% of the PCP disappeared and 40% of the PCP was dehalogenated. Overall PCP degradation in the two media containing LS (2% and 0.23%) was similar (72 to 75%) but was less than that observed when cultures were grown in a glucose medium. Assuming dehalogenation occurs in proportion to disappearance from solution, ~32% of PCP was mineralized within 13 days. It is reasonable to assume that mineralization would follow dehalogenation since aromatic compounds such as phenol have been shown to serve as sole carbon sources for growth of *P. chrysosporium* (Krivobok 1994).

Other investigators have generally observed more rapid removals of PCP by *P. chrysosporium* in liquid cultures (Lamar et al. 1993; Alleman et al. 1995) than observed here. The extent of PCP mineralization has been found to be increased by the presence of LiP enzymes (Mileski et al. 1988; Lin et al. 1990). For example, Mileski et al. (1988) observed 23% PCP mineralization under lignolytic conditions, but only 10% mineralization under non-lignolytic conditions. Since LS inhibits LiP activity we attribute the lower percent PCP dehalogenation observed for cultures grown in LS media compared to nitrogen-deficient glucose medium to be primarily due to the absence of LiP activity at LS concentrations $\geq$0.23%.

It was not possible to prove if LS repressed enzyme expression or inhibited activity. Naturally occurring anionic polysaccharides (APS) have some structural similarities to α-hydroxysulphonates, polysaccharides produced during the delignification processes, and to LS (Rydholm 1965). APS have been found to inhibit enzyme activity, not to repress enzyme expression (Kirkpatrick & Palmer 1989), suggesting that LiP may be produced but inactived by LS. Another possible explanation for an absence of LiP activity is high concentrations of manganese, which causes the preferential production of manganese peroxidase (MnP) over LiP (Bonnarme & Jeffries 1990). Because LS are extracted from wood, we speculated there may have been sufficient manganese to suppress LiP production by this mechanism. However, the concentration of manganese in a 2% LS medium (measured by atomic absorption spectroscopy) was 2.7 mg/l (Table 1). Since LiP production is actually increased at this manganese concentration (Glenn & Gold 1985; Bonnarme and Jeffries 1990), manganese was not likely not a contributing factor in the absence of LiP activity.

There was limited decoloration of LS, likely due to the lack of LiP activity. Lignin degradation studies (Glenn & Gold 1985) suggest that extracellular enzymes are responsible for the initial steps in lignin degradation, particularly in breaking the large structures into smaller congeners. Only 5% lignin mineralization was observed when using a mutant strain of *P. chrysosporium* unable to express LiP (Boominathan et al. 1990); this was in contrast to 33% mineralization by the wild-type grown under lignolytic conditions. LS are 25% hydrolyzed sugars (Table 1) which are caramelized (and hence colored) from the high temperatures used in the process that produces LS. It is possible that the observed decoloration came from the fungi utilizing the sugars in LS, and that the degree of decoloration of the lignin based structures was negligible.

Because we measured only PCP disappearance during our degradation experiments, it was important to establish that PCP disappearance was followed by dehalogenation (Figure 1). The final fate of the PCP had only been partially characterized previously in our laboratory by measuring dehalogenation. Alleman et al. (1995) were able to show fungi released 38% of

Table 2. Biomass production of *P. chrysosporium* based on cell dry weight after eight days of growth with varying concentrations of glucose in either nitrogen-deficient media containing lignosulfonates or nitrogen-sufficient media (2 g/l ammonia).

Glucose	Cell dry weight (g)						
	Lignosulfonate (%)						Ammonia
%	(0)	(0.5)	(1)	(1.5)	(2)	(2.5)	(2 g/l)
0	0.002	0.009	0.022	0.035	0.025	0.044	—
0.5	0.058	0.068	0.061	0.068	0.077	0.044	—
1.0	0.078	0.070	0.139	0.134	0.152	0.097	0.095
1.5	0.058	0.166	0.108	0.170	0.140	0.060	—
2.0	0.123	0.180	0.143	0.158	0.159	0.153	0.138
2.5	0.152	0.193	0.058	0.196	0.035	0.060	—
3.0	0.062	0.208	0.176	0.170	0.174	0.078	—

Experiment not performed (—)

the chlorine in PCP after 8 days of incubation, but as much as 55% of the original chlorine was unaccounted for and was assumed to be organically bound halide (TOX_s). In the current investigation, organically bound halide was measured directly. Based on the day 5 and day 13 measurements (Figure 2), 64 and 44% of the original chlorine was directly measured as TX_s, with only a small percentage (7.5 and 1.8%) remaining as PCP, a range that confirms Alleman et al.'s (1995) assumption. Because both IX_s and TOX_s would be measured as TX_s, we assumed that TX_s=TOX_s since this represents a more conservative estimate of degradation and also since we observed no evidence of mycelium demonstrating halophytic properties. In addition, because recovery of chlorine was high (90–100%), we concluded possible losses due to volatile intermediates, such as pentachloroanisole (Lamar & Dietrich 1992), were insignificant. Once PCP was transformed the resulting non-PCP organic halide intermediates (TOX_s) were only slowly dehalogenated. This slow degradation may be due in part to a characteristic of these fungi when they are grown in a medium deficient in a key nutrient (usually nitrogen). When the limiting nutrient is depleted enzyme production is stimulated but the slower overall metabolic activity can affect the rate of PCP mineralization.

It is also of interest to note that in this study, in a medium of pH=4.5, 75% of the added PCP became immediately cell bound. An earlier study, which used the same fungi and the same medium at pH 6 resulted in only 10% sorption of PCP (Logan et al. 1994). Sorption of PCP onto mycelium is significant because the toxicity of PCP is a function of the chemical dose (Alleman et al. 1992). Chemical dose alone is an incomplete measure of toxicity because only the protonated form of PCP, the relative concentration of which is pH dependant, will partition into the cell's lipid bi-layers, making it more toxic than the phenolate ion form (Lamar & Dietrich 1992). Because contaminant uptake and toxicity are important parameters in designing a treatment system, the pH is a significant design criterion that has not been adequately addressed in the literature.

In spite of the limitations resulting from the presence of lignosulphonates (no LiP enzyme activity and limited color removal), *P. chyrsosporium* was able to degrade a significant fraction of PCP when grown in media containing LS. Since fungi will grow on LS alone, LS could be useful in PCP removal and pretreatment of LS wastestreams.

Acknowledgment

The advice of R.L. Gilbertson and the assistance of R. Casebier was greatly appreciated.

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