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Abstract

Biotransfonnation of atrazine by the white rot fungus *Phanerochaete chrysosporium* was demonstrated by a 48% decrease of the initial herbicide concentration in the growth medium within the first 4 days of incubation, which corresponded to the mycelium-growing phase. Results clearly established the mineralization of the ethyl group of the herbicide. Analysis of the growth medium showed the formation of hydroxylated and/or N-dealkylated metabolites of atrazine during fungal degradation.

Introduction

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine; CAS 1912-24-9; ATZ; see Fig. 1] is one of the most widely used herbicides in the world for the control of annual grasses and broadleaf weeds in corn and sorghum. It is also used as a nonselective herbicide for vegetation control in noncrop land. ATZ microbial and chemical degradation is well documented in soils, where the herbicide often undergoes mineralization and hydrolytic and oxidative reactions (4, 5, 9). Despite that apparent biodegradability, and because of its extensive use, ATZ has led to the contamination of terrestrial ecosystems and can be measured in ground and surface waters in many countries. Because of the public health and environ- mental significance of that pesticide contamination, an under- standing of all of the mechanisms involved in ATZ removal is important. ATZ-containing soils and waters may be amenable to cleanup through bioremediation.

The well-known white rot fungus *Phanerochaete chrysosporium* has been shown to degrade a wide

variety of environmentally persistent pollutants (for reviews, e.g., see references 1, 2, and 6), including the herbicides metolachlor and 2,4,5-trichlorophenoxyacetic acid (10, 13), and offers potential applications in pollution management (2). The ability of *P. chrysosporium* to degrade such a diverse group of pollutants could be due to the lignin-degrading system. This fungal lignin-degrading sys- tem includes two families of extracellular peroxidases designated lignin peroxidases and manganese peroxidases.

The purposes of the present study were (i) to show that *P. chrysosporium* is able to transform ATZ and (ii) to identify the metabolites produced during herbicide transformation.

Materials and methods

High-purity standards of ATZ and metabolites were obtained from Promochem (Molsheim, France). [ethyl-l-14C]ATZ (880 MBq/mmol) and [ring-U-^{14}C]ATZ (425 MBq/mmol) were purchased from Amersham (Les Ullis, France) and Sigma (St. Quentin Fallavier, France), respectively. The radiochemical purity of both 14C-labeled herbicides was at least 98%.

P. chrysosporium BKM-F-1767 (ATCC 24725) was used. The strain was maintained on malt agar slant cultures at 37°C. Spores were collected by washing slants with sterile distilled water and stored at 4°C.

For degradation studies, spores were inoculated in 150-ml Erlenmeyer flasks containing 10 ml of a growth medium described previously (3). It contained 0.5 g of NAT 89, a commercial phospholipid source supplied by Natterman Phospholipid GmbH (Cologne, Germany), per liter. Veratryl alcohol (0.4 mM) was added to the medium. The medium contained 2 μM ATZ consisting of a mixture of unlabeled and labeled (5 kBq) pesticides dissolved in 10 μ,l of acetone. The Erlenmeyer flasks were incubated without shaking in the dark at 37°C in one-liter sealed flasks in the presence of vials containing 10 ml of 1 N NaOH and 10 ml of water, respectively. The headspaces of the flasks were flushed with 100% oxygen for 1 min at the beginning of the experiments and then with air for 45 min every 4 days. The NaOH solutions were also replaced every 4 days.

Growth was measured in terms of the dry weight of mycelium after filtration on glass fiber filters (GF/D, Whatman), which were subsequently rinsed with 10 ml of Milli-Q water and dried for 3 days at 40°C. Eight-milliliter fractions of filtered medium were separated into aqueous and organic phases with 12 ml of ethylene chloride. The disappearance of ATZ was monitored by gas chromatography analysis of 3-μ,l aliquots of the organic phase on a model 3700 gas chromatograph equipped with a Thermionic Specific Detector and a model 4400 integrator (Varian, Les Ullis, France). A J&W Scientific DB-Wax

capillary column (15 m by 0.32 mm [inside diameter]; 0.25-μm film thickness) purchased from Varian was used. The injector, detector, and column temperatures were 220, 300, and 190°C, respectively. Helium was used as the carrier gas at a fixed pressure of 10 lb/in^2. The separation was then repeated two times to optimize the extraction of the chlorinated metabolites. The organic extracts were reduced under vacuum, pooled, and spotted on thin-layer chromatography silica gel 60F254 plates (Merck, Darmstadt, Germany) and chromatographed in n-hexane-ethyl acetate (50:50 [vol/vol]). The chromatograms were first examined under UV light and then exposed to X-ray film for 2 weeks. The radioactivity present in each spot coeluted with reference compounds was quantified by liquid scintillation counting after the silica gel was scraped and eluted with methanol. After filtration on 0.45-μm-pore-size membranes (Millipore, St. Quentin-Yvelines, France) under vacuum, the aqueous phases were subjected to solid-phase extraction. Five hundred milligrams (2.8 ml) of prepacked Bond-Elut SCX cartridges (Varian) were conditioned with 5 ml of methanol and 5 ml of 0.01 N HCl as described by Nelieu et al. (11). The solution (15 ml of the aqueous phase acidified with 15 ml of 0.01 N HCl) was passed through the cartridge at a flow rate of 3 ml/min under vacuum. Elution of triazine compounds was achieved with 7 ml of 0.1 M ammonium acetate dissolved in water-acetonitrile (75:25 [vol/vol]) at pH 8.6. For high-performance liquid chromatography (HPLC) analysis, eluates were spiked with authentic standards of ATZ and metabolites and then concentrated on an MCH-10 C 18 guard column (3 cm by 4 mm [inside diameter]; Varian) at a flow rate of 0.5 ml/min. Partial sample cleanup was obtained by washing the guard column with 10 ml of Milli-Q water. The elution of triazine compounds was then achieved on an ODS- 80TM analytical column (25 cm by 4.6 mm [inside diameter]; Varian) with a pump (Varian 9010) delivering a solvent system composed of acetonitrile and water, each acidified with 0.1% H_3P0_4. The solvent system began with 5% acetonitrile for 4 m1n, and then a linear increase to 50% acetonitrile over 15 min and a stationary phase of 10 min followed. The A_{220} of the column eluate was monitored with a UV-Vis detector (Varian 9050). One-milliliter fractions were collected, and their radioactivity was determined by liquid scintillation counting.

The radioactivity remaining in the cell biomass was deter- mined after the dry weight measurements by catalytic combus- tion of the mycelia in an Intertechnic Oximat 101 oxidizer.

Lignin peroxidase activity in the extracellular fluid was determined by the rate of oxidation of veratryl alcohol to veratraldehyde by the method of Tien and Kirk (14), except that 2 mM veratryl alcohol and 0.27 mM H_20_2 were used. Lignin peroxidase activity was expressed in nanokatals: 1 nkat/ml is equivalent to 60 U/liter. Manganese peroxidase activity was determined spectrophotometrically by the method of Paszczynski et al. (12) with vanillylacetone as a substrate.

Reaction mixtures contained 100 µl of culture fluid, 0.1 mM vanillylacetone, 100 mM sodium tartrate (pH 5), 0.1 mM $MnSO_4$, and 0.05 mM H_2O_2 in a final volume of 1 ml. The reaction was started by adding H_20_2, and the rate of disappearance of vanillylacetone was measured at 334 nm. Manganese peroxidase activity was expressed in nanokatals.

Electron impact mass spectral analysis was performed on a Varian Saturn II ion trap instrument with an ionizing voltage of 70 eV. Samples were introduced by gas chromatography on a Varian 3400 chromatograph. The column was a J&W Scientific DB-5 (Varian; 30 m by 0.25 mm [inside diameter]; 0.25-µm film thickness). The temperature of the septum- equipped programmable injector was programmed from 60 to 220°C at 190°C/min. The temperature of the column was programmed from 100 to 140°C (10°C/min), maintained at 140°C for 4 min, increased from 140 to 200°C (10°C/min), and finally maintained at 200°C for 16 min. Helium carrier pressure was 12 lb/in^2. Confirmation of the identity of the chlorinated metabolites was achieved by gas chromatography-mass spectrometry analysis by using a fragmentation pattern described previously (7).

Each experiment was done in triplicate and repeated two times; the standard deviation was less than 10% of the mean. The nomenclature of the metabolites of ATZ identified in this study is as follows: deethylatrazine [2-chloro-4- (amino)-6-(isopropylamino)-s-triazine], deisopropylatrazine [2- chloro-4-(ethylamino)-6-(amino)-s-triazine], hydroxyatrazine [2-hydroxy-4-(ethylamino)-6-(isopropylamino)-s-triazine], and deethylhydroxyatrazine [2-hydroxy-4-(amino)-6-(isopropyl- amino)-s-triazine]. Their chemical structures are presented in Fig. 1.

Results and discussion

Profiles of ATZ disappearance, lignin and manganese per- oxidase production, and mycelium formation by *P. chrysosporium* arc shown in Fig. 2. After a 1-day lag period, 48% of the initial ATZ content was removed within the first 4 days of incubation, which corresponded to both the ligninolytic-enzyme-producing and the mycelium-growing phases. The ATZ content remained constant in the uninoculated sterile controls (data not shown).

To examine the fungal mechanisms of ATZ degradation, cultures of *P. chrysosporium* were incubated with *ethyl*-1-^{14}C- and ring-U-^{14}C-labeled pesticide. In these experiments, the mycelial growth and ATZ disappearance profiles arc similar to those presented in Fig. 2. Figure 3 shows that 14 CO could be released from cultures incubated with chain-labeled ATZ and clearly established the attack of the ethyl group of the herbicide. Mineralization started after 1 day of incubation and amounted to 23.7% of the

initial radioactivity after 16 days. In the same experimental conditions, ring-labeled ATZ underwent no mineralization. Whatever the labeling position of ATZ, no $^{14}CO_2$ could be trapped from uninoculated sterile controls.

Evolution of the chain-labeled herbicide was calculated from mass-balance analysis. Radioactivity in the aqueous and organic phases of the medium as well as in the cell biomass arc shown in Fig. 4. The amount of $^{14}CO_2$ released is reported for comparison. Water-soluble radioactivity amounting to 13.3% of the initial radioactivity was detected at the end of the experiment. In uninoculated sterile controls, water-soluble radioactivity did not exceed 1% of the initial radioactivity (data not shown). The organic phase contained the highest levels of radioactivity, which decreased markedly between days 1 and 4.

No significant further evolution was noted through the end of the experiment. On the other hand, cell biomass incorporated increasing amounts of radioactive material at the beginning of the experiments. The maximal value of 17.9% noted on day 4 then decreased. The total radioactivity recovered throughout the experiments consisted of between 95.2 and 106.4% of the initial radioactivity.

The evolution of metabolites was monitored in the growth medium during incubation with [ring-U-^{14}C]ATZ. Thin-layer chromatography and HPLC analysis of both the organic and aqueous phases obtained by medium fractionation showed the conversion of the herbicide to more-polar radioactive products having the chromatographic properties of deethylatrazine, deisopropylatrazine, hydroxyatrazine, and deethylhydroxyatrazine. The evolution of labeled ATZ content was in agreement with the profile presented in Fig. 2. The main metabolite, dethylatrazine, accumulated between days 1 and 4 and reached 24% of the initial radioactivity by the end of the experiment (Fig. 5). A similar formation pattern occurred for deisopropylatrazine, although it accumulated at a lower rate (4.5%). Hydroxyatrazine and deethylhydroxyatrazine contents increased much more linearly throughout the experiments; their amounts were 6.8 and 0.5% of the initial radioactivity, respectively. Finally, almost 5% of the initial radioactivity remained unidentified at the end of the experiments and may be attributed to very-polar compounds unretained on SCX or C18 cartridges. Table 1 summarizes the relative abundance of the most characteristic ions of ATZ and its chlorinated metabolites detected by gas chromatography-mass spectrometry analysis.

The present studies provide valuable information on the use of *P. chrysosporium* for the degradation of ATZ. The herbicide is partly converted to polar dechlorinated and/or N-dealkylated metabolites. These results agree with previous studies, in which dealkylation appeared to be the major and first mechanism involved in the microbial degradation of chloro-s-triazines (4, 9). In general, soil fungi removed the ethyl group of ATZ in preference to the isopropyl group (4, 8, 15). Moreover, dealkylated metabolites

were not extensively degraded further and accumulated in the culture medium. Similar results have been already reported (9). Hydroxylated metabolites were also detected in our experiments. Hydroxylation by fungi is reported to be less common than N-dealkylation (8). Our results show that hydroxylation results from fungal activity, either by direct metabolism or by secondary effects due to fungal activity leading to chemical hydrolysis of the herbicide. In addition, in our experimental conditions, evolution of $^{14}CO_2$ occurred only from cultures containing chain-labeled ATZ. Compared with several bacterial strains (4, 9), soil fungi and especially *P. chrysosporium* appeared to be ineffective in carrying out the heterocyclic ring cleavage of triazines, which is somewhat resistant to fungal attack (8, 15).

Nevertheless, on the basis of the ATZ disappearance profile, our results seem to indicate that a rate-limiting step is occur- ring in the transformation reactions, leading to only a partial conversion of ATZ. Further work is needed to shed some light on this question as well as to clarify the biochemistry of ATZ biotransformation by *P. chrysosporium*.

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Table I. Relative abundance of the characteristic mass ions of the chlorinated metabolites produced from atrazine by *P. chrysosporium* cultures and of atrazine

Compound	Characteristic mass ions (m/z)
Deisopropylatrazine	68 (50)[a], 110 (15), 145 (56), 147 (17), 172 (12), 173 [M^+] (100), 174 (20), 175 (31)
Deethylatrazine	68 (16), 110 (8), 145 (23), 147 (7), 172 (100), 173 (9), 174 (36), 187 [M^+] (38), 189 (13)
Atrazine	68 (34), 110 (<5), 145 (7), 147 (<5), 172 (23), 173 (16), 174 (9), 175 (8), 200 (100), 202 (45), 215 [M^+] (89), 217 (43)

[a] The values in parentheses indicate relative intensities.

Fig. 1. Chemical structures of ATZ and its metabolites identified in this study. EtHN, ethylamino; NHiPr, isopropylamino

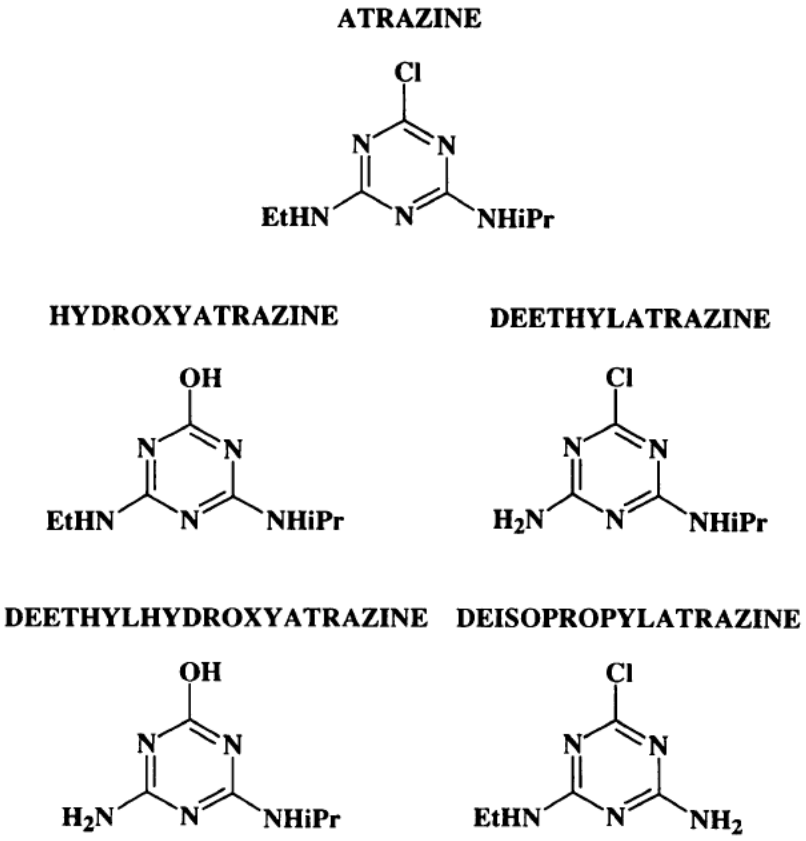

Fig. 2. Profile of ATZ content in the growth medium (O) in relation to lignin (♦) and manganese (◊) peroxidase production and of mycelial formation (●) by *P. chrysosporium*

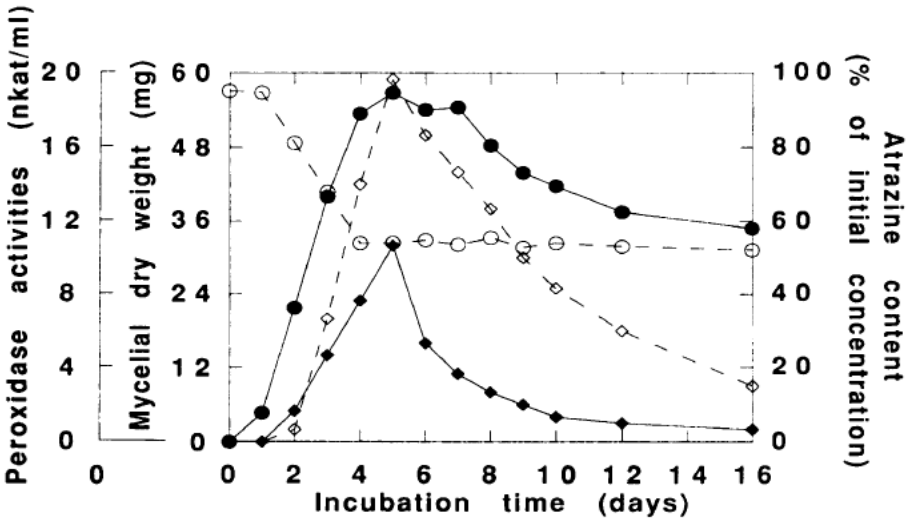

Fig. 3. Mineralization of *ethyl*-l-^{14}C- (●) and ring-U-^{14}C-labeled ATZ (O) by *P. chrysosporium*. X, radioactivity evolved from uninoculated sterile controls

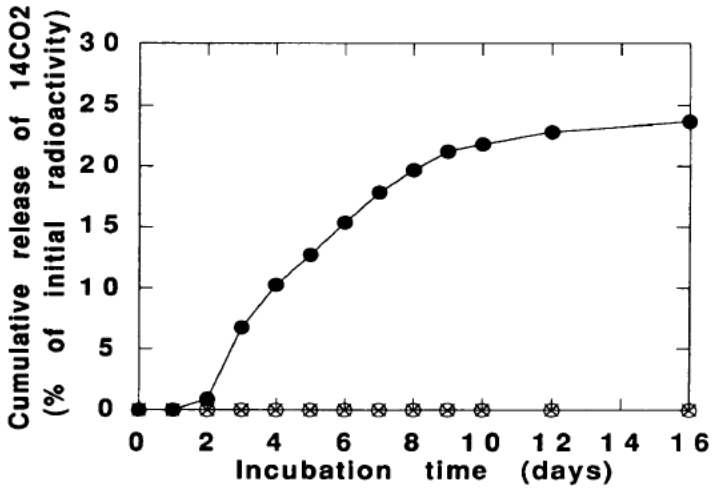

Fig. 4. Mass-balance analysis of *P. chrysosporium* cultures after addition of [*ethyl*-l-^{14}C]atrazine and growth medium fractionation. Symbols: O, medium aqueous phase; ●, medium organic phase; ♦, mycelium; X, $^{14}CO_2$

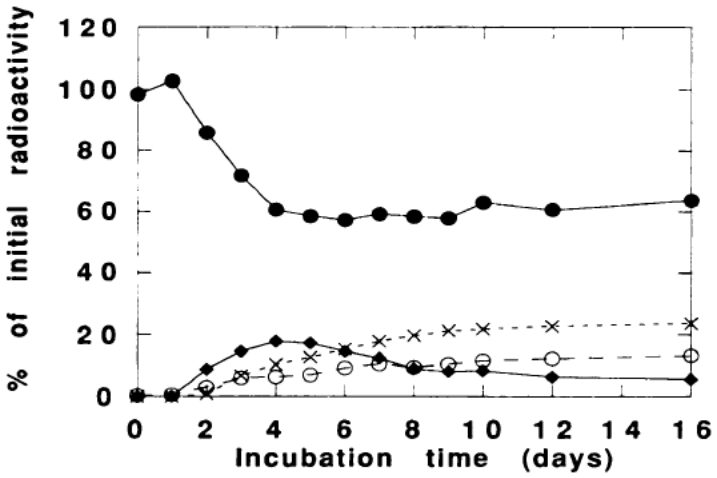

Fig. 5. Evolution of deethylatrazine (●), deisopropylatrazine (♦), hydroxyatrazine (O), deethylhydroxyatrazinc (◊), and unidentified radioactivity (.6) in cultures of *P. chrysosporium*