Role of Mycelium and Extracellular Protein in the Biodegradation of 2,4,6-Trichlorophenol by *Phanerochaete chrysosporium*

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The biodegradation of 2,4,6-trichlorophenol (2,4,6-TCP) by *Phanerochaete chrysosporium* was studied in batch systems. In experiments with mycelial suspension, the degradation of 2,4,6-TCP was found to occur in the absence of ligninase. Chloride ion was recovered in nearly stoichiometric amounts at the end of the process. The microorganism did not retain its degradation ability for more than 6 days under substrate-deficient conditions. Neither the mycelium nor the extracellular protein alone could degrade 2,4,6-TCP; both were required for complete degradation to occur. In experiments in which 2,4,6-TCP was exposed to the culture supernatant separated from its mycelium, negligible degradation was obtained and no chloride ion was recovered. No degradation was observed even when the supernatant was supplemented with hydrogen peroxide as a possible cosubstrate. In experiments performed with washed mycelium separated from its supernatant, no degradation took place until the mycelium released additional extracellular protein 5 to 6 h into the incubation. Additions of washed mycelium separated from its supernatant to active cultures also produced an increase in the rate of degradation in correspondence with the protein release. The protein release was independent of the presence of 2,4,6-TCP. The addition of cycloheximide to inhibit the synthesis of de novo proteins completely suppressed the release of protein by the mycelium and resulted in no 2,4,6-TCP degradation. Additions of culture supernatants containing a high concentration of extracellular protein to active cultures produced an increase in the rate of 2,4,6-TCP degradation. The results of this work indicate that the concentration of extracellular protein is the limiting factor in 2,4,6-TCP degradation but that no degradation can take place unless the mycelium is simultaneously present. This can be interpreted as an indication that sequential steps requiring both extracellular and cellular enzymes are involved in the degradation of 2,4,6-TCP by *P. chrysosporium*.

The basidiomycete *Phanerochaete chrysosporium*, commonly known as a white rot fungus, mineralizes a variety of hazardous organic chemicals (9, 16, 17), including chlorinated phenols (3, 4, 23–25, 27) and polyaromatic hydrocarbons (8, 14, 15, 30). The fungus has been reported to release several extracellular enzymes, such as lignin peroxidases (ligninases), manganese-dependent peroxidases, and glyoxal oxidases, under substrate-limiting conditions (9, 13, 32).

Lignin peroxidase was often indicated as the major enzyme responsible for the degradation of both lignin and many of the chlorinated pollutants that the fungus is capable of attacking (1, 2, 22, 24, 29). Recent investigations have also shown that other fungal enzymes are involved in xenobiotic degradation. For example, Boominathan et al. (5) observed considerable mineralization of synthetic lignin in a ligninase-negative system. Similarly, Dosoretz et al. (11) observed lignin degradation in a batch reactor, although no ligninase was detected in their system. Valli and Gold (34), Valli et al. (33), and Joshi and Gold (19) have shown that the degradation of compounds such as chlorinated phenols and 2,4-dinitrotoluene is catalyzed by several enzymes, including not only lignin peroxidase but also manganese peroxidase and intracellular enzymes contained in fungal extracts.

The lifetime of the enzymes and the ability of the fungus to

carry out degradation over an extended period of time are important factors in the biodegradation of xenobiotics. Faison and Kirk (12) have found that lignin peroxidase activity appeared on the fourth day after inoculation of a batch culture, started decaying after the fifth day, and reached an undetectable limit by the seventh day. Ulmer et al. (32) observed that after a 15-day incubation *P. chrysosporium* ceased completely to degrade lignin. Similarly, Ander and Eriksson (2) concluded that the production of ligninolytic enzymes depends on the availability of both carbon and nitrogen sources and would not continue under substrate-depleted conditions for an extended period of time.

Another important factor in the fungal degradation of xenobiotics is whether the enzymes involved in the process are extracellular or cellular. Some investigators have recently proposed that both the mycelium and extracellular enzymes may be involved in the degradation process (24, 25). The model they proposed includes the formation and decay of an intermediate produced during the degradation of pentachlorophenol. The formation and decay of this intermediate (reported to be 2,3,5,6-tetrachloro-2,5-cyclohexadiene-1,4-dione) are assumed to be the rate-limiting steps. The formation of another intermediate (pentachloroanisole) during pentachlorophenol degradation has been reported by Lamar and Dietrich (22). In a recent study on the degradation of 2,4,5-trichlorophenol, it was shown that *P. chrysosporium* degrades this compound by a pathway involving oxidative dechlorination of 2,4,5-trichlorophenol by either lignin peroxidase or manganese peroxidase (both extracellular enzymes) followed by intra- or extracellular reduction, resulting in the regeneration of a peroxidase substrate (19). This study showed that 2,5-dichloro-1,4-benzoquinone is the primary product of the extra-

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cellular oxidation of 2,4,5-trichlorophenol by either peroxidase and that this product is then converted to 2,5-dichloro-1,4-hydroquinone. The latter reaction was carried out much more efficiently in the presence of washed cells than in spent extracellular medium. These findings suggest that both intra- and extracellular enzymes may be sequentially involved in the degradation of chlorinated phenols. This aspect of the degradation process is especially important in the design of bioreactors for potential industrial uses of the fungus. If both types of enzymes are required to complete the degradation, certain types of reactor configurations that maximize the retention of the extracellular enzymes and the mycelium may be more advantageous than others.

In this study, we examine the contribution of the different components of the mycelial system, namely, the washed mycelium and the culture supernatant containing extracellular protein, on the degradation of 2,4,6-trichlorophenol (2,4,6-TCP), and we demonstrate the involvement of both components in the degradation process.

MATERIALS AND METHODS

Fungal growth in a batch fermentor. *P. chrysosporium* (ATCC 24725) was obtained from the American Type Culture Collection, maintained on yeast malt agar (3, 21), and grown in a growth medium of the following composition, in grams per liter: glucose, 6; KH_2PO_4, 2; $CaSO_4$, 0.2; $MgSO_4$, 0.1; $NaNO_3$, 0.5; thiamine hydrochloride, 0.01; deionized water; made up to 1 liter. The growth medium was supplemented with 5 ml of a mineral salt solution of the following composition, in grams per liter: $MgSO_4 \cdot 7H_2O$, 3; $MnSO_4 \cdot H_2O$, 0.5; NaCl, 1; $FeSO_4 \cdot 7H_2O$, 0.1; $CoSO_4$, 0.1; $CaCl_2$, 0.002; $ZnSO_4$, 0.1; $CuSO_4 \cdot 5H_2O$, 0.01; H_3BO_3, 0.01; $NaMoO_4$, 0.01; $AlK(SO_4)$, 0.01; deionized water; made up to 1 liter. The initial pH of the medium was between 4.2 and 4.4. Except when noted, the pH of the medium was raised to 5.6 with 0.1 N KOH prior to use.

An inoculated 14-liter batch fermentor containing 10 liters of medium (Microferm; New Brunswick Scientific Co.) with built-in aeration, heating, and stirring systems was used to grow the fungus. The aeration rate, agitation rate, and temperature were maintained at 50 ml/min/liter, 80 rpm, and 32°C, respectively. The pH and the concentration of glucose and nitrate were monitored twice a day. On the fifth day, the glucose and nitrate (as N) concentrations were 333 and 71 μM, respectively, and a thick mycelial suspension was observed. A fraction of this suspension (typically about 1 liter) was removed to conduct shake flask experiments or for further processing, as described below.

Production of fresh culture supernatant with a continuous packed-bed reactor. A continuous packed-bed reactor with immobilized fungus was used to produce culture supernatant. The reactor consisted of an acrylic jacketed vessel (length, 72.5 cm; inside diameter, 5.0 cm). Pretreated clear polyethylene terephthalate flakes, irregular in shape and size (cross-sectional area, 2 to 15 mm²; thickness, ≅0.5 mm), were obtained from the Polymer Recycling Plant, Rutgers University, New Brunswick, N.J., and used as random packing material. The void volume was 63%.

About 1 liter of mycelial suspension from the fermentor was transferred to the reactor with the simultaneous addition of the packing material. The fungus was allowed to attach to the packing for 12 h. Aeration was maintained at 50 ml of air per min per liter. The reactor temperature was kept at 32°C by circulating hot water through the jacket. Growth medium (pH 4.2 to 4.4) was continuously fed (1.5 ml/min) to the bottom of the reactor and removed from the top. The retention time was

9.97 h. The reactor was provided with an external top-to-bottom recirculation loop (flow rate, 15 ml/min) for good internal mixing. The fungus was grown for 5 days on growth medium feed and for 4 more days on induction medium feed. The induction medium, having a pH of 4.2 to 4.4, had the same composition as the growth medium except for glucose and $NaNO_3$ concentrations of 0.7 and 0.02 g/liter, respectively. Such a substrate-deficient medium was used to induce the fungal production of the extracellular lignin-degrading enzymes (9). The pH was not controlled during the continuous operation of the reactor. The pH in the reactor outlet stream was found to be constant at 5.6. Fifty milliliters of fungal supernatant from the reactor was removed and used for some of the experiments in shake flasks.

Shake flask experiments. Shake flasks were incubated at 32°C on a gyratory shaker rotating at 45 rpm. A stock solution of 1,010 ± 50 μM 2,4,6-TCP (98% purity; Sigma Chemical Co.) in a 0.1 N KOH solution was used to supplement the flasks with 2,4,6-TCP (range, 150 to 230 μM). The pH in the flasks was adjusted to 5.6 with 0.1 N KOH or 0.1 N tartaric acid.

Samples (2 or 5 ml) were taken periodically to measure the ligninolytic enzyme activity and the concentrations of 2,4,6-TCP, extracellular protein, chloride ion, glucose, and nitrate ion. Controls consisted of either flasks containing deionized water, 2,4,6-TCP, and acid or base as pH adjusters or sterilized flasks with the same concentrations of mycelium, 2,4,6-TCP, and medium as the original flasks.

(i) Experiments with mycelial suspension. A 500-ml portion of mycelial suspension from the batch fermentor was transferred to a 1-liter beaker and supplemented with 228 μM 2,4,6-TCP. After pH adjustment, the suspension was apportioned into four replicate 250-ml Erlenmeyer flasks (100 ml per flask), and the flasks were incubated. Separate incubations of the mycelial suspension under an atmosphere of nitrogen were also conducted.

(ii) Experiments with culture supernatant. Experiments with culture supernatant were carried out in parallel to those with mycelial suspension and with washed mycelium, using the same source of mycelial suspension. A 500-ml portion of the mycelial suspension from the fermentor was transferred to screw-cap centrifuge bottles (approximately 50 ml per bottle). After centrifugation at 6,000 rpm for 20 min, 90% of the clear supernatant from all of the bottles was transferred to a single container and supplemented with 2,4,6-TCP, and the pH was adjusted. The liquid was apportioned into four replicate 250-ml Erlenmeyer flasks (100 ml per flask), and the flasks were incubated.

In a separate series of experiments, the supernatant was separated by centrifugation as before, filtered through a 1.2-μm-pore-size GF/C binder-free glass-fiber filter (Whatman, Inc.), and supplemented with 2,4,6-TCP; the pH was adjusted, and the mixture was apportioned into six replicate flasks. Four of these flasks were supplemented with H_2O_2 (two replicates containing 10 μM and the other two containing 100 μM H_2O_2). All flasks were incubated.

(iii) Experiments with washed mycelium. The mycelium that had been separated from its supernatant from the previous experiment was washed three times with growth medium modified by removing the carbon and nitrogen sources (pH 4.2 to 4.4). The mycelium was resuspended in the modified medium, transferred to a single container, and supplemented with 2,4,6-TCP. After the suspension was brought up to a 1,000-ml final volume by adding modified medium, the pH was adjusted to 5.6, and the washed mycelial suspension was transferred to four 250-ml Erlenmeyer flasks (100 ml per

flask). In another experiment, the mycelial culture in the fermentor was exposed to 227 μM 2,4,6-TCP before the incubations in the flasks with washed mycelium were done.

An experiment in which 2,4,6-TCP was added 10 h after the mycelium was washed and separated from the suspension was conducted in order to test for possible induction of fungal activity by this compound. In a different experiment, 0.25 ml of a 0.18 M trichloroacetic acid solution was added 8 h into the experiment to determine the effect of protein denaturation on 2,4,6-TCP degradation.

In another experimental set, the release of extracellular protein from a previously washed and resuspended mycelium was monitored until completion. Then the mycelium was removed and the supernatant was incubated to determine if the newly released extracellular protein could degrade 2,4,6-TCP independently of the mycelium.

In a separate experiment, cycloheximide (1.46 mM) was added to washed mycelium just after the washing and resuspension had been carried out in order to suppress the de novo synthesis of proteins (6, 7).

(iv) Experiments with additional mycelium. A mycelium wash was conducted as described before, and four pH-adjusted, 250-ml shake flasks, each containing 44 mg of resuspended mycelium per liter in a 100-ml total suspension volume, were incubated at 32°C in the presence of 2,4,6-TCP. After 8 h a new mycelium wash was conducted on fresh mycelial suspension from the batch fermentor. Approximately 2 g of the newly washed, diluted mycelium suspension was added to two of the four shake flasks, bringing the mycelium concentration to 60 mg/liter. All flasks were incubated.

(v) Experiments with additional culture supernatant. Mycelial wash and resuspension were conducted as described before, and four pH-adjusted, 250-ml flasks, each containing 100 ml of mycelial suspension, were incubated in the presence of 2,4,6-TCP. After 8 h 10-ml supernatant aliquots from the continuous packed-bed reactor were added to two of the four flasks, and all flasks were incubated.

Analytical methods. (i) Measurements of nitrate ion, chloride ion, and glucose concentrations. The concentrations of nitrate ion and chloride ion in the samples were measured with specific electrodes (models 93-07 and 96-17B, respectively; Orion Inc.) having a reproducibility of ±2%. Fixed nitrogen was measured as nitrate ion concentration but reported as N. Glucose was assayed by the ortho-toluidine reaction method (35), which had a reproducibility of ±2.5%.

(ii) 2,4,6-TCP assay. Aqueous samples from the flasks were centrifuged for 10 min at 13,000 rpm. 2,4,6-TCP was quantified with a high-performance liquid chromatograph (model 600E; Waters, Inc.) provided with a C_{18} bonded phase column (Alltech Associates Inc.), variable wavelength detector set at 280 nm (model 484; Waters Inc.), and autosampler (715 Ultra-WISP). A 40:60 mixture of methanol and deionized water containing 1% acetic acid was used as the mobile phase. Nelson software was used for data acquisition through an on-line computer. The reproducibility of the analysis was within ±1.5 μM.

(iii) Ligninolytic enzyme assay. Ligninolytic enzyme activity was assayed by measuring the rate of oxidation of veratryl alcohol to veratryl aldehyde at 32°C (31). The increase in absorbance over a period of 5 min from the time the hydrogen peroxide was added was measured at 308 nm, using a Varian DMS 200 spectrophotometer. The enzyme activity was reported in units of ligninase activity (A_{308} units) defined as the amount of enzyme that catalyzes the release of enough veratryl aldehyde to give a change in absorbance of 0.001 U/min at 308 nm and 32°C. This definition is similar to that previously used
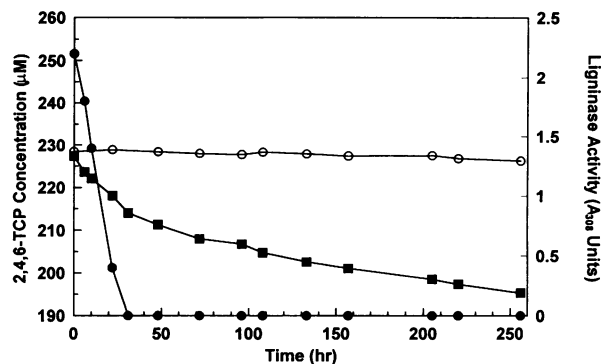

FIG. 1. Degradation of 2,4,6-TCP by mycelial suspension. The mycelial concentration was 240 mg/liter. ■, 2,4,6-TCP (mycelial suspension); ○, 2,4,6-TCP (deionized water); ●, ligninase.

to study the characterization and purification of various enzymes produced by *P. chrysosporium* (10).

(iv) Protein assay and fungal biomass dry weight determination. The protein content in the supernatant of each sample was determined with a Folin protein assay (Pierce Chemicals Co.) calibrated with bovine serum albumin (26). The accuracy of the method was obtained by repeated experiments (typically 10) at three different concentrations and was found to be within ±2% of the measured value.

The concentration of fungal biomass was determined from 100-ml culture suspensions taken from either the batch fermentor or the shake flasks. In the latter case, this volume was collected from all flasks used in a given experiment (conducted in quadruplicate or duplicate). The mycelium in each 100-ml sample was centrifuged at 6,000 rpm for 20 min, washed three times, dried at 90°C for 24 h, and weighed.

RESULTS

Results of experiments with mycelial suspension. Figure 1 shows the change of 2,4,6-TCP concentration as a function of time. Each point in Fig. 1 represents the average of a quadruplicate experiment (standard deviation, ±1.3 μM). 2,4,6-TCP was degraded at an initial rate of 0.42 μmol/h/liter. This value is comparable to those previously reported in the literature (22, 24). The degradation was accompanied by the release of chloride ion in nearly stoichiometric amounts (up to 96.5%; Table 1). An overall 14% 2,4,6-TCP degradation was achieved in the process. Since nutrient-deficient induction medium was used in the experiment, the mycelial biomass concentration was found to be constant and equal to the initial value of 240 mg/liter.

The degradation rate for 2,4,6-TCP decreased with time and became negligible after about 130 h (i.e., 5.5 days after exposure to 2,4,6-TCP and 10.5 days from inoculation). Ulmer et al. (32) also reported that after 6 to 8 days from inoculation the degradation ability of the fungus decreased to 35% of the initial rate and that at the end of the next 7 days degradation ceased completely. These observations confirm that the fungus is incapable of prolonged degradation activity under substrate-depleted conditions.

While 2,4,6-TCP degradation proceeded for about 130 h, the ligninase activity fell below detectable levels after 31 h (Fig. 1). A similar decay in activity has been reported before and has been attributed to the deactivation of lignin peroxidase by inhibitors (17). In other experiments (data not shown) conducted under identical conditions or at different pH values, no

TABLE 1. Degradation of 2,4,6-TCP by mycelial suspension[a]

Time (h)	Glucose (μM)	Nitrate ion (μM)	pH	Protein (mg/liter)	2,4,6-TCP (μM)	Ligninase activity (A_{308} units)	Chloride ion (μM)	$\Delta Cl^-/$ $\Delta 2,4,6$-TCP ratio
0	330	71	5.6	217	227	2.2	226	
6	140	29	5.6	219	224	1.8	NR[b]	NR
10	61	14	5.5	223	222	1.4	234	1.6
22	33	7	5.5	229	218	0.4	248	2.4
31	17	0	5.5	231	214	0.0	259	2.5
48	6	0	5.4	247	211	0.0	271	2.8
72	0	0	5.3	261	208	0.0	276	2.6
96	0	0	5.3	287	207	0.0	279	2.61
108	0	0	5.4	298	205	0.0	282	2.5
133	0	0	5.5	304	203	0.0	296	2.9
157	0	0	5.5	289	201	0.0	302	2.9
205	0	0	5.5	267	199	0.0	307	2.8
220	0	0	5.5	258	197	0.0	310	2.8
256	0	0	5.5	250	195	0.0	316	2.8

[a] The biomass concentration was 240 mg/liter.
[b] NR, not recorded.

ligninase was detected at all during the entire course of the experiments, although 2,4,6-TCP degradation was observed. This confirms that ligninase is not necessary for degradation to occur. This observation is in line with those of other researchers who reported the degradation of compounds such as pentachlorophenol and lignin in ligninase-negative systems (5, 11, 27). No 2,4,6-TCP disappearance was observed when the mycelial suspension was incubated under nitrogen. Ligninase was reported to be active in oxygen-free systems (28). Therefore, the absence of degradation in these experiments further confirms that no ligninase was present.

Results of experiments with culture supernatant. Culture supernatant separated from its mycelium produced only a very slight decrease in 2,4,6-TCP concentration (run 1, Fig. 2a). No chloride ion was recovered. The results of these quadruplicate experiments (standard deviation, 1.2 μM) can be directly compared with those obtained with mycelial suspension since all of these experiments were conducted in parallel, using as starting material the same 5-day culture suspension containing 240 mg of mycelial biomass per liter. As a result, the extracellular protein concentration (including the ligninase concentration) was the same in both types of experiments and was equal to 210 mg/liter. However, the rate of 2,4,6-TCP disappearance in the experiments with culture supernatant was much lower than in the experiments with mycelial suspension.

The chromatograms obtained in the supernatant experiments showed the presence of a small peak having a retention time of 2.35 min (compared with 2.25 min for 2,4,6-TCP). No attempts to identify this compound were made. A larger peak having the same retention time was also observed during the initial phase of the degradation with mycelial suspension. However, in this case the peak height decreased to the vanishing point as the ratio $\Delta Cl^-/\Delta 2,4,6$-TCP (Table 2) increased toward the stoichiometric value of 3.

No appreciable degradation was observed even when H_2O_2 was added to the culture supernatant (run 2, Fig. 2b). However, degradation was observed in the control experiment utilizing mycelium suspension from the same batch from which the supernatant was obtained.

Results of experiments with washed mycelium. Figure 3 shows the results of quadruplicate experiments (standard deviation, 1.4 μM) run in parallel with the previous two sets. During the first 4 h, no significant change in 2,4,6-TCP concentration was observed, while the concentration of protein
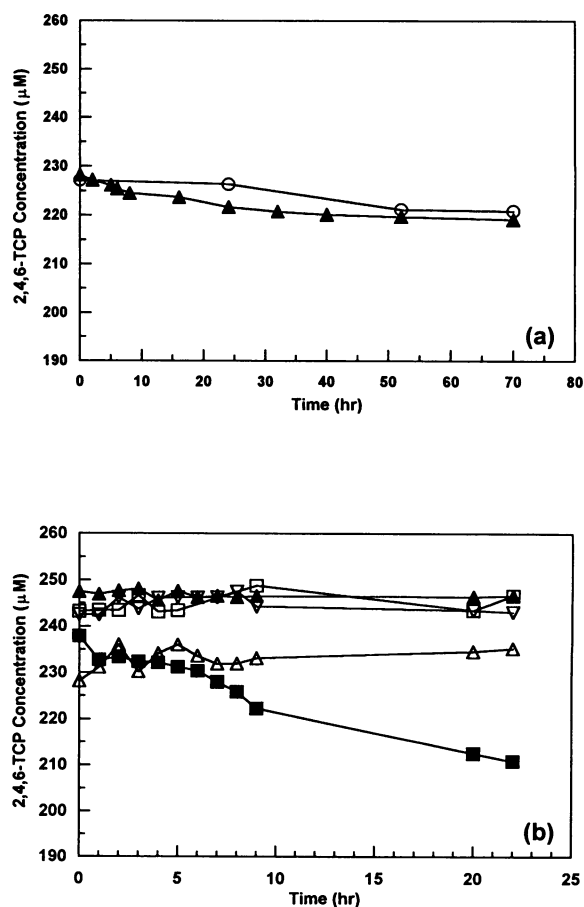

FIG. 2. Degradation of 2,4,6-TCP in experiments with culture supernatant: (a) run 1 (protein concentration, 210 mg/liter); (b) run 2 (protein concentration, 2,880 mg/liter), including experiments with hydrogen peroxide additions. ▲, culture supernatant; △, culture supernatant containing 10 μM H_2O_2; ▽, culture supernatant containing 100 μM H_2O_2; ■, mycelial suspension; □, sterilized mycelial suspension; ○, deionized water.

TABLE 2. Degradation of 2,4,6-TCP by washed mycelium

Time (h)	2,4,6-TCP (μM)	Protein (mg/liter)	pH	Chloride ion (μM)	ΔCl^-/ Δ2,4,6-TCP ratio
0	229	0	5.6	113	0
1	228	0	5.6	113	0
2	227	0	5.6	113	0
3	228	0	5.6	113	0
4	228	0	5.6	113	0
5	227	0.7	5.6	113	0
6	227	1.9	5.6	113	0
8	227	2.2	5.6	117	2.4
9	223	2.2	5.6	120	1.3
10	223	2.3	5.6	121	1.5
12	218	2.3	5.6	123	0.9
14	217	NR[a]	5.6	NR	NR
16	213	2.4	5.6	146	2.1
18	210	NR	5.6	NR	NR
27	206	2.5	5.6	158	2.0
32	203	2.5	5.6	162	1.9
36	202	2.6	5.6	168	2.1
40	200	2.6	5.6	172	2.1
54	200	2.8	5.6	172	2.0
70	200	2.8	5.6	175	2.2

[a] NR, not recorded.

in the supernatant remained at undetectable levels. Between the fifth and the eighth hours, the mycelium released protein at an average rate of 0.55 mg/liter/h, bringing the protein concentration to 2.2 mg/liter. After the eighth hour, the rate of protein release decreased to about 0.01 mg/liter/h. This resulted in an increase in the protein concentration from 2.2 to 2.8 mg/liter over the next 62 h. A number of similar experiments (data not shown) exhibited the same phenomenon of protein appearance a few hours after the mycelium had been washed and resuspended. The protein release coincided with a marked decrease in 2,4,6-TCP concentration, the initial rate of disappearance of which was 1.75 μmol/h/liter. This rate is higher but still comparable to that observed during the initial stage of degradation with mycelial suspension. The percentage of chloride recovery was only a fraction (typically between 32 and 79%) of the stoichiometric amount recoverable from the complete degradation of 2,4,6-TCP (Table 2). The chromatograms obtained at different times during the experiment indicated that an unidentified intermediate having a retention time
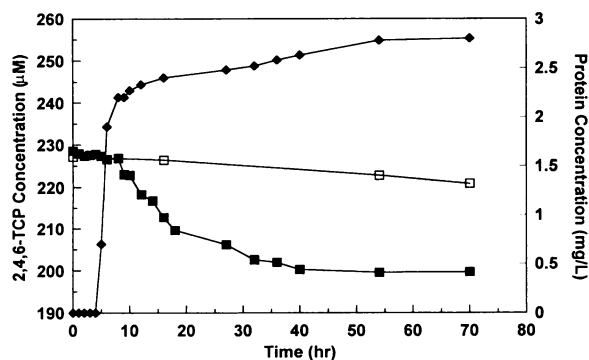

FIG. 3. Degradation of 2,4,6-TCP by washed fungal mycelium. The mycelial concentration was 44 mg/liter. ■, 2,4,6-TCP (washed mycelium); □, 2,4,6-TCP (sterilized mycelium); ◆, protein.
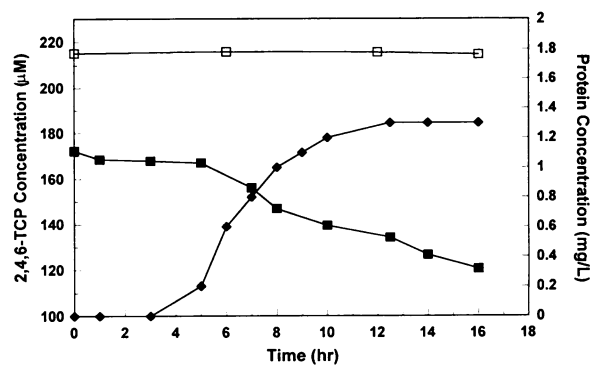

FIG. 4. Degradation of 2,4,6-TCP in experiments with washed mycelium obtained from a culture that had been previously exposed to 2,4,6-TCP. ■, 2,4,6-TCP (washed mycelium); □, 2,4,6-TCP (sterilized mycelium); ◆, protein.

higher than that of 2,4,6-TCP was being produced but not completely degraded.

The rate of 2,4,6-TCP concentration decreased with time as in the experiments with mycelial suspension. No further degradation occurred after 40 h into the incubation (compared with 130 h in the mycelial suspension experiments), implying that the extracellular protein has a finite lifetime. No ligninase was detected throughout the experiment. Fungal degradation of 2,4,6-TCP occurred independently of whether the culture grew in the presence of 2,4,6-TCP in the batch fermentor (Fig. 4) or was not acclimated to 2,4,6-TCP at all, as in all of the other experiments.

To test whether the protein release could be due to induction of some activity by 2,4,6-TCP, this compound was added 10 h into the incubation. At this time, the protein concentration had risen to 1.7 mg/liter from an undetectable level at time zero. The degradation of 2,4,6-TCP started immediately upon addition, bringing the 2,4,6-TCP concentration from 206 μM at t = 10 h to 170 μM at t = 16 h. The decrease in 2,4,6-TCP concentration was accompanied by chloride ion release, as before. Therefore, the rapid release of extracellular protein appears to be an intrinsic characteristic of the mycelial biomass removed from its supernatant. On the other hand, when the trichloroacetic acid solution was added 8 h after the beginning of the experiment (i.e., after the bulk of the protein release had occurred) in order to denature the newly released protein, no degradation of 2,4,6-TCP was observed over the next 60 h. This behavior cannot be attributed to any pH effect since the pH was immediately adjusted to its original value after the addition of trichloroacetic acid.

An experiment in which a previously washed and resuspended mycelium was incubated for 10 h and then removed while the supernatant was further incubated produced the usual pattern of protein release after 5 to 6 h and the associated 2,4,6-TCP degradation (run 1, Fig. 5a). However, after the mycelium was removed at t = 10 h, no further degradation was observed in the supernatant. The same experiment was repeated with a control flask in which the previously washed mycelium was not removed at t = 10 h. 2,4,6-TCP degradation continued in the control flask but ceased completely in the flask from which the mycelium had been removed (run 2, Fig. 5b).

No degradation was observed when cycloheximide was added to the mycelium immediately after washing and resuspension.

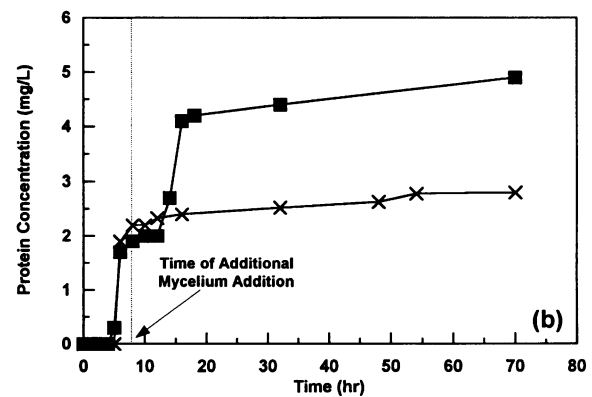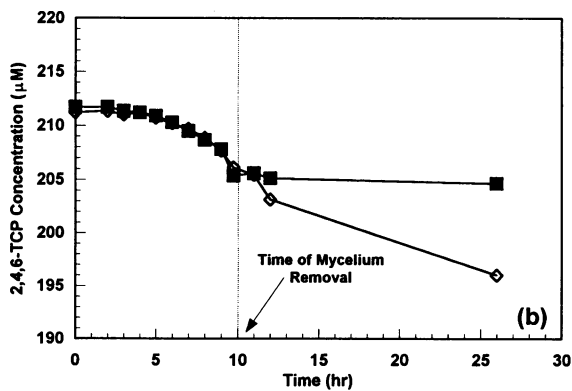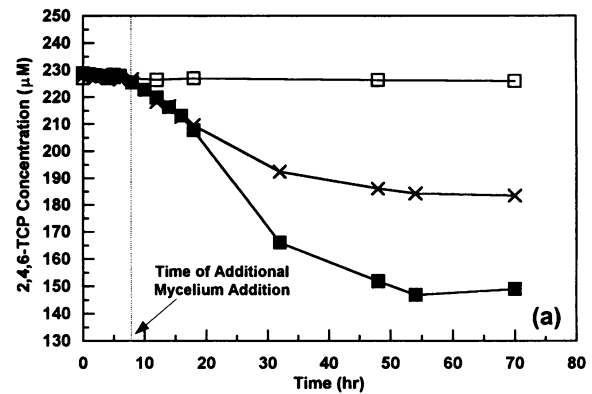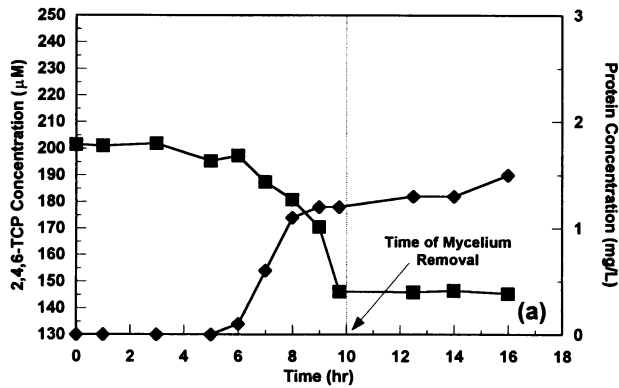**Results of experiments with additional mycelium.** In exper-

FIG. 5. Results of experiments with washed mycelium. After incubation of the previously washed mycelium for 10 h, the mycelium was removed and the supernatant was incubated: (a) run 1; (b) run 2 with control (previously washed mycelium not removed). ■, 2,4,6-TCP (mycelium removed at t = 10 h); ◇, 2,4,6-TCP (no mycelium removed at t = 10 h); ◆, protein.

FIG. 6. Results of experiments with additional mycelium: (a) 2,4,6-TCP degradation; (b) protein concentration. ■, additional mycelium; ×, no additional mycelium; □, sterilized mycelium.

iments with additional mycelium, some flasks were supplemented with additional washed mycelium at t = 8 h. During the first 14 h, all flasks, with or without added mycelium, produced nearly identical 2,4,6-TCP degradation profiles (Fig. 6a) and extracellular protein-time profiles (Fig. 6b), including a rapid protein release some 5 h into the incubation. Starting at t = 14 h, the flasks containing additional mycelium showed a second rapid release of additional extracellular fungal protein accompanied by a marked decrease in 2,4,6-TCP concentration. The lag time between the mycelium addition and the second protein release was nearly the same as that between the beginning of the experiment and the first protein release. 2,4,6-TCP disappearance was accompanied by chloride ion release. The amounts of chloride ion recovered were 74.6% (in the experiments with additional mycelium addition) and 72% (in the experiments with no additional mycelium addition) of the stoichiometric amount recoverable from the complete degradation of the 2,4,6-TCP that disappeared during the experiments.

Results of experiments with additional culture supernatant. Additional supernatant was added to some of the flasks 8 h into the incubation, i.e., after the extracellular protein had been released by the washed mycelium. The small 10-ml additions more than doubled the extracellular protein concen-

tration in the flasks (bringing it to 4 g/liter) since the protein content of the added supernatant (25 mg/liter) was much higher than that originally in the flasks (1.9 mg/liter). The amount of mycelial biomass added to the flasks with the supernatant was negligible since the mycelial concentration in the supernatant from the reactor was 12.0 mg/liter. Figure 7
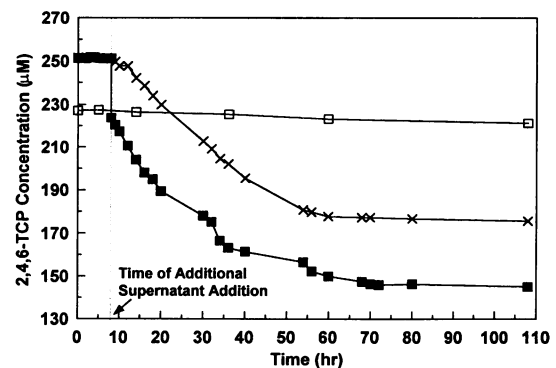

FIG. 7. Degradation of 2,4,6-TCP in experiments with additional culture supernatant. ■, washed mycelium with additional supernatant; ×, washed mycelium with no additional supernatant; □, sterilized mycelium.

shows that those flasks that were supplemented with additional supernatant degraded 2,4,6-TCP at a much faster initial rate than those that did not (3.23 and 2.05 μmol/h/liter, respectively). At 20 h into the incubation (i.e., 12 h after the supernatant addition), the amount of 2,4,6-TCP degraded per unit volume in the flasks supplemented with supernatant was 50% greater than that degraded in the other flasks. This difference became progressively smaller with time and was negligible for times longer than 54 h. The rate of 2,4,6-TCP degradation decreased with time independently of the presence of additional supernatant and ceased almost entirely some 50 h after the extracellular protein was released. These results are similar to those shown in Fig. 3, indicating that the enzyme system contained in the extracellular protein has a finite lifetime.

DISCUSSION

The results obtained in the experiments with washed mycelium show that under the conditions investigated in this work the mycelium alone cannot degrade 2,4,6-TCP unless extracellular fungal protein is present. The results obtained with culture supernatant indicate that the extracellular fungal protein alone is also unable to produce significant degradation. Therefore, both the mycelium and extracellular fungal protein are necessary for 2,4,6-TCP degradation to occur.

It is well established that cometabolism can play an important role in the degradation of xenobiotics by P. chrysosporium. Hence, by totally depriving the fungus of nutrients, as in all of the experiments with washed mycelium, one would expect that minimal or no 2,4,6-TCP degradation would take place. This was indeed the case at the beginning of these experiments, as shown in Fig. 3 and 6. However, this period of inactivity was only temporary since as soon as the mycelium started releasing extracellular protein 2,4,6-TCP degradation began in spite of the absence of any carbon or nitrogen source in the medium. Furthermore, the resulting degradation rates were comparable to or even higher than those observed during the initial stage of degradation by whole mycelial suspension.

The extent of 2,4,6-TCP degradation produced by culture supernatant is very minor and nearly indistinguishable from that in deionized water controls (Fig. 2a). However, the detection of a new peak having a shorter retention time in the chromatograms (whose identification was beyond the scope of this work) and the absence of chloride recovery indicate that the enzymes in the supernatant are capable of attacking 2,4,6-TCP but only to produce a partial degradation of a very small fraction of 2,4,6-TCP. Hence, the extracellular enzymes may only take part in the overall degradation process perhaps by initially attacking the 2,4,6-TCP molecule and/or carrying out one of the degradation steps. This hypothesis is further supported by the data from the experiments with additional supernatant showing a significant increase (50%) in the rate of 2,4,6-TCP degradation when the mycelium is in the presence of a higher concentration of extracellular protein. These results do not support the claim made in recent reports in which it was proposed that fungal degradation results from a combination of sequential or parallel reactions catalyzed by extracellular as well as intracellular enzymes (19, 24, 25, 34).

One could speculate that the fact that 2,4,6-TCP is not degraded by the extracellular fungal protein could be explained by considering that the peroxidases which mediate the initial oxidation require hydrogen peroxide and that the hydrogen peroxide-generating enzymes are intracellular (1). However, the results of the experiments in which hydrogen peroxide was added to the culture supernatant (Fig. 2b) indicate otherwise since the extracellular enzymes were not

able to initiate the 2,4,6-TCP attack even in the presence of H_2O_2. This further confirms that parallel degradation of 2,4,6-TCP by the intracellular and extracellular enzymes is not possible even when a cosubstrate such as hydrogen peroxide is supplied.

The fundamental contribution of the extracellular enzymes to 2,4,6-TCP degradation can be seen from the experiments with washed mycelium, showing that degradation always coincides with the release of extracellular protein 5 to 6 h after mycelial washing and resuspension. When the extracellular protein was denatured (after it had been released and detected), no further degradation occurred, indicating that the protein and not a substrate possibly released by the cells is responsible for 2,4,6-TCP degradation.

The best evidence of the effect of the release of extracellular protein on 2,4,6-TCP degradation came from the experiments with additional mycelium in which two distinct increases in the degradation rate can be observed in correspondence with the two protein releases (Fig. 6). The timing of the protein releases is constant and independent of the presence of extracellular protein. Furthermore, the release does not appear to be induced by the presence of 2,4,6-TCP since degradation occurred immediately, once the extracellular protein had been released, even when the culture had not been previously exposed to 2,4,6-TCP (Fig. 4). The extracellular protein is most likely synthesized de novo by the mycelium, since if an inhibitor of new protein synthesis such as cycloheximide was added, no degradation occurred.

The mycelium appears to play a key role in 2,4,6-TCP degradation not only because it is responsible for the extracellular protein release but also because in its absence the degradation ceases even when extracellular protein is present (Fig. 5a), while degradation proceeds if the mycelium and extracellular protein are both available (Fig. 5b). However, the limiting factor in the degradation process appears to be the concentration of extracellular enzyme rather than the concentration of mycelial biomass. This can be deduced from Fig. 6, which shows that if the mycelial concentration had been limiting, the mycelial addition following the first protein release would have produced an immediate increase in 2,4,6-TCP degradation. This did not occur. Instead, an increase took place only when additional extracellular protein was released by the additional mycelium 6 to 8 h after the new mycelial biomass addition. The same conclusion is also suggested by the data of Fig. 7 showing that the degradation began immediately after additional supernatant was added.

The disappearance of 2,4,6-TCP observed in this work cannot be attributed to physical adsorption onto the mycelium for several reasons: (i) the control experiments (conducted with sterilized mycelial suspension) did not produce any 2,4,6-TCP disappearance; (ii) if adsorption had been responsible for the disappearance of 2,4,6-TCP, then the rate of 2,4,6-TCP removal in the experiments with additional mycelial biomass would have increased immediately after the additional mycelial addition and not some 6 h after that (as shown in Fig. 6); (iii) adsorption is not accompanied by chloride ion release, which was instead consistently observed in all experiments in which mycelium was present.

From the results of this work, it can be concluded that the overall degradation process is a sequential multistep process involving both extracellular and cellular enzymes. In all of the experiments in which degradation took place, the degradation slowed down with time and eventually stopped. This can be interpreted as partial evidence that the extracellular enzyme has a finite lifetime.

This and the other results of this work are especially

significant for the potential industrial applications of this fungus to biodegradation since they impact directly on the engineering design and operation of the bioreactor in which the fungus is contained. If the mycelium and its supernatant must be present simultaneously to degrade a toxic pollutant, batch operations would be favored over continuous operations, at least for immobilized-mycelium plug-flow reactors, because the extracellular enzyme would otherwise be lost with the outflow. In this case, the use of sequencing batch reactors in which the degradation process is conducted in semibatch conditions could be valuable. The limited lifetime of the extracellular enzyme could also make the use of sequencing batch reactors advantageous because of the alternating sequence of the loading, reaction, and discharge operations typical of these reactors. Alternatively, other reactor systems could be designed to maximize the retention of extracellular protein. An example could be the coupling of a continuous immobilized-cell reactor with a membrane pack capable of retaining the extracellular enzyme within the reactor system while allowing the degradation products to be discharged with the effluent.

ACKNOWLEDGMENTS

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