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Mineralization of 2,4-Dichlorophenoxyacetic Acid (2,4-D) and Mixtures of 2,4-D and 2,4,5-Trichlorophenoxyacetic Acid by *Phanerochaete chrysosporium*

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Evidence is presented for mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) in nutrient-rich media (high-nitrogen and malt extract media) by wild-type *Phanerochaete chrysosporium* and by a peroxidase-negative mutant of this organism. Mass balance analysis of [U-*ring*-^{14}C]2,4-D mineralization in malt extract cultures showed 82.7% recovery of radioactivity. Of this, 38.6% was released as $^{14}CO_2$ and 27.0, 11.2, and 5.9% were present in the aqueous, methylene chloride, and mycelial fractions, respectively. 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were simultaneously mineralized when presented as a mixture, and mutual inhibition of degradation was not observed. In contrast, a relatively higher rate of mineralization of 2,4-D and 2,4,5-T was observed when these compounds were tested as mixtures than when they were tested alone.

2,4-Dichlorophenoxyacetic Acid (2,4-D) is one of the most commonly used phenoxyalkanoic herbicides for selective control of weeds and for defoliation (27). 2,4-D and other phenoxyalkanoic acids have also been reported to be mutagenic agents (22). Since these toxic chemicals are manufactured and used each year in massive quantities, effective handling of their production wastes and the contaminated environment is needed. 2,4-D does not persist for long in the environment (half-life in soil, 1 to 6 weeks) because it is susceptible to microbial degradation (21, 23, 27); however, adverse conditions such as low pH and low temperature are known to promote its longevity (25). 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), on the other hand, is degraded relatively slowly by soil bacterial populations (19).

Biodegradation of 2,4-D by microorganisms has received considerable attention lately (6, 11, 15), not only because of its extensive use but also because it serves as a model compound for understanding the mechanism of biodegradation of other, structurally related, environmentally significant haloaromatic compounds (23). A number of bacterial genera are known to degrade 2,4-D, both in mixed and in pure cultures (5–8, 15, 18, 23). However, no naturally occurring bacterium is known to be capable of mineralizing 2,4,5-T. Moreover, mutual inhibition of degradation has been reported when 2,4-D and 2,4,5-T are presented as a mixture to the degrading bacterium (3). In a recent study, even a combined culture of a 2,4-D degrader (*Alcaligenes eutrophus* JMP134) and a 2,4,5-T degrader (*Pseudomonas cepacia* AC1100) failed to effectively metabolize a mixture of these two herbicides (10).

P. chrysosporium, the lignin-degrading white rot fungus, has received worldwide attention for its reported ability to degrade and mineralize a wide range of haloaromatic environmental pollutants such as polychlorinated biphenyls, dioxins, and 2,4,5-T (1, 2, 9, 20, 29, 30). However, there have been no reports to date on the degradation of 2,4-D or more importantly, mixtures of 2,4-D and 2,4,5-T by this organism. In the present study, we investigated the ability of *P. chrysosporium* to mineralize 2,4-D individually and in combination with 2,4,5-T.

MATERIALS AND METHODS

Strains. *P. chrysosporium* ME-446 (ATCC 34541) was used in the present study and was maintained on malt extract agar slants as previously described (31).

Chemicals. 2,4-D (99% pure) and 2,4,5-T (98% pure) were obtained from Aldrich Chemical Co., Milwaukee, Wis. Stock solution of 2,4-D (20 mg/ml) in 0.1 M NaH_2PO_4 buffer (pH 7.0) was stored at 4°C until use. Stock solution of 2,4,5-T (10 mg/ml) was freshly prepared in a similar manner.

Radiochemicals. [U-*ring*-^{14}C]2,4-D (specific activity, 16.6 mCi/mmol; radiochemical purity >98% based on supplier's high-pressure liquid chromatography [HPLC] analysis) was obtained from Sigma Chemical Co., St. Louis, Mo., and [*side-chain*-1-^{14}C]2,4,5-T (specific activity, 0.2 mCi/mmol; radiochemical purity >98%) was a gift from A. M. Chakrabarty, University of Illinois, Chicago, Ill.

Media and inoculum. Low-nitrogen basal III medium (low-N medium), high-N medium, and malt extract (ME) medium used in this study were described previously (31). The compositions of low-N medium and high-N medium were identical, except that the high-N medium contained a 10-fold-higher nitrogen content (24 mM nitrogen). ME medium contained 2% malt extract (Difco Laboratories, Detroit, Mich.), 2% glucose, and 0.1% Bacto peptone (pH 4.5). A blended mycelial inoculum was prepared in low-N medium (without Tween 80) as described elsewhere (31) and was used at 10% (vol/vol) for both the static and the shaken cultures described below.

Culture conditions. The organism was grown as static cultures (10 ml) in 125-ml Erlenmeyer flasks, as previously described (31). Each flask contained 25 mg of 2,4-D per liter, unless otherwise indicated, in an appropriate medium. Following the addition of 10% (vol/vol) inoculum, the flasks were oxygenated as previously described (4) and incubated at 37°C. Cultures were reoxygenated at 3-day intervals. The heat-killed controls were prepared by autoclaving cultures pregrown under culture conditions identical to those used for

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the experimental cultures. Hence, the biomass in heat-killed controls was comparable to that of the corresponding experimental cultures.

HPLC. Degradation of 2,4-D was monitored by HPLC analysis of the methylene chloride extracts of the cultures. Each culture (10 ml) was acidified to pH 2.0 with concentrated HCl, blended for 3 min at speed 5 in an Omni mixer (model 17150; Ivan Sorvall Inc., Newtown, Conn.), mixed with 10 ml of methanol, and extracted three times with methylene chloride (20 ml). The pooled methylene chloride extracts were dried over Na_2SO_4 and evaporated to dryness followed by resuspension in methanol (10 ml). Aliquots (1 ml) of these methylene chloride extracts were filtered through Millex-LCR$_4$ syringe filter units (pore size, 0.5 μm) purchased from Millipore Corp., Bedford, Mass. The filtrates were analyzed for 2,4-D by using Hewlett-Packard series 1050 HPLC equipped with Lichrosorb RP-18 column (Anspec Co., Ann Arbor, Mich.) and a UV detector (set at 230 nm). Methanol–0.1% phosphoric acid (60:40) was used as eluant.

Mineralization. [U-*ring*-^{14}C]2,4-D (10^5 cpm; 3.77 nmol), along with 5 mg of the unlabeled 2,4-D per liter, was added to the liquid cultures (10 ml). At specified intervals during incubation (37°C), the $^{14}CO_2$ generated by mineralization of [^{14}C]2,4-D was trapped by flushing the culture headspace with CO_2-free air and quantified as described elsewhere (31). After each $^{14}CO_2$ trapping, the cultures were reoxygenated as described above.

Mineralization of mixtures of 2,4-D and 2,4,5-T was studied by using four combinations of these compounds: [^{14}C]2,4-D alone; [^{14}C]2,4-D plus unlabeled 2,4,5-T; [^{14}C]2,4,5-T alone; and unlabeled 2,4-D plus [^{14}C]2,4,5-T. In each treatment containing [^{14}C]2,4-D, cold 2,4-D was added to give 5 mg/liter while in treatments containing [^{14}C]2,4,5-T, cold 2,4,5-T was added to give 10 mg/liter. All mineralization experiments were done in triplicate cultures, and parallel uninoculated controls were included. Values plotted represent means ± standard deviations and have been corrected for the values obtained with the uninoculated controls.

Mass balance analysis. After quantifying the $^{14}CO_2$ from [^{14}C]2,4-D mineralization, each culture (10 ml) was acidified (pH 2.0), homogenized, and extracted three times with 10 ml of methylene chloride, and the pooled extracts were referred to as the CH_2Cl_2 fraction. The remaining aqueous suspension was then centrifuged to separate the aqueous and mycelial fractions. To quantify ^{14}C, 1-ml aliquots of the aqueous and CH_2Cl_2 fractions, and the total mycelial fraction were mixed with Safety Solve (15 ml each) and the radioactivity was counted as described above.

Other analyses. Lignin peroxidase (LIP) and manganese-dependent peroxidase (MNP) activities were estimated, respectively, by the procedures of Tien and Kirk (26) and Paszczynski et al. (16). Mycelial dry weights were estimated as described by Michel et al. (14).

RESULTS

Degradation and mineralization of 2,4-D. Mineralization of [U-*ring*-^{14}C]2,4-D to $^{14}CO_2$ was seen in low-N cultures (Fig. 1). When [^{14}C]2,4-D was added to 6-day pregrown cultures, a relatively rapid release of $^{14}CO_2$ was seen in the first 3 days (Fig. 1B), compared with that seen in parallel flasks inoculated at zero time (Fig. 1A). These results indicated that the enzymes responsible for the mineralization of 2,4-D are produced constitutively and that prior acclimation of the organism for 2,4-D degradation is not required. The rate of

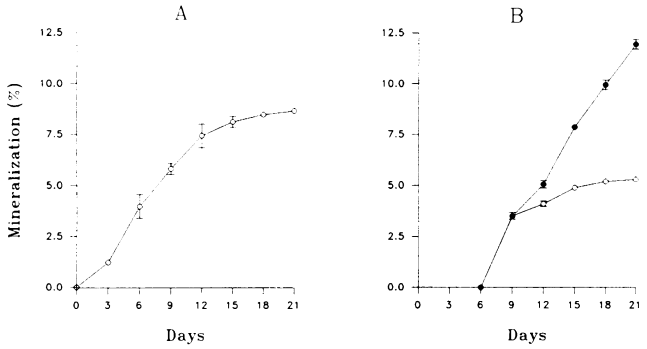

FIG. 1. Mineralization of 2,4-D by *P. chrysosporium* in low-N medium. (A) Rate of mineralization of 2,4-D. Cultures (10 ml) were grown in 125-ml flasks under static conditions at 37°C in low-N medium as described in Materials and Methods. [U-*ring*-^{14}C]2,4-D (100,000 cpm) was added to each culture, along with 5 mg of unlabeled 2,4-D per liter. Percent mineralization represents the percentage of the initial radioactivity released as $^{14}CO_2$. (B) Effect of glucose supplementation on the mineralization of 2,4-D. [^{14}C]2,4-D plus unlabeled 2,4-D was added to 6-day pregrown static low-N cultures, as described for panel A. One group of cultures (●) was supplemented with glucose (14 mg per culture) on day 9 and every 3 days thereafter, and the second group of cultures (○) was not supplemented with glucose. Values plotted represent means ± standard deviations for triplicate cultures.

mineralization of 2,4-D in low-N cultures dropped off substantially after 9 days of incubation. However, supplementation of these cultures with glucose (14 mg/10 ml of culture) at 3-day intervals starting on day 9 led to a steep increase in the observed rate of mineralization (Fig. 1B). These results indicated that the cultures become nutrient limited in the later stages of incubation. This was further supported by the observation that both degradation and mineralization of 2,4-D occurred to a greater extent in ME medium and in high-N medium than in low-N medium (Fig. 2A and B).

Since the enzyme assays showed that LIPs and MNPs are produced in low-N medium but not in high-N and ME media (data not shown), the above results further indicated that LIPs and MNPs are not required for 2,4-D mineralization. Mineralization of 2,4-D by the *per* mutant of *P. chrysosporium*, which lacks the ability to produce LIPs and MNPs, is consistent with this idea (Fig. 2C).

Mass balance analysis. The mass balance analysis for [^{14}C]2,4-D degradation in ME medium showed total radioactivity recovery of 87.5% ± 2.8% and 82.7% ± 2.3% for 20- and 42-day-old cultures, respectively (Table 1). Fractional distribution of the recovered label showed that the release of water-soluble compounds, as well as $^{14}CO_2$, was considerably higher in 42-day-old cultures than in 20-day-old cultures.

Kinetics of mineralization. Since ME medium is relatively simple and gave higher rates of mineralization of [^{14}C]2,4-D, this medium was used in all further studies. To better understand the kinetics of 2,4-D mineralization, the amount of $^{14}CO_2$ produced from [^{14}C]2,4-D during each 3-day sampling period (rather than the cumulative $^{14}CO_2$ produced with time) was monitored (Fig. 3A). The results showed that the rate of $^{14}CO_2$ production follows a biphasic pattern with two peaks of mineralization activity around day 6 and day 30 of incubation. This biphasic pattern was consistently seen in different experiments. The extent of 2,4-D mineralization was higher in shaken cultures (48%) than in static cultures (36%) (Fig. 3B).

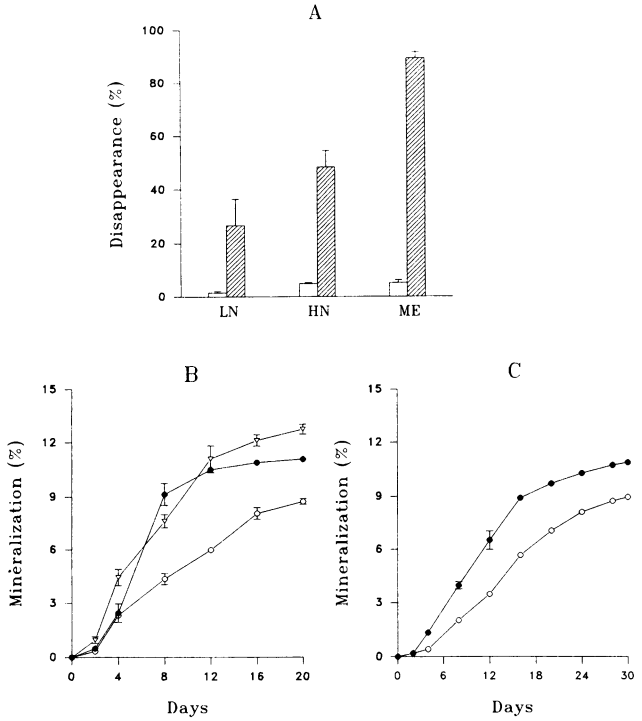

FIG. 2. Comparative degradation and mineralization of 2,4-D by *P. chrysosporium*. (A) Total disappearance of 2,4-D in low-N medium (LN), high-N medium (HN), and malt extract medium (ME). Cultures (10 ml each) were grown for 14 days in the presence of 2,4-D (25 mg/liter), and the degradation was monitored by HPLC analysis of the methylene chloride extracts of the cultures, as described in Materials and Methods. Symbols: ▨, experimental culture; □, heat-killed controls. (B) Mineralization of [U-*ring*-^{14}C]2,4-D in LN medium (○), HN medium (●), and ME medium (▽). (C) Mineralization by a peroxidase-negative (*per*) mutant (●) versus the wild-type strain (ME-446) (○) in low-N medium. Cultures (10 ml each) were grown and percent mineralization of [^{14}C]2,4-D was determined as described in the legend to Fig. 1A.

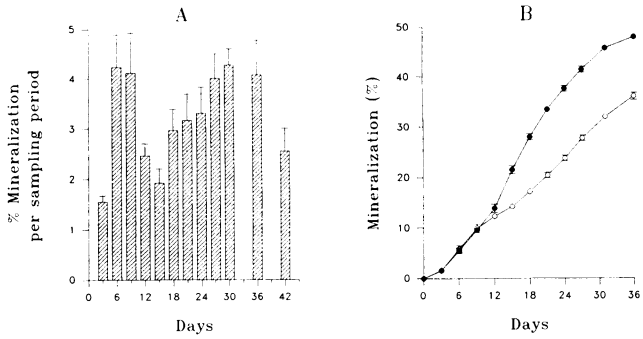

FIG. 3. (A) Rate of mineralization of [U-*ring*-^{14}C]2,4-D to $^{14}CO_2$ by *P. chrysosporium* in ME medium. Cultures were grown in the presence of [U-*ring*-^{14}C]2,4-D (100,000 cpm) plus unlabeled 2,4-D (5 mg/liter). The percentage of initial radioactivity released as $^{14}CO_2$ in each sampling period is plotted on the *y* axis. (B) Comparative rate of mineralization of 2,4-D in static (○) and shaken (●) cultures. The total cumulative mineralization of [^{14}C]2,4-D to $^{14}CO_2$ at each time point is plotted (as described in the legend to Fig. 1A).

Effect of 2,4-D concentration on mineralization. The initial concentration of 2,4-D in the malt extract medium was varied from 0 to 5,000 ppm, and the effect of these variations on growth (mycelial dry weight) and percent degradation (of the initial 2,4-D added) was determined. Substantial levels of degradation and little inhibition of growth were observed when the initial concentration of 2,4-D was below 1,500 ppm, but marked inhibition of growth along with a substantial decrease in 2,4-D degradation were observed at concentrations of 2,000 ppm and above (data not shown).

Mineralization of mixtures of 2,4-D and 2,4,5-T. Mineralization of 2,4-D and 2,4,5-T mixtures by *P. chrysosporium* in ME medium was studied by mixing the ^{14}C-labeled form of one of these compounds with the unlabeled form of the other (Fig. 4). The results indicated that 2,4-D and 2,4,5-T are mineralized simultaneously and that there is no mutual inhibition of degradation. On the contrary, both the rate and the extent of mineralization of [^{14}C]2,4-D and [^{14}C]2,4,5-T were somewhat higher when these compounds were tested as a mixture than when they were tested singly (Fig. 4). For example, 36% of the [^{14}C]2,4-D was mineralized when tested alone, compared with 46.5% mineralization when [^{14}C]2,4-D was tested in combination with 2,4,5-T. The corresponding values for [^{14}C]2,4,5-T mineralization were 24.9 and 36.8%, respectively.

DISCUSSION

P. chrysosporium has been known to mineralize a wide variety of structurally diverse, environmentally significant organopollutants including chlorinated phenols, PCBs, dioxins, mono- and polyaromatic hydrocarbons, and nitroaromatics (1, 9, 17, 28–31). Our results on 2,4-D mineralization alone or in mixtures with 2,4,5-T further extend the range of pollutants degraded by this organism. Many of the aromatic

TABLE 1. Mass balance analysis of [^{14}C]2,4-D degradation

Culture[a]	% Distribution of recovered ^{14}C in fraction[b]:				Total ^{14}C recovery[b] (%)
	CO_2	CH_2Cl_2	Aqueous	Mycelial	
20-day-old cultures					
Control	0.0 ± 0.0	92.5 ± 0.8	3.2 ± 0.6		95.7 ± 0.9
Experimental	12.7 ± 1.4	55.2 ± 1.7	7.5 ± 1.0	12.1 ± 1.7	87.5 ± 2.8
42-day-old cultures					
Control	0.0 ± 0.0	92.6 ± 1.8	2.3 ± 0.2		94.9 ± 1.2
Experimental	38.6 ± 3.4	11.2 ± 2.2	27.0 ± 2.3	5.9 ± 1.5	82.7 ± 2.3

[a] Experimental cultures (10 ml each) were grown in ME medium as described in the legend to Fig. 1A. Then 10^5 cpm of [U-*ring*-^{14}C]2,4-D was added to each culture along with 5 mg of the cold 2,4-D per liter. Uninoculated controls were also included in each experiment.

[b] Values presented are means ± standard deviations for experimental (triplicate) and control (duplicate) flasks.

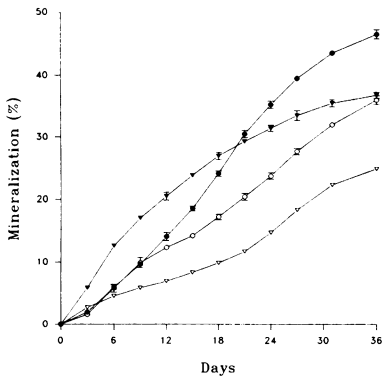

FIG. 4. Mineralization of mixtures of 2,4-D and 2,4,5-T by *P. chrysosporium*. Cultures were grown in ME medium as described in the legend to Fig. 1A. Each flask contained 100,000 cpm of [U-*ring*-^{14}C]2,4-D or [*side-chain*-1-^{14}C]2,4,5-T and an appropriate amount of the unlabeled form of these compounds to give a final concentration of 5 mg/liter for 2,4-D and/or 10 mg/liter for 2,4,5-T. The following four combinations were compared: [^{14}C]2,4-D alone (○); [^{14}C]2,4-D plus unlabeled 2,4,5-T (●); [^{14}C]2,4,5-T alone (▽); and [^{14}C]2,4,5-T plus unlabeled 2,4-D (▼).

pollutants degraded by *P. chrysosporium* have chemical linkages similar to those found in lignin, and the involvement of LIPs and MNPs, the key components of the lignin-degrading enzyme system of this organism, in the degradation of a number of aromatic pollutants has been demonstrated (9, 14, 28, 29). However, other studies indicated that LIPs and MNPs of *P. chrysosporium* are not involved in the degradation of certain aromatic pollutants such as DDT (12), phenanthrene (24), and benzene, toluene, ethylbenzene, and xylene (BTEX) compounds (31). The results of this study and other recent results (30) show that LIPs and MNPs are not required for 2,4-D and 2,4,5-T degradation by *P. chrysosporium*.

The rate of 2,4-D mineralization by *P. chrysosporium* increased with an increase in the level of nitrogen or carbon, as evident from a comparison of the extent of mineralization in high-N medium versus low-N medium (Fig. 2B) and in glucose-supplemented versus unsupplemented low-N cultures (Fig. 1B). These results are consistent with the recent finding that supplementation of low-N medium cultures of *P. chrysosporium* gave 45% degradation of 2,4,5-T, compared with the 36% degradation observed by unsupplemented cultures (30).

It is of interest that 2,4-D mineralization in malt extract cultures consistently exhibited a biphasic pattern (Fig. 3A), with two peaks of activity, one around day 6 and the other around day 30 of incubation. The second phase of increased mineralization activity, beginning around day 20, could be due to (i) induction of enzyme(s) in response to nutrient starvation and/or induction of enzymes involved in catalyzing one or more rate-limiting steps in 2,4-D metabolism or (ii) a metabolic switch to the breakdown of intermediary compounds in the 2,4-D mineralization pathway. The latter phenomenon has also been observed during the degradation of pentachlorophenol by *P. chrysosporium* (13), in which pentachloroanisole transiently accumulated during the first stage and was degraded during the second stage, beginning after 9 days of incubation. Studies to identify metabolic intermediates produced during 2,4-D mineralization are in progress.

Since the contaminated environmental sites or the industrial wastes generally contain more than one class of chlorophenoxyalkanoic acids, it is desirable to identify organisms with broader degradative ability. It is significant that *P. chrysosporium* is capable of simultaneously mineralizing both 2,4-D and 2,4,5-T. This is rather unusual when compared with degradation of a mixture of these two compounds by bacterial systems (3). For example, *A. eutrophus* JMP134, which efficiently degrades 2,4-D, failed to do so in the presence of 2,4,5-T. Likewise, *Pseudomonas cepacia* AC1100, which degrades both 2,4,5-T and 2,4-D individually, degraded these compounds poorly when they were presented as a mixture. Even a mixed culture of the above two strains was inefficient in simultaneously degrading 2,4-D and 2,4,5-T. However, a recombinant strain has recently been constructed by transferring a 2,4-D-degradative plasmid, pJP4, from *A. eutrophus* JMP134 to the 2,4,5-T-degrading *Pseudomonas cepacia* AC1100 (10). This genetically engineered strain, designated RHJ1, has been reported to simultaneously degrade both 2,4-D and 2,4,5-T in controlled liquid cultures.

In conclusion, *P. chrysosporium*, an organism widely distributed in nature, effectively mineralizes 2,4-D alone as well as in combination with 2,4,5-T.

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