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Research Paper

**Biotransformation of acetamiprid by the white-rot fungus *Phanerochaete sordida***

**YK-624**

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## Abstract

Acetamiprid (ACE) belongs to the neonicotinoid class of systemic broad-spectrum insecticides, which are the most highly effective and largest selling insecticides worldwide for crop protection. As neonicotinoid insecticides persist in crops, biotransformation of these insecticides represents a promising approach for improving the safety of foods. Here, the elimination of ACE from liquid medium by the white-rot fungus *Phanerochaete sordida* YK-624 was examined. Under ligninolytic and non-ligninolytic conditions, 45% and 30% of ACE were eliminated, respectively, after 15 days of incubation. HR-ESI-MS and NMR analyses of a metabolite identified in the culture supernatant suggested that ACE was *N*-demethylated to (*E*)-*N*<sup>1</sup>-[(6-chloro-3-pyridyl)-methyl]-*N*<sup>2</sup>-cyano-acetamidine, which has a much lower toxicity than ACE. In addition, we investigated the effect of the cytochrome P450 inhibitor piperonyl butoxide (PB) on the elimination of ACE. The elimination rate of ACE by *P. sordida* YK-624 was markedly reduced by the addition of either 0.01 and 0.1 mM PB to culture medium. These results suggest that cytochrome P450 plays an important role in the *N*-demethylation of ACE by *P. sordida* YK-624.

Keywords: Acetamiprid · Detoxification · *Phanerochaete sordida* YK-624 · *N*-demethylation · White-rot fungi

## Introduction

Neonicotinoid insecticides, which are one of the most important classes of commercial insecticides worldwide, are systemic in plants and animals and are used to manage crop pests and control fleas on cats and dogs (Arther et al. 1997; Jacobs et al. 1997; Yamamoto and Casida 1999). Acetamiprid (ACE; Fig. 1) belongs to the class of chloronicotinyl neonicotinoid insecticides and is used to control Hemiptera, particularly aphids, Thysanoptera, and Lepidoptera, on a wide range of crop species (Mateu-Sanchez et al. 2003; Tokieda et al. 1997). In recent years, ACE residues in crops are receiving considerable attention due to their potential toxicity to humans (Pramanik et al. 2006; Sanyal et al. 2008), and methods for the biotransformation of neonicotinoids are being actively researched. A number of microorganisms that are capable of degrading neonicotinoids have been identified, including the yeast *Rhodotorula mucilaginosa* strain IM-2, which is able to hydrolyze ACE and thiacloprid, and the bacterium *Stenotrophomonas maltophilia* CGMCC 1.1788, which is able to hydroxylate imidacloprid and *N*-demethylate ACE (Chen et al. 2008; Dai et al. 2006; Dai et al. 2010).

Lignin-degrading white-rot fungi, which have the unique ability to degrade lignin

to the level of CO<sub>2</sub> (Kirk and Farrell 1987), and their ligninolytic enzymes have also attracted interest for the biotransformation of contaminants because of their industrial potential for degrading recalcitrant environmental pollutants, such as polychlorinated dibenzodioxin (Kamei et al. 2005), lindane (Bumpus et al. 1985), chlorophenols (Joshi and Gold 1993), and polycyclic aromatic hydrocarbons (Bezalel et al. 1996; Collins et al. 1996). More recently, we reported that the white-rot fungus *Phanerochaete sordida* YK-624 produces manganese peroxidase (MnP) that is capable of detoxifying aflatoxin B<sub>1</sub>, which is a frequent contaminant of food products (Wang et al. 2011).

Here, we examined the elimination of ACE by *P. sordida* YK-624 in order to develop a bioremediation system for ACE-contaminated foods. We also detected the metabolite from ACE and proposed a metabolic pathway for the metabolism of ACE by *P. sordida* YK-624. This represents the first report describing the biotransformation of ACE by a white-rot fungus.

## Materials and methods

### Fungal strain and culture conditions

*P. sordida* YK-624 (ATCC 90872), which has been isolated from rotted wood

(Hirai et al. 1994), was used in the present study. The fungus was maintained on potato dextrose agar (PDA) slants at 4°C.

## Chemicals

ACE and piperonyl butoxide (PB) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were analytical-pure grade and were used without further purification.

## Fungal treatment of ACE

Nitrogen-limited (NL) medium described by Tien and Kirk (1988) and potato dextrose broth (PDB) medium (4% potato starch, 2% dextrose, pH 4.5) were used for ACE elimination experiments. The fungus was incubated on a PDA plate at 30 °C for 3 days, and then 10-mm-diameter disks were punched out from the growing edge of mycelium. Two disks were each placed into a 100-mL Erlenmeyer flask containing 10 mL of liquid medium (NL or PDB medium, pH 4.5). After statically incubating the flasks at 30 °C for 7 days, 100 µL of 1 mM ACE (final concentration: 10 µM) was added to the cultures, which were then further incubated for 5, 10, 15 and 20 days (each in triplicate). The culture was filtrated with a 0.2-µm membrane filter, and the filtrate

was then subjected to high-performance liquid chromatography (HPLC) for the quantification of ACE under the following conditions: column, Wakosil-II 5C18HG (4.6 x 150 mm; Wako Pure Chemical Industries); mobile phase, 30% MeOH aq.; flow rate, 0.5 mL/min; and UV wavelength, 246 nm.

#### Metabolite identification

Inoculated cultures (5 L NL medium) of *P. sordida* YK-624 were prepared under the conditions described above. After static incubation at 30 °C for 7 days, 5 ml of 100 mM ACE (final concentration: 100 µM) was added to these cultures. The cultures were further incubated for 15 days and then filtrated with a 0.2-µm membrane filter. Distilled water (100 mL) was added to the filtrate after evaporation to dryness, and the resulting solution was extracted twice with 100 mL ethyl acetate (EtOAc). The EtOAc extract was dried over anhydrous sodium sulfate and then evaporated to dryness. The residue was cleaned on a silica gel flash column chromatography (silica gel 60N, φ40 × 600 mm) and eluted with dichloromethane/EtOAc/MeOH (10/0/0, 9/1/0, 8/2/0, 7/3/0, 5/5/0, 3/7/0, 0/10/0, 0/9/1, 0/8/2, 0/7/3, 0/5/5, and 0/0/10; vol/vol/vol) to obtain 14 fractions. Each fraction was analyzed by thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), high-resolution-electrospray ionization-mass

(HR-ESI-MS), and  $^1\text{H}$ - nuclear magnetic resonance (NMR). Silica gel plates (Merck F254; Merck, Darmstadt, Germany) and silica gel 60N (Merck 100-200 mesh; Merck) were used for analytical TLC and flash column chromatography. The metabolite was further separated by HPLC (column: Develosil C30-UG-5; Nomura Chemistry, Seto, Japan) using 70% MeOH. The purified metabolite was analyzed by HR-ESI-MS and NMR, including correlation spectroscopy (COSY), hetero-nuclear multiple quantum coherence (HMQC), and hetero-nuclear multiple-bond connectivity (HMBC) spectroscopies. The HR-ESI-MS data were measured using a JMS-T100LC mass spectrometer.  $^1\text{H}$ -NMR spectra were recorded using a Jeol Lambda-500 spectrometer at 500 MHz, while  $^{13}\text{C}$ -NMR spectra were recorded on the same instrument at 125 MHz.

#### Cytochrome P450 inhibitor experiment

After pre-culturing *P. sordida* YK-624 for 5 days, ACE (final concentration: 10  $\mu\text{M}$ ) and the cytochrome P450 inhibitor PB (final concentration: 0, 0.01, and 0.1 mM) were added to cultures. The cultures were further incubated for 5, 10, and 15 days, and each culture was then subjected to HPLC for the quantification of ACE, as described above (column, Wakosil-II 5C18HG; mobile phase, 30% MeOH aq.; flow rate, 0.5 mL/min; and UV wavelength, 246 nm.). All experiments were performed in triplicate.

## Results

### Elimination of ACE by *P. sordida* YK-624

In the present study, NL and PDB media were used for the elimination experiment of ACE. When *P. sordida* YK-624 was cultured in NL medium, ACE was reduced by approximately 45% after 20 days of incubation (Fig. 2). In PDB medium, the eliminated concentration of ACE was approximately 28% after 20 days of incubation. Moreover, purified lignin peroxidase (LiP) and MnP from *P. sordida* YK-624 could not degrade ACE (data not shown). These results suggested that *P. sordida* YK-624 had a higher ability for the elimination of ACE in NL medium and that ligninolytic enzymes (LiP and MnP) were not involved in the elimination of ACE, as ACE was also eliminated under the non-ligninolytic conditions provided by PDB medium. In fact,

### Identification of the metabolite from ACE

As shown in Fig. 3, main metabolite was detected in HPLC analysis of 10-day NL culture fluid inoculated with *P. sordida* YK-624, and minor metabolites were hardly detected. To determine the structure of the metabolite produced during the degradation

of ACE, 15-day cultures of *P. sordida* YK-624 in NL medium supplemented with 100  $\mu$ M ACE were subjected to TLC and HPLC. The purified metabolite was then subjected to HR-ESI-MS analysis, which yielded a molecular ion at  $m/z$  231.0428  $[M+Na]^+$  (calculated for  $C_9H_9ClN_4Na$ , 231.0413), indicating that the molecular formula of this compound was  $C_9H_9ClN_4$ . This formula suggested that the metabolite might be a demethylated form of ACE. The structure of the purified metabolite was further characterized by NMR analyses. Table 1 lists the chemical-shift assignment data for the metabolite of ACE. HMBC correlations (Fig. 4) (H-7/C-2, H-7/C-3, H-7/C-4; H-7/C-9, H-13/C-9) confirmed that the metabolite was *N*-demethylated ACE, (*E*)-*N*<sup>1</sup>-[(6-chloro-3-pyridyl)-methyl]-*N*<sup>2</sup>-cyano-acetamidine, which is commonly known as IM 2-1 (Fig. 1).

#### Effect of cytochrome P450 inhibitors

The effect of cytochrome P450 inhibitors on the elimination of ACE by *P. sordida* YK-624 were investigated using PB. The elimination rate of ACE after the addition of 0.01 and 0.1 mM PB into cultures of *P. sordida* YK-624 is shown in Fig. 5. In contrast to the elimination rate of ACE in cultures without PB, markedly lower elimination activity of ACE was observed in cultures containing PB. In the presence of 0.01 and 0.1

mM PB, only 16% and 7% of ACE were eliminated after 15 days of incubation, respectively, compared to 45% in cultures lacking PB.

## Discussion

White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. However, prior to the present study, the degradation of neonicotinoid insecticides by white-rot fungi had not been reported. Here, we demonstrated the elimination of ACE from liquid cultures of the white-rot fungus *P. sordida* YK-624. Under ligninolytic and non-ligninolytic conditions, 45% and 30% of ACE, respectively, were eliminated after 15 days of incubation (Fig. 2). Although the fungal growth in PD media is much faster than in NL media, the elimination rate of ACE in PD media was almost same or lower than in NL medium. Moreover, no decrease was observed in PD medium containing 10  $\mu$ M ACE without fungal inoculation for 20 days. These results suggest that ACE was biotransformed to other compounds by fungal treatment, not abiotic elimination or adsorption on biomass.

We detected the generation of the ACE metabolite IM 2-1 by *P. sordida* YK-624

(Fig. 3). The metabolic pathway of ACE has been studied in honeybee, mice, spinach, and soil bacteria (Brunet et al. 2005; Ford and Casida 2006; Ford and Casida 2008; Tokieda et al. 1999). In these studies, the main metabolic pathway involved the *N*-demethylation of ACE to yield IM 2-1. In honeybee, the nitromethylene bond of IM 2-1 might be subsequently oxidized to yield 6-chloronicotinic acid (Brunet et al. 2005). In addition to the metabolite IM 2-1, we also detected other compounds by HPLC and TLC analyses of *P. sordida* YK-624 culture supernatant. However, we were unable to determine the structures of these additional metabolites due to their low concentrations. The lipophilic *N*-methyl group of neonicotinoids plays an important role in the bioefficacy of these insecticides, with the loss of this group reducing the insecticidal activity by more than 10-fold or even leading to complete inactivation (Chen et al. 2008). Although ACE is reported to have a relatively high toxicity in the honeybee, with an LD<sub>50</sub> value of 7.1 µg/bee, the metabolite IM 2-1 resulted in no mortality at 50 µg/bee (Iwasa et al. 2004). Since the metabolite IM 2-1 has lower toxicity than ACE, the detoxification of ACE is possible using *P. sordida* YK-624.

The functional diversity of cytochrome P450s in white-rot fungi has been studied because cytochrome P450s play an important role on degrading a wide variety of recalcitrant aromatic compounds (Hiratsuka et al. 2001; Ichinose et al. 1999; Masaphy

et al. 1996). Recently, Hata et al. (2010) suggested that hydroxylation catalyzed by cytochrome P450 in *P. sordida* YK-624 might be involved in the elimination and detoxification of diclofenac and mefenamic acid. The involvement of cytochrome P450 in the *N*-demethylation of a few drugs has also been reported in human cells (Ghahramani et al. 1997; Sutton et al. 1997). Our present study showed that the elimination of ACE was efficiently inhibited by the addition of PB, which is a common inhibitor of cytochrome P450 and is often used for demonstrating whether a reaction is catalyzed by cytochrome P450 enzymes (Kamei et al. 2005; Mori and Kondo 2002; Mori et al. 2003). Since *N*-demethylation of ACE was inhibited by PB, we propose that cytochrome P450s are involved in the *N*-demethylation of ACE.

In conclusion, we have described for the first time the biotransformation of ACE by a white-rot fungus.

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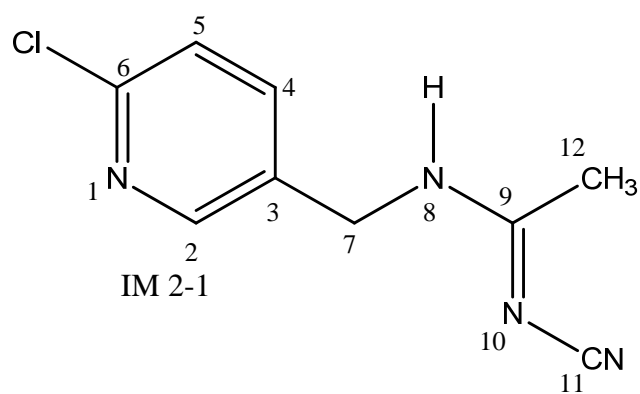
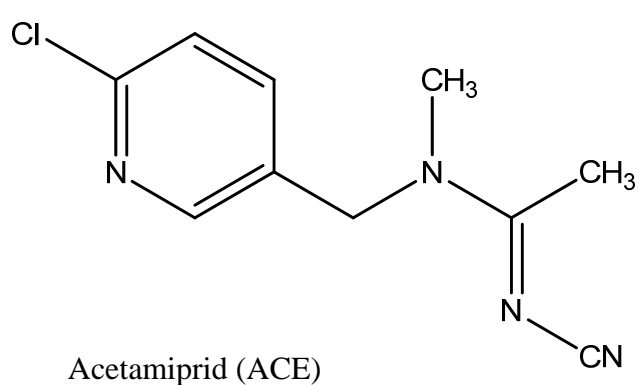
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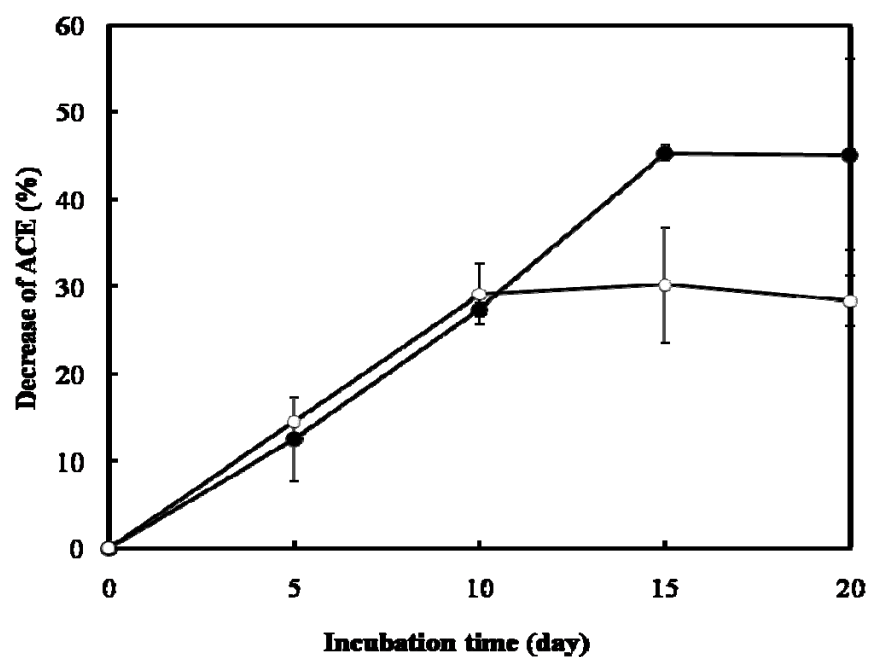
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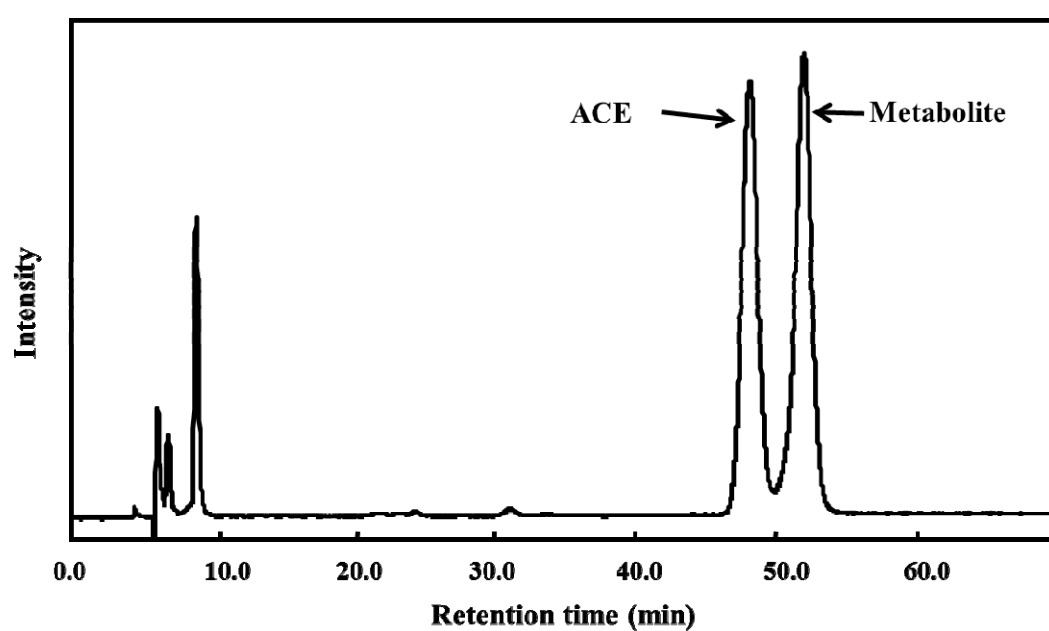
266 acetylcholine receptor. Springer, Tokyo.



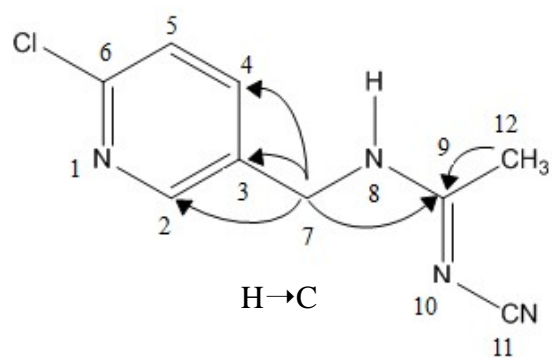
**Fig. 1** Structures of acetamiprid and the metabolite IM 2-1



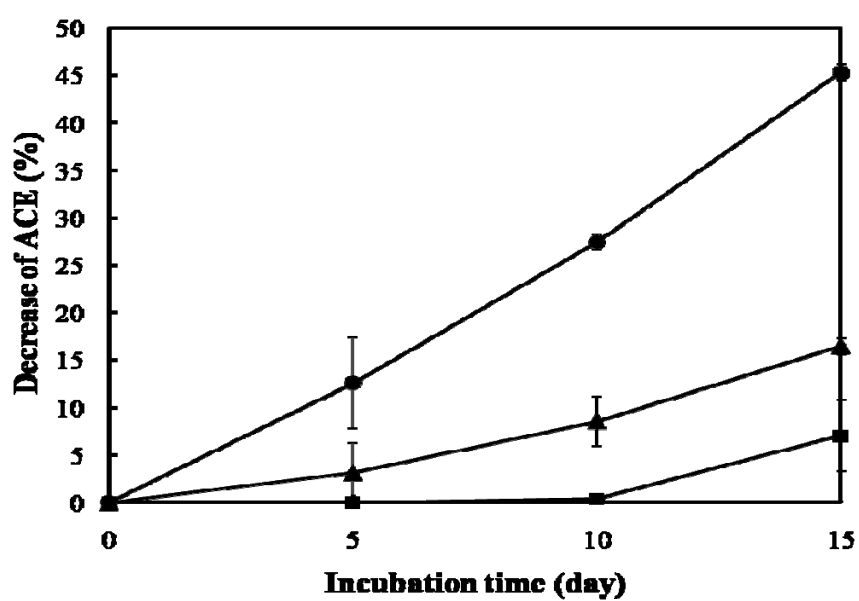
**Fig. 2** Time course for ACE elimination by *P. sordida* YK-624. Closed circles, NL medium; open circles, PDB medium. Values are the means  $\pm$  SD of triplicate samples



**Fig. 3** Detection of the ACE metabolite in the 10-day NL culture fluid by HPLC. These compounds were detected by HPLC under the following conditions: column, Wakosil-II 5C18HG; mobile phase, 30% aqueous MeOH; flow rate, 0.5 mL/min; and detection wavelength, 246 nm.



**Fig. 4** HMBC correlations of the identified ACE metabolite



**Fig. 5** Effect of the cytochrome P450 inhibitor PB on the elimination of ACE by *P. sordida* YK-624 in NL medium. Circles, without PB; triangles, 0.01 mM PB; squares, 0.1 mM PB. Values are the means  $\pm$  SD of triplicate samples

Table 1 NMR data for the metabolite of ACE in CD<sub>3</sub>OD

Position	<sup>1</sup> H	<sup>13</sup> C
	δ <sub>H</sub> (mult, <i>J</i> in Hz)	δ <sub>C</sub>
2	8.22 (d 2.6)	149.9
3	-	124.0
4	7.69 (dd 8.2, 2.6)	139.2
5	7.34 (d 8.2)	132.4
6	-	148.7
7	4.39	41.6
9	-	172.5
11	-	117.5
12	2.21	19.1