J. ENVIRON. SCI. HEALTH, A36(7), 1145–1162 (2001)

PCB BIODEGRADATION IN AGED CONTAMINATED SOIL: INTERACTIONS BETWEEN EXOGENOUS *PHANEROCHAETE CHRYSOSPORIUM* AND INDIGENOUS MICROORGANISMS

J. M. Fernández-Sánchez,[1] R. Rodríguez-Vázquez,[1,*] G. Ruiz-Aguilar,[1] and P. J. J. Alvarez[2]

[1]CINVESTAV-IPN, Depto. de Biotecnología y Bioingeniería, Av. Instituto Politécnico Nacional 2508, San Pedro Zacatenco, 07360, México, D.F.
[2]The University of Iowa, Dept. of Civil and Environmental Engineering, Iowa City, IA 52242-1527, USA

ABSTRACT

This work investigated whether the interaction between the white-rot fungus *Phanerochaete chrysosporium* and indigenous microorganisms could enhance polychlorinated biphenyl (PCB) removal from historically contaminated soil in aerobic microcosms. The PCB mixture was composed mainly of 14% tri-, 20% tetra-, 9% penta-, 17% hexa-, 26% hepta-, 11% octa-, and 3% nona-chlorobiphenyl (CB) congeners, determined by GC/MS. The fungus, which was grown on sugarcane bagasse and added via this solid substrate, successfully colonized the contaminated soil. The added fungi and the indigenous soil community biodegraded most PCB congeners, with removing efficiencies ranging from 13% to 100% for the 45-day incubation period. The interaction between the fungus and the microorganisms present in the added bagasse inhibited both heterotrophic activity (measured by CO_2 evolution) and PCB degradation, suggesting a possible antagonism. In contrast, analysis of variance

*Corresponding author. E-mail: rrodrig@mail.cinvestav.mx

(ANOVA) inferred a synergistic effect between fungus and soil microorganisms, which resulted in a heterotrophic activity above 2.5 mg-CO_2/g-initial dry matter/day. The statistical analyses also showed that the presence of fungus alone was particularly beneficial for the removal of penta- and hepta-CB.

Key Words: Polychlorinated biphenyls; *Phanerochaete chrysosporium*; Bioaugmentation; Contaminated soil; Sugar cane bagasse; Solid culture.

INTRODUCTION

Soil contamination by polychlorinated biphenyls (PCBs) is of great concern because of the persistence, bioaccumulation, and adverse health effects of these ubiquitous xenobiotics (1). PCBs have been released to the environment by improper disposal and by accidental leaks from transformers, heating exchangers, and hydraulic systems (2). Bioremediation nearly always makes the short list of remedial alternatives, as result of its relatively low cost, effectiveness and simplicity (3). However, soil bioremediation, can be slow and difficult to implement for aged PCB contamination, where the increased partitioning of PCB into soil micropores reduces its bioavailability (4).

One promising approach for improving the performance of bioremediation of historically contaminated soil is by addition of white-rot fungi (5). Bioremediation systems based on addition of these fungi can enhance the degradation of recalcitrant compounds, resulting in faster and more complete elimination of contaminants than bioremediation system relying solely on indigenous microorganisms.

White-rot fungi such as *Trametes versicolor*, *Pleurotus ostreatus*, and Phanerochaete chrysosporium have been widely reported to degrade and detoxify many different types of pollutants in liquid cultures (6, 7, 8). However, only a few studies have addressed the potential for these fungi to degrade hydrophobic pollutants in contaminated soil. Some of these latter studies have used white-rot fungi to remediate soil artificially spiked with standard PCB mixtures (9), but studies with complex PCB mixtures in historically contaminated soil (which are more difficult to bioremediate) are very difficult to find.

The success of soil bioremediation with white-rot fungi depends on fungal colonization and survival in the contaminated soil, and catabolic enzyme production. This can be enhanced by growing the lignin-degrading fungi on solid organic substrates (e.g., wood chips, wheat straw, corncobs, commercial mushroom spawn, and other agricultural products) and mixing these substrates thoroughly with the contaminated soil (10, 11). It is known that fungi differ widely in their abilities to use different carbon sources, and that their ability to utilize a particular substrate may be altered by the combination of available nutrients. Little is known, however, about how exogenous fungi interact with microorganisms present in the contaminated soil or the solid substrate(s), and how such interactions affect bioremediation.

Lang et al. (12) reported that white-rot fungi such as *Pleurotus* sp., *Dichomitus squalens* and *Ganoderma applanatum* were highly competitive with the soil microbiota. Interactions between white-rot fungi and soil microorganisms have been reported to improve the degradation of cellulose, hemicellulose, and lignin (7, 12, 13, 14, 15). This raises the possibility that such interactions may affect the activity and survival of the added fungus. However, to our knowledge no studies have addressed the interactions between indigenous microorganisms and white-rot fungi within the context of bioremediation of complex PCB mixtures from historically contaminated soils.

This study investigated the effect of bioaugmentation with the white-rot fungus, *P. chrysosporium*, on the biotransformation of a complex PCB mixture in an aged contaminated soil. Emphasis was placed on investigating whether the combined action of *P. chrysosporium* and microorganisms indigenous to the soil or the added bagasse had a synergistic or antagonistic effect on heterotrophic activity and PCB removal efficiency.

MATERIALS AND METHODS

Microorganisms

Phanerochaete chrysosporium, strain H-298 (CDBB-500) from the Microbial Cultures Collection of CINVESTAV-IPN, was used as a model white-rot fungus for this study. This strain was maintained on malt extract agar (Merck-México) and stored at 4°C. Before each experiment the fungus was recultivated on malt extract agar at 39°C for 2 days.

Sugarcane Bagasse

Sugarcane bagasse was provided by Casa Blanca Sugar Mill, Morelos, Mexico. The bagasse was used as a support medium and carbon source for fungal growth. The bagasse was air-dried and sieved through an 860–590 µm mesh, and stored for further experiments at 4°C. For treatments requiring sterilized bagasse, the bagasse was autoclaved twice at 121°C for 40 min.

Soil

A historically PCB-contaminated soil sample (660-mg PCB/Kg soil) was taken from 15 cm below the surface near an electrical transformer manufacturing facility located 25-km north of Mexico City. The soil was

air-dried, stored at room temperature and passed through a 0.03-in sieve before use. The soil texture analysis showed a composition of 78% sand, 15% silt and 4% clay. The total organic content was 1.9% and the total phosphorus and nitrogen contents were 0.007 and 0.098% mg/Kg of soil, respectively. The cation exchange capacity was 5.5-mEq/100 g and the pH was 6.5. For treatments with autoclaved soil, 50 g samples were placed in 125-ml flasks and autoclaved three times for 45 min at 121°C with intermediate incubations at 25°C for 3 days.

Experimental Design

A 2^3 factorial experimental design (16) was used to evaluate the effects of the fungus, baggase and soil microorganisms on heterotrophic activity (reported as CO_2 evolution) and PCB removal efficiency (Table 1). Eight treatments were prepared in duplicate: microcosm sets 1–4 without fungus (F −) and sets 5–8 with fungus (F +). All microcosms were prepared using 125-ml serological flasks. Thus, three independent variables were considered (fungus, bagasse and soil microorganisms) at two levels (present [+], and absent [−]). PCB congener concentrations were measured initially at day 11 (when contaminated soil was added) and at the end of the experiment to calculate PCB removal efficiencies. CO_2 evolution was measured daily to quantify the heterotrophic activity.

Treatments in the absence (B −) and presence (B +) of bagasse microflora were amended with 1.5 g (dry weight) of autoclaved and non-autoclaved bagasse, respectively. The material was moistened with 1.5-ml Kirk's medium (17) without glucose, to reach 60% of moisture content. The medium consisted of (in mg/L): KH_2PO_4 (200), $MgSO_4 \cdot 7H_2O$ (50), $CaCl_2$ (80), NH_4NO_3 (48), L-asparagine (79), 0.5-ml of vitamins and 1-ml trace mineral solutions. The vitamins solution consisted of (in mg per L dist. H_2O): biotin (2), folic acid (2), thiamine HCl (5), riboflavin (5), pyridoxine HCl (10), cyanocobalamine (0.1), nicotinic acid (5), DL-calcium pantothenate (5), *p*-aminobenzoic acid (5), thioctic acid (5). Trace mineral solution contained (in g per L dist. H_2O): nitrilotriacetate (1.5), $MgSO_4 \cdot 7H_2O$ (3.0), $MnSO_4H_2O$ (0.5), NaCl (1.0), $FeSO_4 \cdot 7H_2O$ (100), $CoSO_4$, 100 mg, CaCl (0.082), $ZnSO_4$ (0.1),

Table 1. 2^3 Factorial Experimental Design Matrix

Variable	Level	
	Absence	Presence
Bagasse microorganisms (B)	−1	+1
Soil microorganisms (S)	−1	+1
White-rot fungus (F)	−1	+1

$CuSO_4$ $5H_2O$ (10), $AlK(SO_4)_2$ (0.10), H_3BO_3 (0.1), $NaMoO_4$ (0.1). The pH of the medium was adjusted to 4.5 with HCl, and the microcosms were acclimated at 39°C for 11 days prior to soil addition. At this time, 8.5 g of soil with (S +) or without (S −) indigenous microflora, (non-autoclaved and autoclaved PCB-contaminated soil, respectively) were added to the microcosms. To improve the contact between soil and bagasse, 1 ml of autoclaved Ringer's solution (18) was applied to the soil surface. The microcosms were then sealed and incubated at 39°C in the dark during 34 days. To preserve aerobic conditions and avoid carbon dioxide accumulation, each flask was flushed daily with autoclaved moistened air for 15 min.

Analysis of variance (ANOVA) was performed to evaluate the effect of type of microorganism on the removal of different PCB isomers. This analysis was performed using Design Expert (version 6.0). Statistical significance was set at the 95% confidence level.

Fluorescence Microscopy

Bagasse (0.5 g) with fungal growth was added to 50-ml of autoclaved Ringer's solution (18) and mixed for 30 s. The extract was diluted as necessary in the solution and 10-ml aliquots were filtered through 0.8 μm pore-size polycarbonate membrane filter (Millipore). Fluorescein diacetate (10 μg/ml) was used as the staining agent. The filter was covered with the stain solution for 10 min, and suction was applied to remove the stain solution. The filters were then washed with distilled water, placed on microscope slides, and secured with a cover slip.

Figure 1 shows the fluorescence micrography of *P. chrysosporium* growth. Viable hyphae were detected based on fluorescein production by esterase activity. This visual technique was used to monitor microbial survival during treatments, and it has the advantage of being rapid and flexible, permitting differentiation between viable and dead hyphae (18).

Heterotrophic Activity Measurement

To determine heterotrophic activity, 2-ml headspace samples were taken from the flasks and analyzed for CO_2 evolution. This analysis was performed using a Gow-Mac chromatograph equipped with thermal conductivity detector and a concentric column CTR1 (Alltech, USA). The GC detector and injector temperature were set at 100 and 40°C respectively, the column was kept at room temperature and the detector potential was set at 125 volts. Helium was used as the carrier gas at a flow rate of 55-ml/min. Runtime for each sample was 4 min approximately. The CO_2 quantitation was performed as described by Saucedo et al. (19). Microbial CO_2 evolution was monitored

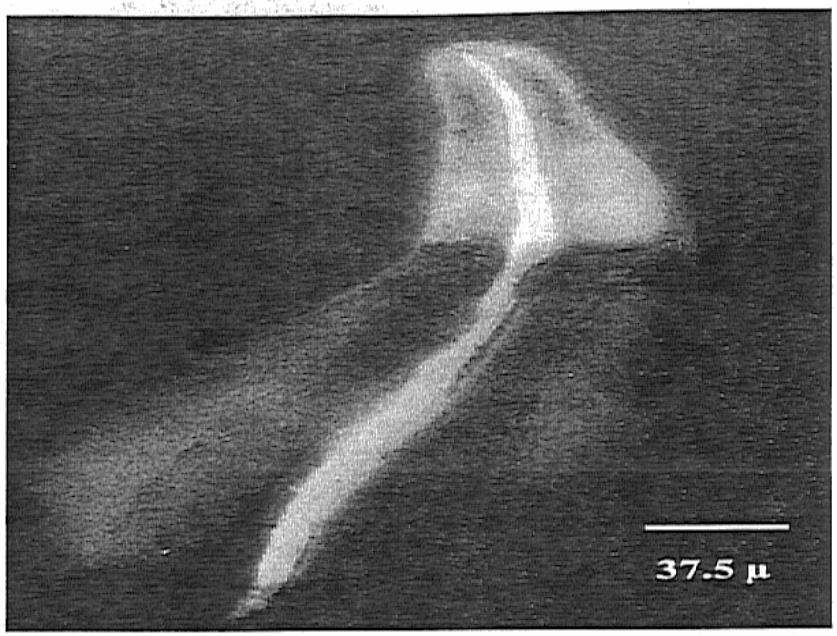


Figure 1. Micrography illustrating staining technique for the visualization of viable *P. chrysosporium* hyphae on bagasse.

daily during the 45-day incubation period. To preserve aerobic conditions and to avoid carbon dioxide accumulation, each flask was flushed daily with autoclaved moist air for 15 min.

PCB Analysis

Analysis of PCBs was based on EPA method 8080. Soil samples (10 g) were extracted in a Soxhlet apparatus for 9 h with 125-ml (1:1, v/v) of n-hexane/acetone (chromatography grade) (20). The extracts were concentrated down to 2-ml in a rotary evaporator. Organic matter was removed by acid digestion (H_2SO_4:H_2O 1:1, v/v), this procedure was repeated up for each sample under the same culture conditions. Extracts were added to Florisil (magnesium silicate, RP 60/100 mesh, 900 cc, Supelco, Bellafonte, USA) in a 30 cm × 3 cm column. Florisil was previously washed with water and acetone and activated overnight at 650°C for 24 h. The column was eluted with 250-ml of hexane and the first 50-ml were collected in a vessel evaporator and concentrated to 2-ml (20).

Individual PCB congeners were measured using a GC/MS. The concentrated sample (1 μl) was injected to a GC-MS (Varian 3000) equipped with a DB-5 fused silica column (30 m × 0.32 mm, 2.5 μm film thickness; J&W Scientist). The operating conditions were: temperature of injector 250°C, initial temperature 90°C held for 3 min and increased to 280°C during 45 min. Helium was used as a carrier gas. The detection limit was 0.25 mg/kg. The mass spectrometer was operated in ion-monitoring mode.

The two most abundant ions of the chlorine isotope cluster, M^+ and M^{+2}, were recorded for each molecular ion of PCBs congeners (Table 2). Identification of PCB was performed by matching the retention times of standards (Supelco, Supelco Park, Bellefonte, P. A.) and using the GC-MS library. This method involves the simultaneous acquisition of gas chromatography-mass spectrometry (GC-MS) data for the molecular ions of each PCB group. A PCB group is defined as all the PCBs that have the same molecular weight. Mass chromatograms are successively displayed by the data system for the molecular ions of each PCB group in the appropriate retention time for the group. The intensity ratios of the molecular ions in the appropriate retention time are used to determine the presence of PCB in each PCB group. Areas beneath PCB peaks in the mass chromatograms are used to determine concentrations.

The GC extract analyses showed the presence of 32 PCB congeners, which were numbered according to Ballschmiter and Zell, (21) (Table 4). In addition, several *meta*, *ortho* and *para* isomers were identified by differences in elution time. The numbers of chlorine atoms were determined considering the approximate m/e values of molecular ions, and verified by their relative peak intensities. The mass spectrometer was prepared and tuned according to the manufacturer's instructions. To optimize the sensitivity for high and low PCB molecular weight, each sample was analyzed twice, from mono-to nona-CB groups, in all treatments. No significant losses of PCB congeners were observed due to autoclaving of the control treatments.

Table 2. Multiple Mass/Ion PCB Congeners Detected by Mass Spectrometry in Soil Extract

PCB Group	Mass Range Scanned	Identified Ions	Quantified Ions
Tri-CB	254–260	256, 258, 260	258
Tetra-CB	288–294	290, 292, 294	292 (294)
Penta-CB	322–328	324, 326, 328	326 (328)
Hexa-CB	356–364	358, 360, 362	360 (364)
Hepta-CB	386–400	394, 396, 398	394 (398)
Octa-CB	420–434	428, 430, 432	430 (432)
Nona-CB	460–468	462, 464, 466, 468	464 (468)

CB = Chlorobiphenyl.

RESULTS AND DISCUSSION

Effect of Indigenous Microflora on *Phanerochaete chrysosporium* Growth

Soil inoculation with white-rot fungi is often carried out through addition of solid growth substrates such as wheat straw and wood chips (10, 11, 12). Rodriguez-Vázquez et al. (22) grew *P. chrysosporium* on sugarcane bagasse to enhance its resistance to soil microflora attack. In addition, Lamar et al. (14) showed that the activity of white-rot fungi inoculated to sterile soil could be improved by addition of glucose and minerals salts. This procedure was employed in our work, but without glucose addition and without soil sterilization. Thus, *P. chrysosporium* was first grown on bagasse and then mixed with the PCB-contaminated soil. This inoculum effectively colonized the soil. The highest level of fungal colonization was observed in microcosms with autoclaved bagasse and soil (B−S−F+), with abundant mycelia observed within 15 days. In experiments without added white-rot fungus (S+B+F−), indigenous bagasse microorganisms rapidly proliferated and black spores of saprophytic fungi were observed in the bagasse. Lang et al. (12) had noticed these kinds of spores also in straw.

During the experiment, the soil pH decreased from 6.5 to 5.0. This probably enhanced the growth of *P. chrysosporium*, which growths optimally at pH 4.5 (23). The decrease in pH was attributed to *P. chrysosporium* metabolism, which releases oxalic (24) and acetic acids (22).

Heterotrophic Activity

Aerobic metabolic activity was measured as CO_2 production in microcosms with and without added fungus. These data are presented in Figures 2 A and 2 B respectively, and in Table 3. Except for controls with sterile bagasse without added fungus (B−S+F− and B−S−F−), all treatments showed an increase in CO_2 production (2.5 mg-CO_2/g-IDM/day) after 4 days (Figure 2 A). This increase might be explained by the high carbohydrates content of bagasse (25) being consumed by the microorganisms. When contaminated soil was spiked at 11 days, the activity decreased in microcosms without fungus (B+S−F− and B+S+F−), and with fungus plus bagasse microorganisms (B+S−F+), with corresponding cumulative CO_2 values of about 13, 14, and 16 mg/g IDM after 45 days (Table 3). In all other treatments with added fungus (B−S+F+, B−S−F+, and B+S+F+) the relatively high CO_2 production was maintained after adding the soil (2.5 mg-CO_2/g-IDM/day), and decreased after 25 days, yielding cumulative CO_2 values from 16 to 21 mg-CO_2/g-IDM/day (Table 3). This high activity seems to be mainly due to the added fungus, with some participation by the microorganisms initially present in the soil and/or the bagasse.

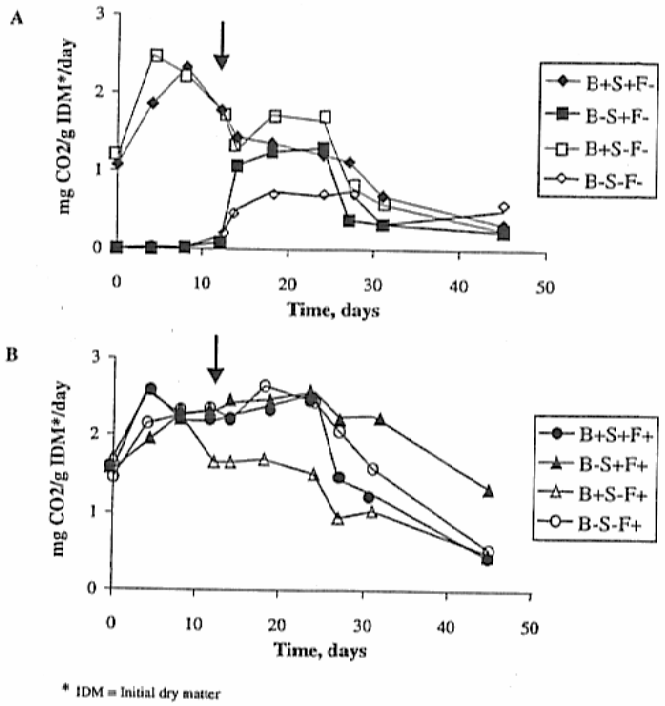


Figure 2. Heterotropic activity during treatment of historically PCB-contaminated soil. Daily CO_2 production is shown for microcosms (A) without added fungus, and (B) with fungus. Arrow indicates the time that the PCB-contaminated soil (with and without indigeneous microorganisms) was added.

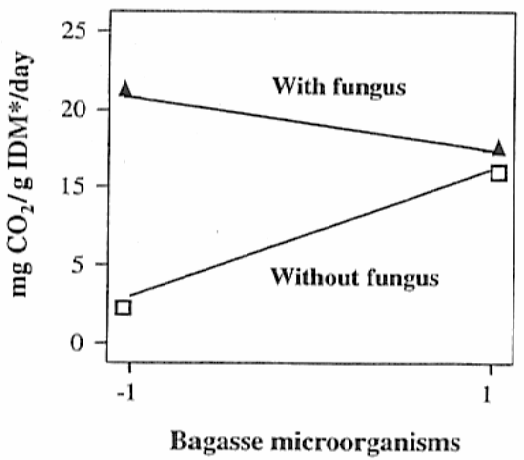
Table 3. Cumulative CO_2 Production in Microcosms

Microcosm Set	Bagasse Microorganisms (B)	Soil Microorganisms (S)	Added Fungus (F)	Cumulative mg-CO_2/g-IDM
1. (B+S+F−)	+1	+1	−1	13
2. (B−S+F−)	−1	+1	−1	5
3. (B+S−F−)	+1	−1	−1	14
4. (B−S−F−)	−1	−1	−1	4
5. (B+S+F+)	+1	+1	+1	19
6. (B−S+F+)	−1	+1	+1	21
7. (B+S−F+)	+1	−1	+1	16
8. (B−S−F+)	−1	−1	+1	20

Incubated at 39°C for 45 days.
+1 indicates presence of microorganism.
−1 indicates absence of microorganism.

CO_2 production was observed in controls with autoclaved bagasse without added fungus (B−S+F− and B−S−F−) after 11 days, with cumulative values of about 4 and 5 mg CO_2/g IDM, respectively. This activity may be partly due to incomplete soil sterilization. Sylvia et al. (26) reported that fungi spores and endospores of *Bacillus* could be present as resistant forms that can germinate later. Rojas-Avelizapa et al. (27) found such endospores in the same PCB-contaminated soil used in these experiments.

The statistical analysis of the 2^3 factorial experimental design showed no significant difference in cumulative CO_2 evolution between bagasse microorganisms alone and with added fungus (14 and 16 mg CO_2/g IDM, respectively) (Table 3). In fact, the presence of bagasse microorganisms slightly hindered CO_2 evolution in systems where fungi were present. The interaction of both variables is shown in Figure 3. These regressed values suggest a possible antagonism between bagasse microorganisms and the added fungus. Antagonistic interactions between bacteria and fungi have been widely reported (28). Radtke et al. (29) observed that bacteria isolated from benzo(a)pyrene-contaminated soils strongly inhibited *P. chrysosporium* growth. Many fungi and bacteria have the potential to produce antimicrobial growth substances that may serve to inhibit other microorganisms (26), although the nature of fungistasis is not well understood.



* IDM = Initial dry matter

Figure 3. Effect of dual interaction between bagasse microorganisms and *P. chrysosporium* on cumulative CO_2 production, calculated by ANOVA. The presence and absence of bagasse microorganisms is denoted as (+1) and (−1), respectively. Only bagasse microorganisms and fungus variables are considered in this analysis.

Initial PCB Characterization

Extracts from the PCB-contaminated soil were matched with the standard of Aroclor 1260 and the substation library. The initial PCB concentration was about 660 mg/kg in the soil, and the PCB was composed mainly of 14% tri-, 20% tetra-, 9% penta-, 17% hexa-, 26% hepta-, 11% octa-, and 3% nona-CB congeners. A total of 32 congeners were detected (Table 4).

PCB Biodegradation in Contaminated Soil

Table 4 summarizes the PCB congeners that were present at the beginning and after 45 days of aerobic treatment with the indigenous microflora alone (B−S+F−). All congeners that were initially present were also detected at the end of the incubation period in microcosms without added fungi. In contrast, some of tri- to octa-CB congeners were not found in systems bioaugmented with fungus (B−S−F+). The lack of significant PCB removal in sterile controls suggests that biodegradation by fungi was a key factor in the disappearance of these congeners.

Figure 4 presents the average PCB removal efficiency for the duplicate treatments described in Table 1. Except for the high removal efficiency (100%) of hepta-CB in microcosms with added fungus plus bagasse and soil microorganisms (Figure 4D), the combination of white-rot fungus and bagasse microorganisms was generally antagonistic and resulted in a relatively low heterotrophic activity (Figure 2B). PCB removal efficiencies were also hindered when both fungus and bagasse microorganisms were present. This is evident when one compares the relatively high removal efficiencies for treatments with added fungus containing sterile versus non-sterile bagasse (Figures 4A versus 4B, and 4C versus 4D). This bagasse had a sizable population of both fungi and bacteria (1.38×10^7 colony-forming units [CFU]/g-IDM) (30). Some of these microorganisms could inhibit *P. chrysosporium* by competing for limiting substrates or by exerting fungistasis when producing phenazines (29).

The statistical analyses of these results were focused on hexa- and hepta-CB removal, because these highly-chlorinated isomers were initially present in relatively high concentrations. When analyses of dual interactions were performed, regressed removal efficiencies for hexa-CB decreased significantly for the interaction of fungus and bagasse microorganisms (Figure 5A). This suggests an antagonistic interaction between these microorganisms. On the other hand, combining soil and baggase microorganisms enhanced the regressed PCB removal efficiencies for hepta-CB (Figure 5B). The statistical analysis infers that these isomers are more efficiently removed when soil and bagasse microorganisms are combined compared to treatments without bagasse microorganisms. Enhancement of hepta-CB removal was also inferred

Table 4. PCB Congeners Detected by Gas Chromatography Initially and After Treatment with Indigenous Microflora or *P. chrysosporium* H-298 Alone

Pea No	R.t. (min)	Initially Present	Detected After 45 Days of Incubation with: Indigenous Microflora Alone (B−S+ F−)	Detected After 45 Days of Incubation with: *P. chrysosporium* H-298 Alone (B−S−F+)
1	17.99	2,2′,5 (18)	2,2′,5 (18)	2,2′,5 (18)
2	18.53	2,2′,5 (18)	2,2′,5 (18)	ND
3	19.26	2,2′,5 (18)	2,3,6 (24)	2,3,6 (24)
4	19.53	2,3,6 (24)	2,3,6 (24)	ND
5	19.73	2,3,6 (24)	2,3,6 (24)	ND
6	20.19	2,2′,3,3′ (40)	2,2′,3,4′ (41)	2,2′,3,4′ (41)
7	20.33	2,2′,3,3′ (40)	2,2′,3,3′(40)	2,2′,3,3′ (40)
8	20.73	2,2′,3,4′ (41)	2,2′,3,4′ (41)	ND
9	21.06	2,2′,3,4′ (41)	2,2′,3,4′ (41)	ND
10	21.26	2,2′,3,3′ (40)	2,2′,3,4′ (41)	ND
11	21.86	2,2′,3,3′,6′ (84)	2,2′,3,3′,6′ (84)	ND
12	22.26	2,2′,5,6 (53)	2,2′,5,6 (53)	2,2′,5,6 (53)
13	22.46	2,2′,4,4′,5 (99)	2,2′,4,5,5′ (101)	2,2′,4,5,5′ (101)
14	23.13	2,2′,4,5,5′ (101)	2,2′,4,4′,5 (99)	ND
15	23.33	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,4′ (128)
16	23.66	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5′ 129)
17	23.93	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)
18	24.53	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,4′ (128)
19	24.86	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)
20	25.19	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)	2,2′,3,3′,4,5 (129)
21	25.39	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	ND
22	25.59	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)
23	25.93	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	ND
24	26.13	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)
25	26.33	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	ND
26	26.73	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)	2,3,3′,4′,5,5′,6 (193)
27	27.39	2,2′,3,3′,5,5′,6′ (178)	2,2′,3,3′,5,5′,6 (178)	2,2′,3,3′,5,5′,6 (178)
28	27.53	2,2′,3,3′,4,5′,6,6′ (201)	2,2′,3,3′,4,5,6,6′ (201)	ND
29	27.66	2,2′,3,3′,4,5′,6,6′ (201)	2,2′,3,3′,4,5,6,6′ (201)	2,2′,3,3′,4,5′,6,6′ (201)
30	28.39	2,2′,3,3′,4,5′,6,6′ (201)	2,2′,3,3′,4,5,6,6′ (201)	2,2′,3,3′,4,5′,6,6′ (201)
31	28.86	2,2′,3,3′,4,5′,6,6′(201)	2,2′,3,3′,4,5,6,6′ (201)	2,2′,3,3′,4,5′,6,6′ (201)
32	29.9	2,2′,3,3′,4,4,5,5′,6 (206)	2,2′,3,3′,4,4,5,5′,6 (206)	2,2′,3,3′,4,4,5,5′,6 (206)

Number in parenthesis is used as a synonym for the corresponding PCB compound according by Ballschmiter and Zell (21). ND = Not detected after treatment.

for treatments combining soil microflora and fungus, although to a lesser extent (Figure 5C), suggesting a synergistic interaction between these microorganisms.

In treatments with indigenous microflora (Figure 4C and 4D), a transformation of the high- to low-chlorinated congeners occurred. An increase in the area of peaks corresponding to lesser-chlorinated congeners was observed on GC-MS chromatograms (data not shown). This suggests that some reductive dechlorination occurred, perhaps in anaerobic microniches created by the high biochemical oxygen demand of the added substrates.

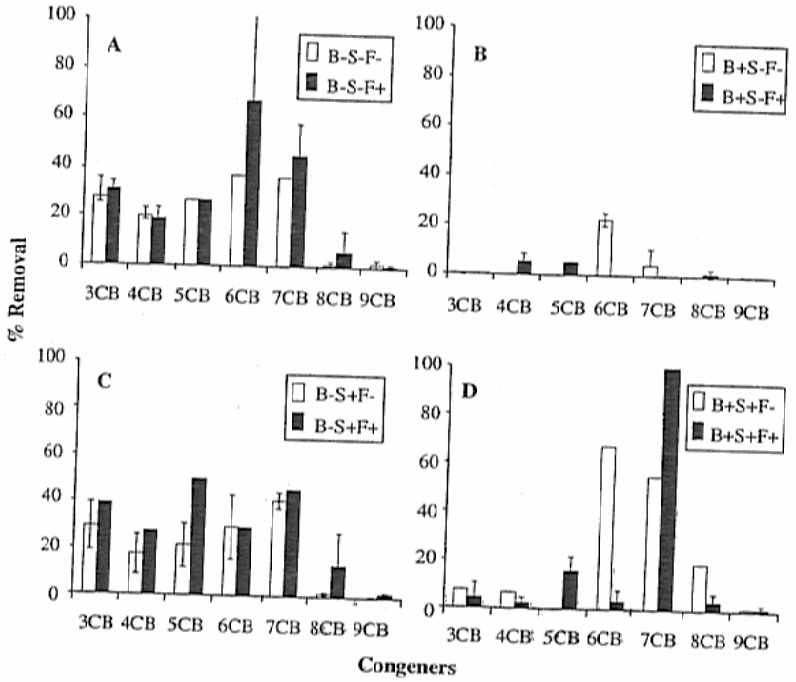


Figure 4. PCB removal efficiency in different microcosms with (black bars) and without (white bars) *P. chrysosporium*. Culture conditions: temperature 29°C, moisture contents 60%, pH 5, and 45 days of treatment, soil/bagasse relationship (15:85). Error bars represent one standard deviation from the mean of duplicate microcosms.

Some of the highly chlorinated congeners that tend to persist in aerobic environments were not detected after treatment with fungus (Table 4). The possibility that fungi removed these congeners is supported by the fact that white-rot fungi degrade a wide variety of chlorinated pollutants cometabolically, mainly during secondary metabolism under nutrient limitations (31). PCBs have an aromatic structure like lignin, a common substrate for fungi, which makes them susceptible to fungal attack (31, 32), and such ligninolytic enzymes were detected in our laboratory when *P. chrysosporium* was grown under similar conditions (33). It should be kept in mind, however, that highly-chlorinated congeners are often reported to be removed by reductive dechlorination under anaerobic conditions (9). Although care was taken to ensure that aerobic conditions prevailed in our experiments, we can not rule out the potential development of anaerobic microniches in soil particles (34). This raises the possibility that some removal of hepta- and octa-chlorinated congeners was due to bacterial reductive dechlorination.

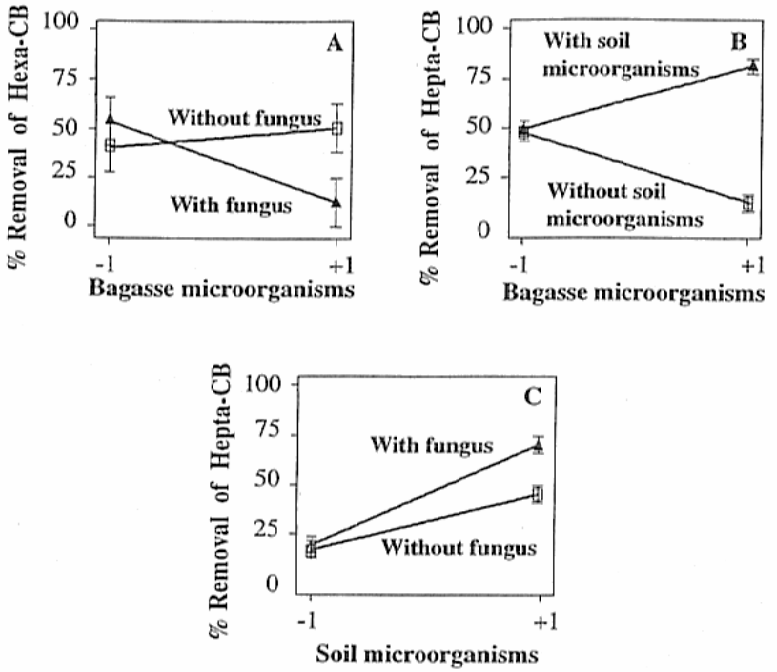


Figure 5. Effect of dual microbial interactions interactions on hexa- and hepta-CB removal, calculated by ANOVA. Panel A shows the interaction between fungus and bagasse microorganisms for hexa-CB removal, Panel B depicts the removal of soil and bagasse microorganisms for hepta-CB removal, and Panel C corresponds to the fungus and soil microorganisms interaction for hepta-CB removal. The presence or absence of the group shown in the x-axis is indicated by (+1) or (−1). respectively.

Zeddel et al. (35) found that white-rot fungi exhibit some specificity during the degradation of penta- and hexa-CB, depending on the aromatic ring chlorine substitution. Aerobic soil bacteria also exhibit different affinities to degrade a particular type of PCB isomer (4). Therefore, the combination of bacteria and fungi could expand the range of PCB congeners that can be attacked, resulting in more PCB degradation than can be accomplished by fungi and bacteria separately.

Table 5 summarizes the statistical analyses of the effect of the presence of three types of microorganisms (and their interactions) on the removal of different PCB congeners. Specifically, this table shows the attained level of significance (p-value) for the factorial ANOVA of isomer-specific removal efficiencies corresponding to the experimental matrix shown in Table 1. The presence of bagasse microorganisms had a significant negative effect ($p < 0.05$) on the removal of less chlorinated isomers (i.e., tri-, and tetra-CBs) and

Table 5. Statistical Analysis of Effect of Different Types of Microorganisms on the Removal of Different PCB Isomers

Variation Source	Attained Level of Significance (p value)						
	Tri-CB	Tetra-CB	Penta-CB	Hexa-CB	Hepta-CB	Octa-CB	Nona-CB
Model	0.0003	0.0009	< 0.0001	0.029	0.0001	0.083	0.61
B	< 0.0001 (−)	< 0.0001 (−)	< 0.0001 (−)	0.079	0.47	0.86	0.44
S	0.07	0.27	0.0057 (−)	0.95	< 0.0001 (+)	0.036 (+)	0.44
F	0.36	0.22	0.0002 (+)	0.14	0.0007 (+)	0.79	0.70
BS	0.89	0.75	0.36	0.0279 (+)	< 0.0001 (+)	0.27	0.31
BF	0.16	0.34	0.34	0.0097 (+)	0.0296 (+)	0.027 (−)	0.70
SF	0.74	0.86	0.001 (+)	0.069	0.0023 (+)	0.44	0.24
BSF	0.37	0.03 (−)	0.050	0.77	0.0008 (+)	0.08	0.33

Signs in parenthesis represent significant effect ($p < 0.05$), (−) negative effect (decrease of removal) and (+) positive effect (increase in removal). B = Bagasse microorganims, S = soil microorganisms, F = fungus, BS = Interaction between Bagasse microorganisms and soil microorganisms, BF = interaction between bagasse microorganisms and fungus, SF = interaction between soil microorganisms and fungus, interaction among soil microorganisms, bagasse microorganisms and fungus.

penta-CBs. Soil microorganisms had a significant ($p < 0.05$) overall positive effect on the removal of highly chlorinated isomers (i.e., penta-, hepta-, and octa-CBs). The added fungus contributed to the removal of selected congeners below detection limits (Table 4), and also had a significant ($p < 0.05$) overall positive effect on the removal of penta- and hepta-CBs isomers (Table 5), which suggests the feasibility of bioaugmentation.

In summary, PCB congeners present in soil were degraded by white-rot fungus *P. chrysosporium* in tandem with indigenous microorganisms. Fungus grown and added in a solid matrix proved to be an effective approach to enhance the aerobic biodegradation of (even highly chlorinated) PCB congeners present in a historically contaminated soil.

CONCLUSIONS

These results suggest that bioaugmentation with *P. chrysosporium* can enhance the remediation of soil historically contaminated with complex PCB mixtures. Specifically the fungus alone significantly removed penta- and hepta-CB congeners, as shown by ANOVA analyses. Two types of microbial interactions were inferred during PCB removal. Antagonism was found between the bagasse microflora and white-rot fungus when grown together, which hindered heterotrophic activity and PCB removal efficiency. Synergism was found between fungus and soil microflora. This combination resulted in higher heterotrophic activity and higher PCB removal efficiency. These results suggest that the effects of the matrix on metabolic activity of different

microbial populations need to be considered in bioaugmentation attempts, because growth of bacteria antagonistic to *P. chrysosporium* might be inadvertently stimulated.

ACKNOWLEDGMENTS

The funding for this research was supported, in part by CONACYT and IMP (FIES 95-106-VI). We thank Michael Pickard (University of Alberta), Dolores Díaz-Cervantes, Ania Mendoza-Cantu and Alfredo Medina-Dávila for excellent technical assistance. Stat-ease, Inc, generously donated Designs Expert (version 6.0).

REFERENCES

1. Erickson, M.D., Analytical Chemistry of PCBs, 2nd Ed.; Butterworth Publishers: Stoneham, M.A., Ed.; Press: Boca Raton, Florida, 1986; 34.
2. Grittini, C.; Malcomson, M.; Fernando, Q.; Korte, N. Rapid Dechlorination of Polyclorinated Biphenyls on the Surface of a Pd/Fe Bimetallic System. Environmental Science Technology **1995**, *29* (11), 2898–2900.
3. Phillips, J.P.; Hindawi, M.; Phillips A.; Baily, R.V. Viewing Bioremediation from a Multimedia Perspective. Pollution Engineering 1998, (accessed June 2000).
4. Wiegel, J.; Wu, Q. Microbial Reductive Dehalogenation of Polychlorinated Biphenyls. FEMS Microbiology Ecology **2000**, *32*, 1–15.
5. Stroo, R.; Jurek, M.A.; Bumpus, J.A.; Torpy, M.F.; Aust, S.D. Bioremediation of Wood Preserving Waste Using the White-rot Fungus *Phanerochaete chrysosporium*. American Wood-Preservers Association **1989**, 1–7.
6. Andersson, B.E.; Henrysson, T. Accumulation and Degradation of Dead-end Metabolites during Treatment of Soil Contaminated with Polycyclic Aromatic Hydrocarbons with Five Strains of White-rot Fungi. Applied Microbiology Biotechnology **1996**, *46* (5/6), 647–652.
7. Boonchan, S.; Britz, M.L.; Stanley, G.A. Degradation and Mineralization of High-molecular-weight Polycyclic Aromatic Hydrocarbons by Defined Fungal-bacterial Cocultures. Applied Environmental Microbiology **2000**, *66* (3), 1007–1019.
8. Braun-Lüllemann, A.; Majcherczyk, A.; Hüttermann, A. Degradation of Styrene by White-rot Fungi. Applied Microbiology Biotechnology **1997**, *47* (2), 150–155.
9. Tiedje, J.M.; Quensen III, J.F.; Chee-Sanford, J.; Schimel, J.P.; Cole, J.A.; Boyd, S.A. Microbial Reductive Dechlorination of PCBs. Biodegradation **1993**, *4*, 231–240.
10. Fernando, T.; Bumpus, J.A.; Aust, S.D. Biodegradation of TNT (2,4,6-Trinitrotoluene) by *Phanerochaete chrysosporium*. Applied Environmental Microbiology **1990**, *56*, 1666–1671.

11. Loske, D.; Hutterman, A.; Majcherczyk, A.; Zadrazil, F.; Lorsen, H.; Waldinger, P. Use of White-rot Fungi for the Clean-up of Contaminated Site. In Advances in Biological Treatment of Lignocellulosic Materials; Coughlan M.P.; Amaral-Collaco M.T., Eds.; Elservier: London, 1990; 311–322.
12. Lang, E.; Eller, G.; Zadrazil, F. Lignocellulose Decomposition and Production of Ligninolytic Enzymes during Interaction of White-rot Fungi with Soil Microorganisms. Microbiology Ecology **1997**, *34*, 1–10.
13. Huttermann, A.; Trojanowski, J.; Loske, D. Verfahren zum abbau schwer abbaubarer aromaten in kontaminierten boden bzw. Deponiestaffen mit mikroorganismen. German Patent no. DE3,731,816, June 25, 1989.
14. Lamar, R.T.; Glaser, J.A.; Kirk, T.K. Fate of Pentachlorophenol in Sterile Soils Inoculated with the White-rot Basidiomycete *Phanerochaete chrysosporium*: Mineralization, Volatilization and Depletion of PCP. Soil Biology Biochemical **1990**, *22*, 433–440.
15. Morgan, P.; Lee, S.A.; Lewis, S.T.; Sheppard A.N.; Watkinson, R.J. Growth and Biodegradation by White-rot Fungi Inoculated into Soil. Soil Biology Biochemical **1993**, *25* (2), 279–287.
16. Montgomery, D.C. Factorial Design 2^k. In *Design and Analysis of Experiments*, 3rd Ed.; John Wiley & Sons Inc. (Ed.). USA, 1991; 241–297.
17. Kirk, T.K.; Connors, W.J.; Zeikus, T.G. Requeriment for a Growth Substrate during Lignin Descomposition by Two Wood-rotting Fungi. Applied Environmental Microbiology **1976**, *32* (1), 192–194.
18. Morgan, P.; Cooper, C.J.; Battersby, N.S.; Lee, S.A.; Lewis, S.T.; Machin, T.M.; Graham, S.C.; Watkinson, R.J. An Automated Image Analysis Method for the Determination of Fungal Biomass in Soils and on Solid Matrices. Soil Biology Biochemical **1991**, *23* (7), 609–616.
19. Saucedo-Castañeda, G.; Trejo-Hernández, M.R.; Lonsane, B.K.; Navarro, J.M.; Roussos, S.; Dufour, D.; Raimbault, M. On-line Automated Monitoring and Control Systems for CO_2 and O_2 in Aerobic and Anaerobic Solid-state fermentations. Process. Biochemical **1994**, *29*, 13–24.
20. Fernández-Sánchez, J.M.; Ruiz-Aguilar, G.; Rodríguez-Vázquez, R. Polychlorinated Biphenyl Transformation by Bioaugmentation in Solid Culture, Proceedings of the Fifth International *In situ* and *On site* Bioremediation Symposium, San Diego California, U.S.A., April 19–22, 1999; Battelle Press., Columbus, Ohio 1999, *5*, 173–178.
21. Ballschiter, K.; Zell, M. Analysis of Polychlorinated Biphenyls (PCBs) by Glass Capillary Gas Chromatography. Composition of Technical Aroclor and Clophen PCBs Mixtures. Fresenius Z Analytical Chemical **1980**, *302*, 20–31.
22. Rodríguez-Vázquez, R.; Cruz-Córdova, T.; Fernández-Sánchez, J.M.; Roldán-Carrillo, T.; Mendoza-Cantí, A.; Saucedo-Castañeda, G.; Tomasini-Campocosio, A. Use of Sagarcane Bagasse Pith as Solid Substrate for *Phanerochaete chrysosporium* Growth. Folia Microbiology **1999**, *44*, 213–218.
23. Bumpus, J.A.; Tien, M.; Wright, D.; Aust, S.D. Oxidation of Persistent Environmental Pollutants by a White-rot Fungus. Science **1985**, *288*, 1434–1436.
24. Agosin, E.; Odier, E. Solid-state Fermentation, Lignin Degradation and Resulting Digestibility of Wheat Straw Fermented by Selected White-rot Fungi. Applied Microbiology Biotechnology **1985**, *21*, 397–403.

25. Rodríguez-Vázquez, R.; Díaz-Cervantes, D. Effect of Chemical Solutions Sprayed on Sugarcane Bagasse pith to Produce Single Cell Protein Physical and Chemical Analysis of Pith. Bioresource Technology **1994**, *47*, 159–164.
26. Sylvia, D.M.; Fuhrmann, J.J.; Hartel, P.G.; Zuberer, D.A. Habitat and Organisms. *In Principles and Applications of soil Microbiology*. Prentice Hall Inc., Edit. Upper Saddle River, New Jersey 07458, 1999, 72–92.
27. Rojas-Avelizapa, N.G.; Rodríguez-Vázquez, R.; Enriquez-Villanueva, F.; Martínez-Cruz, J.; Poggi-Varaldo, H.M. Transformer Oil Degradation by an Indigenous Microflora Isolated from a Contaminated Soil. Resources, Conservation and Recycling **1999**, *27*, 15–26.
28. Atlas, R.M.; Bartha, R. Population Interactions. *In Microbial Ecology. Fundamentals and Applications*. 4th edition. Benjamin/Cummings Science Publishing., 2725 Sand Hill Road, Menlo Park, California, 1999; 60–93.
29. Radtke, C.; Cook, W.S.; Anderson, A. Factors Affecting of the Growth of *Phanerochaete chrysosporium* by Bacteria Isolated from Soils. Applied Microbiology Biotechnology **1994**, *41*, 274–280.
30. Pérez-Armendáriz, B.; Rodríguez-Vázquez, R.; Loera-Corral, O.; Fernández-Línares, L. Hydrocarbon Removal by Native Microbial Communities in Weathering Tropical Soil Using Solid State Culture. Microbiology of Composting and other Biodegradation Processes. Congress Innsbruck, Austria. October 18–20, 2000, 19.
31. Bumpus, J.A.; Brock, B. Biodegradation of Crystal Violet. Applied Environmental Microbiology **1988**, *54*, 1143–1150
32. Yadav, J.S.; Quensen, J.F.; Tiedje, J.M.; Reddy, C.A. Degradation of Polychlorinated Biphenyls (PCB) Mixtures Aroclor 1242, 1254, 1260 by the White-rot Fungus *Phanerochaete chrysosporium* as Evidenced by Congener Specific Analysis. Applied Environmental Microbiology **1995**, *61*, 2560–2565.
33. Ruiz-Aguilar, G.; Fernández-Sánchez, J.M.; Rodríguez-Vázquez, R.; Poggi-Varaldo, H.M.; Esparza-García, F.; Vázquez-Duhalt, R. PCBs Biotransformation by a White-rot Fungus under Composting and Liquid Culture Conditions. Microbiology of composting and other Biodegradation Processes. Congress Innsbruck, Austria. October 18–20, 2000; 20.
34. Madigan, M.T.; Martinko, J.M.; Parker, J. Brock Biology of Microorganisms, 9th Ed.; Prentice Hall: Upper Saddle River, NJ, 2000; 645.
35. Zeddel, A.; Majcherczyk, A.; Huttermann, A. Degradation of Polychlorinated Biphenyls by White-rot Fungi *Pleurotus-ostreatus* and *Trametes-versicolor* in a Solid-State System. Toxicology Environmental Chemical **1993**, *40* (1–4), 255–266.

Received January 16, 2000