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Potency of *Phlebia* species of white rot fungi for the aerobic degradation, transformation and mineralization of lindane[§]

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The widespread use of the organochlorine insecticide lindane in the world has caused serious environmental problems. The main purpose of this paper is to investigate the potency of several *Phlebia* species of white rot fungi to degrade, transform and mineralize lindane, and to provide the feasibility of using white rot fungi for bioremediation at contaminated sites. Based on tolerance experiment results, *Phlebia brevispora* and *Phlebia lindtneri* had the highest tolerance to lindane and were screened by degradation tests. After 25 days of incubation, *P. brevispora* and *P. lindtneri* degraded 87.2 and 73.3% of lindane in low nitrogen medium and 75.8 and 64.9% of lindane in high nitrogen medium, respectively. Several unreported hydroxylation metabolites, including monohydroxylated, dehydroxylated, and trihydroxylated products, were detected and identified by GC/MS as metabolites of lindane. More than 10% of [^{14}C] lindane was mineralized to $^{14}CO_2$ by two fungi after 60 days of incubation, and the mineralization was slightly promoted by the addition of glucose. Additionally, the degradation of lindane and the formation of metabolites were efficiently inhibited by piperonyl butoxide, demonstrating that cytochrome P450 enzymes are involved in the fungal transformation of lindane. The present study showed that *P. brevispora* and *P. lindtneri* were efficient degraders of lindane; hence, they can be applied in the bioremediation process of lindane-contaminated sites.

***Keywords*:** biodegradation, *Phlebia* species, lindane, metabolic pathway, mineralization, cytochrome P450 enzymes

Introduction

From 1950 to 2000, an estimated 600,000 tons of lindane (γ-hexachlorocyclohexane) was produced worldwide for use as an organochlorine insecticide and globally for the control of agricultural pests and disease vector pests (Madaj *et al.*, 2018). The production process of lindane and its extensive use in agriculture are the primary causes of environmental pollution (Kumar and Pannu, 2018). According to a survey, approximately 12–30% of lindane volatilizes from agricultural soil into the atmosphere, where it undergoes long-term and long-distance transport and can be deposited through rainfall (Waite *et al.*, 2001). Lindane is persistent in the environment, has a tendency to bioaccumulate and may be toxic to nontarget organisms including humans (Lal and Saxena, 1982); thus, it was listed as one of the persistent organic pollutants to be reduced and finally eliminated by the Stockholm Convention on Persistent Organic Pollutants (Madaj *et al.*, 2018). Although the use of lindane in agriculture has been banned or restricted in many developed countries due to its toxicities, persistence, and bioaccumulative properties, lindane is still frequently detected in the environment where it is produced and/or used. Moreover, in some developing countries, the insecticide is currently still being produced and permitted to be used restrictively.

The issue of environmental pollution caused by organochlorine pesticides such as lindane has received considerable critical attention. Considering the potential adverse effects of lindane, an effective method for remediation of lindane should be developed. In recent years, the biodegradation of pollutants by bacteria and fungi has received increased attention due to its low cost, environmental friendliness, reduced risks of exposure and public acceptance (Kaur *et al.*, 2016). There are some advantages of using fungi for the biodegradation of pollutants, including their ability to secrete extracellular enzymes, and the large surface area and strong permeability of their mycelia. Among fungi, only white rot species have shown excellent degradation efficiency for a wide variety of recalcitrant pollutants; their extracellular enzymes with low specificity of and their ability to generate colossal mycelial networks are important reasons why white rot species are suitable for bioremediation processes (Asif *et al.*, 2017). Furthermore, white rot fungi are very valuable for removing very low concentrations of a pollutant without any pretreatment because their degradation systems are usually induced by nutrient depletion rather than by specific pollutants.

Previous studies in this area of research have reported the biodegradation of lindane using white rot fungi including *Phanerochaete chrysosporium* (Bumpus *et al.*, 1985; Mougin *et al.*, 1996, 1997), *Pleurotus sajor-caju*, *Pleurotus eryngii* (Arisoy, 1998), *Pleurotus citrinopileatus* (Mohapatra *et al.*, 2012), *Pleurotus florida* (Mohapatra and Pandey, 2015), *Pleurotus ostreatus* (Rigas *et al.*, 2005, 2009; Papadopoulou *et al.*, 2006; Sadiq *et al.*, 2015, 2018), *Cyathus bulleri*, *Trametes hirsutus*, *Phanerochaete sordida* (Singh and Kuhad, 1999, 2000), *Ganoderma australe* (Rigas *et al.*, 2007), *Bjerkandera adusta* (Quin-

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[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956
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tero *et al.*, 2007), *Polyporus ciliates*, *Irpex lacteus* (Quintero *et al.*, 2008), *Trametes versicolor* (Ulčnik *et al.*, 2012, 2013; Sari *et al.*, 2017; Saez *et al.*, 2018), *Hypoxylon fragiforme* (Ulčnik *et al.*, 2012) and *Ganoderma lucidum* (Kaur *et al.*, 2016). The majority of the studies were performed using *P. chrysosporium* and *Pleurotus* strains. In these reports, the ability of different white rot fungi to degrade lindane in liquid medium showed great differences, with degradation rates ranging between more 90 and 10%. Although significant research has been carried out on the efficient removal of lindane by white rot fungi, the degradation pathways and mineralization of lindane by white rot fungi largely remain to be elucidated. Only a few studies on the degradation of lindane using white rot fungi have reported the metabolites and partial degradation pathways of lindane (Mougin *et al.*, 1996; Singh and Kuhad, 1999, 2000). The mechanisms of lindane degradation by white rot fungi seems to be similar, and tetrachlorocyclohexene (TCCH), tetrachlorocyclohexene epoxide (TCCE), and tetrachlorocyclohexenol (TCCH) are the major initial degradation products in these fungi. In a recent study by Sari *et al.* (2017), *T. versicolor* U97 degraded 0.1 and 1.0 mmol/L lindane by approximately 78 and 81% in malt extract liquid medium during a 30-day incubation period, respectively. In *T. versicolor* U97, lindane transformation begins with the dehydrochlorination of lindane to 1,3,4,5,6-pentachlorocyclohexene and ends with the formation of dichlorophenols including 2,4-dichlorophenol and 2,5-dichlorophenol. Currently, information on the complete transformation of lindane using white rot fungal cultures is scarce, and it is still unclear whether the intermediates mentioned above can be further mineralized into CO_2 by these degrading fungi.

In our previous studies, some white rot fungi of the genus *Phlebia* were used to evaluate their potency to degrade organochlorine compounds, including polychlorinated dioxin, polychlorinated biphenyls, polychlorinated naphthalenes and organohalogen pesticides (Mori *et al.*, 2003, 2009; Kamei and Kondo, 2005; Kamei *et al.*, 2006, 2010; Xiao *et al.*, 2011a, 2011b, 2011c; Xiao and Kondo, 2019). It was observed that these *Phlebia* fungi could efficiently degrade and transform these compounds into dechlorinated and hydroxylated products in liquid medium, and cytochrome P450 enzymes play an indispensable role in the transformation of these compounds. Among the *Phlebia* fungi, *Phlebia acanthocystis*, *Phlebia brevispora*, *Phlebia aurea*, and *Phlebia lindtneri* showed the best degradation activity and may represent useful and promising tools for the bioremediation of organochlorine-contaminated environments; however, there is a lack of research on the degradation and transformation of lindane by *Phlebia* species.

Our previous studies have demonstrated that *Phlebia* species possess an extraordinary ability to degrade organochlorine compounds; this result has attracted our attention to the degradation of lindane by these fungi. The main purposes of this study were to evaluate the tolerance, degradation and mineralization ability of *Phlebia* species to lindane in liquid medium and to identify the metabolites of lindane produced from fungal cultures using GC/MS. Additionally, the effects of cytochrome P450 inhibitors on the fungal transformation of lindane were also investigated. Ultimately, we expect to provide candidate fungi for the bioremediation of lindane in contaminated sites.

Materials and Methods

Chemicals

Lindane (98% pure), anhydride, pyridine, and piperonyl butoxide (PB) were purchased from Wako Pure Chemical Industries. A stock solution with a specific activity of 32 kBq/ml was obtained by dissolving labelled [^{14}C] lindane (purity > 98%, Sigma Chemical Co.) in ethanol. All other chemicals and reagents used were of analytical-reagent grade or higher purity.

Fungi and culture conditions

Phlebia acanthocystis TMIC34875 and *Phlebia aurea* TMIC-33908 were obtained from the Tottori Mycological Institute. *Phlebia lindtneri* GB1027 was obtained from the Forest Products Laboratory of the United States Department of Agriculture. *Phlebia brevispora* TMIC34596 was isolated from butt rot of Japanese cypress (Suhara *et al.*, 2002). These fungi were inoculated on potato dextrose agar (PDA) in 9-cm-diameter Petri dish plates and cultured at 30°C for 5 days. Two mycelium mats were transferred from the PDA plates to a blender cup containing 50 ml of sterilized water and were homogenized for 30 sec. Immediately, two ml of this homogenate was then inoculated into 100-ml Erlenmeyer flasks containing 10 ml of low-nitrogen (LN) or high-nitrogen (HN) medium as described by Tien and Kirk (1988). The medium was composed of 1% (w/v) glucose, 20 mmol/L sodium acetate, 0.1% Tween 80, and 1.2 (LN) or 12 mmol/L (HN) ammonium tartrate as a nitrogen source. The fungal cultures were preincubated statically at 30°C in a thermostat.

Tolerance experiments

The tolerance of the four strains to lindane was examined in a 9-cm-diameter Petri dish with PDA solid medium. Solutions of lindane dissolved in acetone were added to PDA medium which was autoclaved (121°C, 20 min) and cooled to 50°C. After volatilization of acetone, 5-mm mycelium plugs were punched from 5 d-old fungal colonies and placed in the centre of a plate containing PDA medium supplemented with lindane at different concentrations. Cultures were incubated at 30°C in darkness, and the fungal colony diameter was measured every day to evaluate the tolerance of fungal strains to different concentrations of lindane until the colony completely covered the plates. Plates without lindane were considered as a control.

Biodegradation experiments

After preincubation in LN and HN media for 5 days, lindane solution was added to *P. lindtneri* and *P. brevispora* cultures to a final concentration of 0.1 mmol/L. Immediately, oxygen was added to the headspace of each flask for 30 sec, and the flasks were sealed with glass stoppers and sealing tape. The cultures were incubated statically at 30°C in dark conditions for 25 days. In the control sample, the fungal cultures were sterilized by autoclaving before adding lindane. Phenanthrene

was added to the cultures as an internal standard to determine the concentration of lindane after incubation. The cultures were homogenized with 20 ml of acetone, and the supernatant obtained after centrifugation for 10 min at 3,000 × *g* was evaporated at 45°C for 10 min to remove acetone. The aqueous phase was acidified to pH 2.0 and extracted three times with 50 ml of ethyl acetate. The residual biomass after centrifugation was also air-dried and extracted using a Soxhlet extractor for 12 h with ethyl acetate. The ethyl acetate extracts were blended and dehydrated with anhydrous sodium sulfate and further concentrated to approximately 0.5 ml under decompression. The acetic anhydride in pyridine was used for acetyl derivatization analysis to investigate the metabolites of lindane, as described previously by Xiao *et al.* (2011a).

GC/MS analysis

Residual lindane and intermediate metabolites from derivatized and underivatized extracts were analysed by gas chromatography/mass spectrometry (GC/MS) performed on an HP 6890 GC system linked to an HP 5973 mass selective detector and a 30-m fused DB-5MS column (0.25 mm × 0.25 μm). Helium was used as a carrier gas at a flow rate of 1.0 ml/min. The initial column temperature started at 80°C, held for 3 min, ramped to 300°C at a rate of 20°C/min, and finally held for 5 min. The injector temperature was set at 250°C, and a 1 μl splitless injection was used. The mass spectra were recorded at 70 eV using the full scan mode.

Mineralization experiments

The fungal culture conditions were the same as described above. After preincubation for 5 days, 50 μl of labelled [^{14}C] lindane stock solution was added as a substrate to each inoculated flask containing 10 ml of fungal culture with LN medium. Incubation was performed in a dark chamber at 30°C. As a control, the fungal cultures were sterilized by autoclaving (121°C, 20 min) to monitor the abiotic degradation of lindane before [^{14}C] lindane was added. The $^{14}CO_2$ was captured using the methods described by Mori and Kondo (2002). Oxygen was used to flush the headspace of each flask every 5 days, and $^{14}CO_2$ was captured in 10 ml of ethanolamine containing scintillation fluid (ethanolamine:methanol:Ultima gold EX = 1:4:5). Captured $^{14}CO_2$ was measured according to the ^{14}C radioactivity on a liquid scintillation counter (Accuflex7400, Aloka).

Cytochrome P450 inhibitor experiments

The inhibition studies of cytochrome P450 monooxygenase (Cyt P450) were conducted as previously described (Xiao and Kondo, 2019). The cultures of white rot fungi were incubated in LN medium at 30°C in the dark for 5 days, and then the Cyt P450 inhibitor piperonyl butoxide (PB) was added at a concentration of 0.2 or 1.0 mmol/L. The flasks were agitated gently to mix the inhibitor solution with the medium and then, incubated for 120 min. Lindane solution was added to the culture, and the final concentration of lindane was 0.1 mmol/L. Subsequently, fungal cultures were further incubated for 20 days, and the recovery of lindane was measured using the above methods. To clarify the effects of PB on lindane degradation, a parallel trial without the addition of PB was also were performed.

All experiments in this study were performed in triplicate, and the results were reported as the average of triplicate determinations.

Results

Fungal tolerance to lindane

In general, the tolerance experiment is the primary method to screen acceptable degrading species for application in bioremediation. The growth of four *Phlebia* strains in PDA medium containing different concentrations of lindane is shown in Fig. 1. As evident from the data, the presence of lindane at a concentration of 1.0 mg/L did not have any adverse effects on the growth of the four fungi compared with the control without lindane. When the concentration of lindane in the PDA medium was 10 mg/L, the colony diameter of the four strains was inhibited by a range of 5.9 to 20.5%, indicating that the transverse growth of all strains was partially inhibited by increasing the lindane concentration. The diameter of the fungal colony in PDA medium containing 50 mg/L lindane was more decreased than that in the medium with 10 mg/L lindane. Although all fungal growth was generally inhibited by the addition of lindane, the fungi showed different tolerances to lindane. Among the four strains, *P. brevispora* and *P. lindtneri* showed relatively higher tolerance to lindane at high concentrations, and their growth was inhibited by less than 30%, while the other two strains *P. acanthocystis* and *P. aurea* were inhibited by 47.7 and 60.3%, respectively.

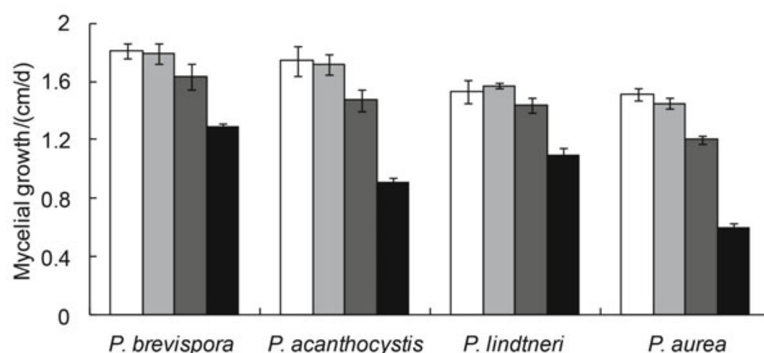

Fig. 1. Effect of lindane on mycelial growth of *P. brevispora* and *P. lindtneri* in PDA medium. White bar = control without lindane, light grey bar = 1 mg/L, dark grey bar = 10 mg/L, and black bar = 50 mg/L. Values are means ± SD of triplicate samples.

Biodegradation of lindane by fungi

P. lindtneri and *P. brevispora* with relatively strong lindane tolerance were investigated for their ability to degrade lindane in liquid cultures. The recovery rate of lindane from the control culture was higher than 97.3% of the initial concentration, which indicated that the loss of lindane, such as abiotic degradation or mycelial adsorption, was not significant in the experiments. It can be clearly seen from Fig. 2 that *P. brevispora* and *P. lindtneri* were able to degrade lindane in both liquid media, although there were differences in the degradation efficiency of lindane between the two fungi. The decrease in lindane in cultures with LN medium was observed after 5 days of incubation, and then lindane continued to be degraded rapidly until the end of incubation, when the degradation rate reached 87.2% for *P. brevispora* and 73.3% for *P. lindtneri*. On the other hand, 75.8 and 64.9% of the initial amount of lindane had disappeared from *P. brevispora* and *P. lindtneri* cultures after 25 days of incubation, respectively, when fungi were cultured in HN medium. These results indicated that these two fungi have the ability to degrade lindane efficiently, and LN medium is more favourable than HN medium for the degradation of lindane by fungi.

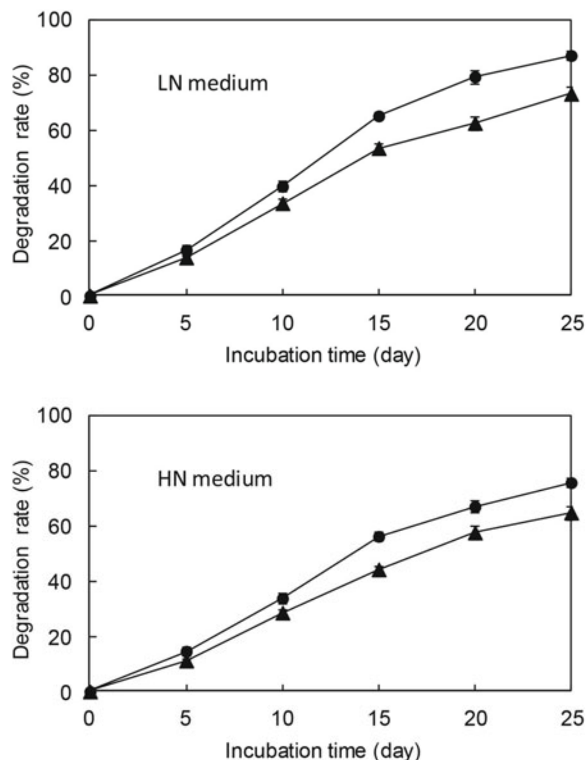

Fig. 2. Biodegradation of lindane by *P. brevispora* (*closed circles*) and *P. lindtneri* (*closed triangles*) in LN and NH media. Values are means ± SD of triplicate samples.

Identification of metabolites by GC/MS

The decrease in lindane concentration was accompanied by the formation of metabolites during the incubation period. The underivatized extracts of two fungal cultures were analysed by GC/MS analysis, and three new compounds were detected (Supplementary data Fig. S1). Among them, tetrachlorocyclohexene (TCCH; Supplementary data Fig. S2) and tetrachlorocyclohexenol (TCCOL; Supplementary data Fig. S3) were identified by comparing the mass spectra with published data (Mougin *et al*., 1996; Singh and Kuhad, 2000). The mass spectrum of another metabolite, which showed ion peaks at *m/z* 199 $[M\text{-}Cl\text{-}HCl]^+$ (Cl = 35), 181 $[M\text{-}2HCl\text{-}OH]^+$, 169 $[C_5H_4Cl_3]^+$, 163 $[199\text{-}HCl]^+$, 156 $[C_4H_3Cl_3]^+$, 135 $[C_5H_5Cl_2]^+$, 109 $[C_3H_3Cl_2]^+$ and 99 $[C_5H_4Cl]^+$ (Fig. 3), matched well with the spectrum of pentachlorocyclohexanol (PCCOL), reported to be formed after the degradation of β-hexachlorocyclohexane in soil and bacteria (Kumar *et al*., 2005; Nagata *et al*., 2005).

Four other metabolites (M1–M4) were detected by GC/MS after the culture extracts were acetyl derivatized. As shown in Fig. 4A, the mass spectrum of acetylated metabolite M1 from cultures of both fungi shows that the heaviest ion peak at *m/z* 301 with three chlorine atoms is an even-electron ion; thus, it is not a molecular ion. The ion peaks at *m/z* 259 and *m/z* 241 have isotopic clusters of three chlorine atoms, which represent the loss of $COCH_2$ and CH_3COOH from the ion peak at *m/z* 301, respectively. The difference in *m/z* 36 between *m/z* 259 and *m/z* 223 and between *m/z* 241 and *m/z* 205 is very typical for the mass spectra of chloroalkanes, and represents a loss of hydrogen chloride molecules. The detection of fragment ions at *m/z* 198 $[241\text{-}COCH_3]^+$, 180 $[223\text{-}COCH_3]^+$, and 163 $[205\text{-}COCH_2]^+$ (or $[198\text{-}HCl]^+$) confirmed the existence of two hydroxyl groups in the molecule of the metabolite. Based on the above analysis, metabolite M1 was tentatively identified as tetrachlorocyclohexanediol (TCCDL), which explains that why the ion peak of

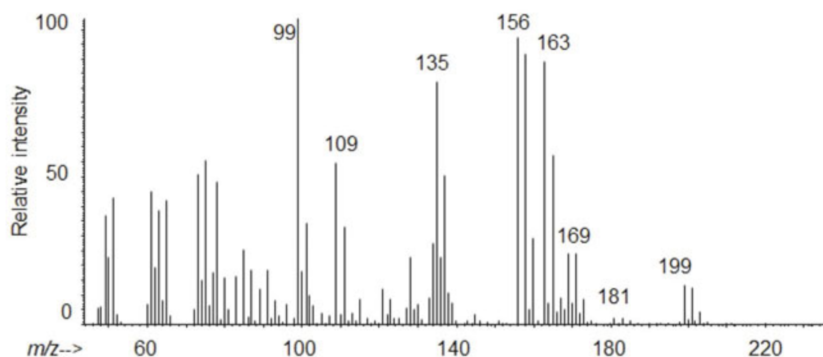

Fig. 3. Mass spectrum of PCCOL from *P. brevispora* and *P. lindtneri* cultures with lindane.

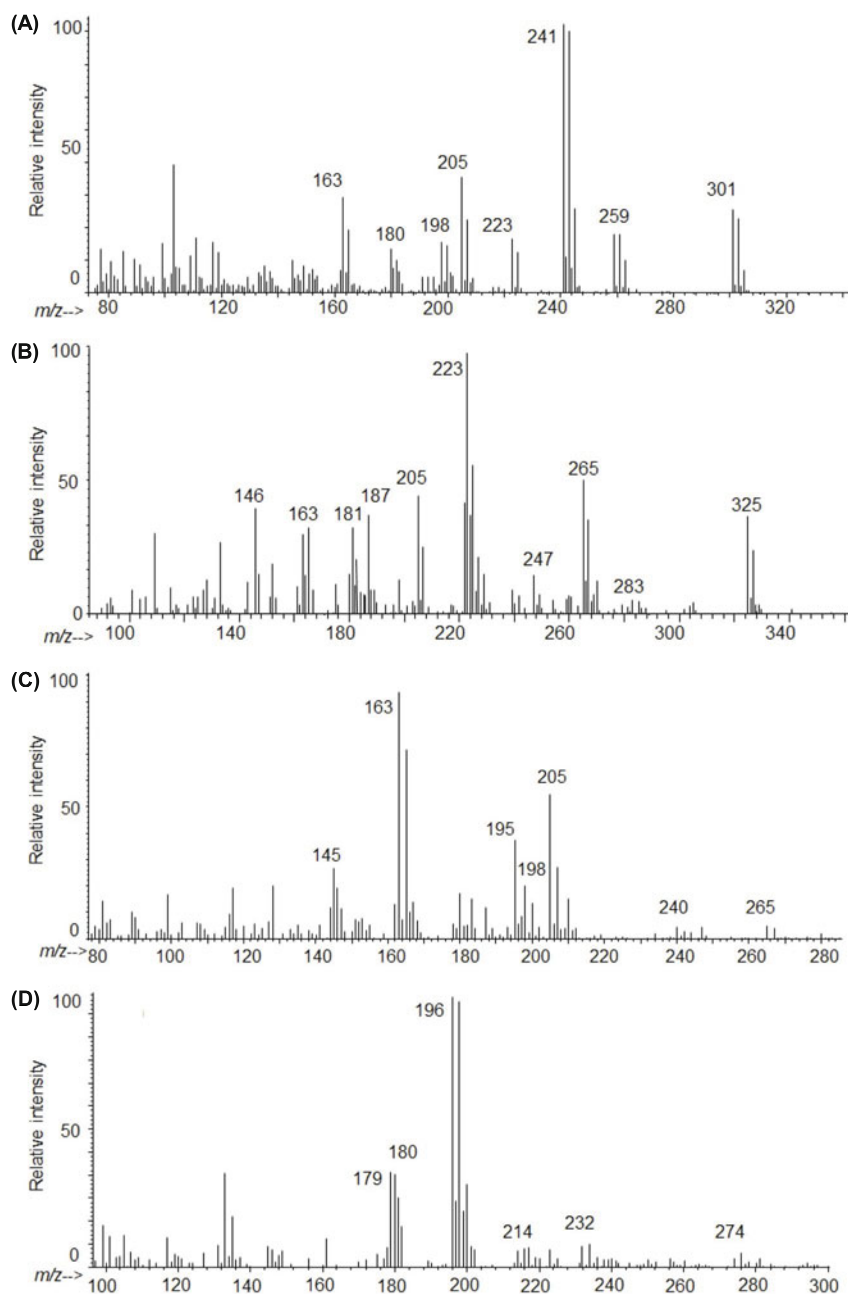

Fig. 4. Mass spectra of acetylated metabolites M1 (A), M2 (B), M3 (C), and M4 (D) from fungal cultures with lindane. Acetylated M1, M2, and M4 were detected from *P. brevispora* and *P. lindtneri* cultures, while acetylated M3 was only detected from *P. brevispora* culture.

m/z 301 was formed by the loss of chlorine atoms from the molecular ion of diacetyl-TCCDL (molecular weight of 336). The acetylated metabolite M2 was also obtained from the two fungal cultures. It can be seen from Fig. 4B that the heaviest ion at *m/z* 325 in the mass spectrum of M2 was an even-electron ion, indicating that the molecular ion peak was not displayed on the mass spectrum. Similar to the ion at *m/z* 325, the major ion at *m/z* 265 and *m/z* 205 also contains the isotopic cluster of two chlorine atoms, and the difference of *m/z* 60 between these three ions can be accounted for by the sequential loss of two CH_3COOH groups from the ion at *m/z* 325. The ions at *m/z* 223 and *m/z* 163 with two chlorine atoms correspond to the loss of $COCH_2$ groups from the ions at *m/z* 265 and *m/z* 205, respectively. Moreover, the loss of HCl and $COCH_2$ groups from the base peak of *m/z* 223 gives rise to the ions at *m/z* 187 and *m/z* 181 with one and two chlorine atoms, respectively. The ions at *m/z* 283 $[325\text{-}COCH_2]^+$, 247 $[283\text{-}HCl]^+$, and 146 $[181\text{-}Cl]^+$ are other noticeable fragment ions that are obvious on the mass spectrum. It can be inferred from the discovery of *m/z* 181, 163, and 146 fragment ions that there are three hydroxyl groups in metabolite M2, which should be trihydroxytrichlorocyclohexane (THTCH). In this way, the ion at *m/z* 325 is generated by the molecular ion of triacetyl-THTCH (molecular weight: 360) through the loss of a chlorine atom.

The acetylated metabolite M3 was only detected in *P. brevispora* culture, and its mass spectrum can be found in Fig. 4C. The mass difference between two weak ions at *m/z* 265

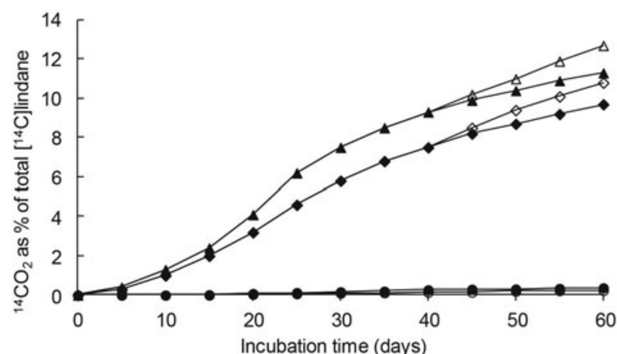

Fig. 5. Release of $^{14}CO_2$ from *P. brevispora* (*triangles*) and *P. lindtneri* (*diamonds*) cultures without glucose supplement (*closed*) and with 1% (w/v) glucose supplement (*open*) within 60 days incubation. Release of $^{14}CO_2$ from the sterilized cultures of *P. brevispora* (*closed circles*) and *P. lindtneri* (*open circles*) are also shown.

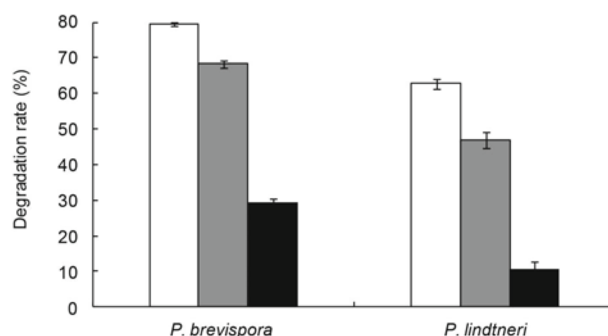

Fig. 6. Effect of Cyt P450 inhibitor PB on fungal degradation of lindane in LN medium after 20 days of incubation. White bar = 0 mmol/L PB, grey bar = 0.2 mmol/L PB, and black bar = 1.0 mmol/L PB. Values are means ± SD of triplicate samples.

and *m/z* 240 was *m/z* 25, which suggests that the ion at *m/z* 265 may represent the loss of a chlorine atom from the molecular ion, while the ion at *m/z* 240 results from the loss of CH_3COOH from the molecular ion, although the molecular ion peak was not observed at *m/z* 300. Other intense ions include *m/z* 205 $[265\text{-}CH_3COOH]^+$ (or $[240\text{-}Cl]^+$), 198 $[240\text{-}COCH_2]^+$, 163 $[205\text{-}COCH_2]^+$ (or $[198\text{-}Cl]^+$), and 145 $[205\text{-}CH_3COOH]^+$ with 2, 3, 2, and 2 chlorine atoms, respectively. The information obtained from the mass spectrum of acetylated metabolite M3 indicates the presence of two oxygen atoms and three chlorine atoms in the molecule, and the most likely structure to account for this spectrum is diacetylated trichlorocyclohexenediol (Tri-CCDL) with *m/z* 300. This estimation would be further supported by the finding of fragment ions at *m/z* 195. The isotope cluster ions have not been found at *m/z* 195, suggesting that there was no chlorine atom in the ion at *m/z* 195, which may result from the loss of three chlorine atoms from the molecule.

As shown in Fig. 4D, the acetylated metabolite M4 from the two fungal cultures showed three weak ion peaks at *m/z* 274, 232, and 214 in its mass spectrum. It is proposed that the loss of $COCH_2$ or CH_3COOH from the ion at *m/z* 274 leads to the formation of the ions at *m/z* 232 and 214, respectively, though it is not possible to determine the number of chlorine atoms in two ions due to low relative intensity. However, the fragment ion cluster at *m/z* 196 clearly showed that they had three chlorine atoms, probably from the ion at *m/z* 232 by the loss of HCl. Furthermore, the ion at *m/z* 214 loses the chlorine atom or HCl, forming the ions at *m/z* 179 or *m/z* 180 with three chlorine atoms in the molecule. Based on these limited mass spectral data, it can be inferred that there the M4 molecule contains one hydroxyl group and four chlorine atoms.

Mineralization of [^{14}C] lindane by fungi

The total evolution of $^{14}CO_2$ from [^{14}C] lindane in LN medium by *P. brevispora* and *P. lindtneri* after 60 days of incubation is shown in Fig. 5. The fungal mineralization of [^{14}C] lindane was detected after 5 days of incubation and peaked between 20 and 25 days of incubation. Subsequently, the formation of $^{14}CO_2$ steadily increased until the end of incubation. After 30 days of incubation, 7.5 and 5.8% of the total [^{14}C] lindane was converted to $^{14}CO_2$ by *P. brevispora* and *P. lindtneri*, while 11.3 and 9.7% of [^{14}C] lindane was mineralized to $^{14}CO_2$, respectively, after 60 days. Mineralization in the sterilized control was only less than 0.37% of total [^{14}C] lindane during the experimental period. Within 60 days of incubation, the mineralization rate was 0.19% of [^{14}C] lindane per day in the *P. brevispora* culture whereas it was 0.16% of [^{14}C] lindane per day in the *P. lindtneri* culture. In addition, considering the decrease in glucose concentration due to fungal consumption during incubation, 1% (w/v) glucose was added to some of the flasks on the 40th day of incubation to investigate the influence of glucose on the mineralization of lindane by fungi. As a result, it was observed that the formation of $^{14}CO_2$ increased by 1.1 to 1.4% in the glucose-supplemented culture of the two species *P. brevispora* and *P. lindtneri*, respectively, compared with the treatment without glucose supplementation at the end of incubation.

Table 1. Effect of Cyt P450 inhibitor PB on the formation of metabolites from fungal cultures after lindane degradation

Strains	Treatments	Formation of metabolites						
		TCCH	TCCOL	PCCOL	M1	M2	M3	M4
P. brevispora	Control	+	+	+	+	+	+	+
	0.2 mmol/L PB	+	+	+	+	–	–	–
	1.0 mmol/L PB	+	–	+	–	–	–	–
P. lindtneri	Control	+	+	+	+	+	–	+
	0.2 mmol/L PB	+	+	+	+	–	–	–
	1.0 mmol/L PB	+	–	–	–	–	–	–

+, detectable ; –, undetectable

Effects of Cyt P450 inhibitor on the biodegradation of lindane

As shown in Fig. 6, the addition of PB effectively inhibited the degradation of lindane by two fungi in LN liquid medium within 20 days. When 0.2 mmol/L PB was added to the fungal cultures, the degradation rates of lindane decreased from 79.5 to 68.3% in the *P. brevispora* culture and from 62.7 to 47.0% in the *P. lindtneri* culture. Moreover, when the PB concentration in the fungal culture was 1 mmol/L, the degradation rate of lindane decreased to only 29.3 and 10.6% in the *P. brevispora* and *P. lindtneri* cultures, respectively, indicating that the inhibition of lindane degradation increased with the increasing PB concentration. In addition, a clear difference was observed in the formation of lindane metabolites between the PB-treated cultures and uninhibited control cultures (Table 1). Especially in fungal cultures with high concentrations (1 mmol/L) of PB, no other metabolites, such as dihydroxylated or trihydroxylated products were detected except TCCH and PCCOL in the *P. brevispora* culture and TCCH in the *P. lindtneri* culture (Supplementary data Figs. S4 and S5). In addition, no effect of PB toxicity on the growth of fungi was found by measuring the mycelial biomass (Supplementary data Fig. S6).

Discussion

White rot fungi are more tolerant to high concentrations of toxic pesticides than other fungi, and hold considerable promise in environmental remediation technology (Sadiq *et al.*, 2018). To develop an effective biological resource to degrade lindane in contaminated sites, this is the first work to screen for degrading strains that can grow efficiently in the presence of high concentrations of lindane. Quintero *et al.* (2008) investigated the tolerance of several white rot fungi to four isomers of hexachlorocyclohexane and found that the inhibition rate of mycelial growth of *Phlebia radiata* and *P. sordida* by 10 mg/L lindane was 60%, while that of *P. ciliates* and *B. adusta* by lindane of the same concentration was 40%. Dritsa and Rigas (2013) reported that the growth rate of *P. ostreatus* in PDA medium containing 20 mg/L lindane decreased to less than half of that in the control without lindane. In the present study, the tolerance of four strains of *Phlebia* white rot fungi were validated by comparing their colony diameters in solid medium containing different concentrations of lindane. It has been proposed that the presence of lindane induces the production of protective proteins to counteract the adverse effects of lindane on the growth of fungi (Kaur *et al.*, 2016). In fact, the concentration of lindane in the actual environment is much lower than that of lindane used in this study. For example, Gao *et al.* (2008) reported that the concentration range of lindane in the surface water of China, including seven major river basins, is 0.17 ng/L to 368 ng/L, with an average concentration of 31.3 ng/L. Thus, *P. brevispora* and *P. lindtneri* with high tolerance to 1.0 and 10 mg/L lindane appear to be useful as tools for subsequent research, including the degradation or mineralization of lindane.

The earliest research on the degradation of lindane using white rot fungi was reported by Bumpus *et al.* (1985). Over the next three decades, a number of studies have been car-

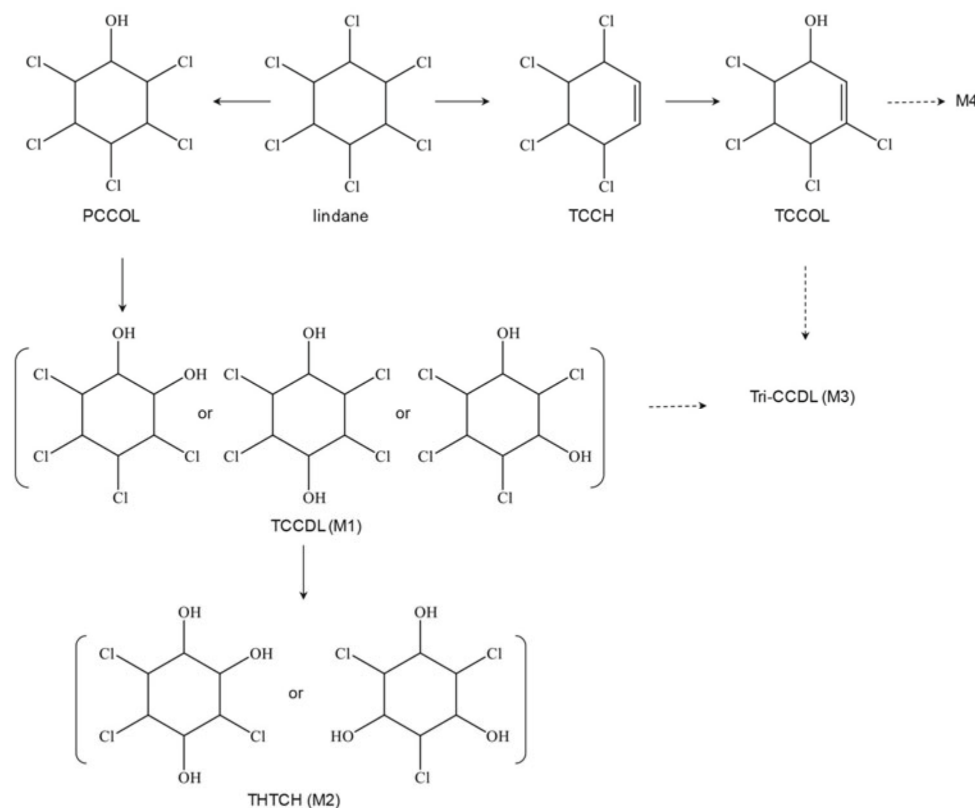

Fig. 7. Proposed partial pathways for degradation of lindane by *P. brevispora* and *P. lindtneri*. The dashed arrows represent possible metabolic routes.

ried out on the degradation of lindane using white rot fungi (Singh *et al*., 2000; Phillips *et al*., 2005; Girish and Kunhi, 2013). The complete degradation of lindane by all four cultures of *Pleurotus* species was observed irrespective of the concentration after 30 days (Mohapatra *et al*., 2012). Ulčnik *et al*. (2012) reported that over 90% of lindane was degraded in the liquid cultures by *T. versicolor*, *H. fragiforme*, and *P. ostreatus* after 21 days of exposure. Recently, lindane degradation was studied by using *G. lucidum* grown on rice bran substrate in liquid fermentation, and the maximum degradation of 75.5% was obtained after 28 days of incubation (Kaur *et al*., 2016). However, studies on the metabolic pathway of lindane by white rot fungi are still lacking. In this study, a part of lindane was converted into TCCH by a reductive dechlorination reaction and then hydroxylated to TCCOL as the first route in the metabolic pathway of lindane by selected fungi. The same pathway was reported to be confirmed in *P. chrysosporium* (Mougin *et al*., 1996), *T. hirsutus*, *C. bulleri*, and *P. sordida* (Singh and Kuhad, 1999, 2000), suggesting that this pathway might be common in white rot fungi. Interestingly, a novel second route for lindane transformation was found in our study. It is clear from the present results that *Phlebia* strains convert a part of lindane to form PCCOL by hydroxylation as first reaction step and, in a second step, to TCCDL, which continues to undergo a hydroxylation reaction to form THTCH. This indicates that in *P. brevispora* and *P. lindtneri*, chlorine atoms at carbon ring of the lindane molecule are continuously substituted by hydroxyl groups to produce polyhydroxylated products. The degradation reactions that successively hydroxylated both β- and δ-hexachlorocyclohexane to the respective TCCDL isomers were also described in the soil microorganism *Sphingobium indicum* B90A (Sharma *et al*., 2006; Raina *et al*., 2007). However, the second route found in this study has not been previously reported in similar studies. Another noticeable finding from the detection of metabolites was the formation of Tri-CCDL (M3) and M4. There are two possible routes for the formation of Tri-CCDL: by dehydrochlorination of TCCDL or hydroxylation of TCCOL. The latter reaction was also observed in the degradation pathway of lindane by the haloalkane dehalogenase linB from the soil bacterium *S. indicum* B90A (Raina *et al*., 2008). Although the mass spectral data alone do not prove the structures of metabolite M4 unequivocally, it seems likely that M4 is produced by the dehydrogenation of TCCOL in the first route of the metabolic pathway of lindane because these compounds have the same number of hydroxyl and chlorine atoms, and the molecular weight (232) of M4 is only two less than that of TCCOL. Based on the information obtained from the results, it is considered that the metabolic pathway of lindane in *P. brevispora* and *P. lindtneri* is similar and is proposed in Fig. 7.

As is well known, the continuous introduction of hydroxyl groups can increase the hydrophilicity and bioavailability of compounds, and polyhydroxylated compounds are unstable and may be further degraded or even mineralized. It is worth mentioning that this inference is partially confirmed by the results of mineralization experiments in the present study, which showed that approximately 10% of ^{14}C was detected from fungal cultures with [^{14}C] lindane within 60 days. From this result, it is clear that the two fungi can continuously mineralize lindane into CO_2 in LN medium during the incubation period. A few studies on the mineralization of lindane by white rot fungi have focused only on *P. chrysosporum*. An early study confirmed that in a nitrogen-deficient, ligninolytic culture of *P. chrysosporum* containing 1.25 nmol of ^{14}C-labeled lindane, 190.8 pmol of lindane was converted to $^{14}CO_2$ during a 30-days incubation period (Bumpus *et al*., 1985). After that, Kennedy *et al*. (1990) observed a significant mineralization of lindane in liquid cultures and soil-corncob cultures inoculated with *P. chrysospo*rium under ligninolytic conditions. Mougin *et al*. (1996) reported that between 3.0 and 4.5% of lindane is mineralized by *P. chrysosporum* in liquid culture during 14 days of incubation. They further reported that the mineralization rate of lindane increased from 21.6 to 49.1% within 9 weeks after inoculating this strain into non-sterilized soil (Mougin *et al*., 1997). In comparison, the lindane mineralization ability of *P. brevispora* and *P. lindtneri* in liquid culture seems to be similar to that of *P. chrysosporum*. Meanwhile, it was demonstrated in this study that the supplementation of glucose in fungal cultures could significantly stimulated the production of $^{14}CO_2$. This shows that glucose in a suitable carbon source for both *Phlebia* strains and may be one of the important factors for fungal mineralization of lindane in environment or biological treatment systems. A similar phenomenon was also reported by Xu and Wang (2014), who pointed out that glucose not only is a good carbon source for fungal growth but also could markedly promote the degradation of decabromodiphenyl ether by *P. lindtneri*. The effects of carbon sources on lindane mineralization can be summarized in two reasons: one is to promote fungal growth and enhance the degradation of lindane, and the other is to act as a co-substrate in cometabolism by inducing certain enzymatic reaction (Hadibarata and Kristanti, 2012).

It should be mentioned that extracellular ligninolytic enzymes were thought to be expressed in the secondary metabolism process of white rot fungi, and this process is triggered by nitrogen concentration. Generally, the activity of ligninolytic enzymes is stimulated by nitrogen limitation and is inhibited under high-nitrogen conditions. Surprisingly, the degradation of lindane was not significantly affected by the amount of nitrogen in fungal cultures in our experiment. It is presumed, therefore, that the degradation of lindane is catalyzed by enzymes that are not regulated by nitrogen concentration and/or have no direct relationship with extracellular ligninolytic enzymes. This finding further supports the ideas of Mougin *et al*. (1996), who claimed that the peroxidases from *P. chrysosporium* were not involved in the transformation of lindane. In contrast, different conclusions have also been reported. Rigas *et al*. (2005) thought that manganese peroxidase and laccase were related to the highest biodegradation of lindane in *P. ostreatus.* Studies carried out by Singh and Kuhad (1999, 2000) pointed out that at least the initial steps in lindane degradation do not involve intracellular enzyme systems because lindane was only found in mycelial extracts of fungi. Sadiq *et al*. (2015) found that the degradation efficacy of lindane was different between local and exotic strains of *P. ostreatus*, which could be linked with lignolytic enzyme production ability.

On the other hand, the involvement of Cyt P450 in the

degradation of lindane by *P. brevispora* and *P. lindtneri* has been confirmed in our experiments by the fact that the addition of PB strongly inhibited lindane degradation as well as the formation of hydroxylated metabolites. This indicates that one or more steps of lindane transformation, such as hydroxylation reactions in the second route, are catalysed by Cyt P450 systems of selected fungi. This is not a surprising result because Cyt P450 is known to be responsible for the transformation of many kinds of refractory organic pollutants, such as polychlorinated naphthalenes, DDT, chlordane and endrin, in white rot fungi, including *P. brevispora* and *P. lindtneri* (Mori *et al*., 2009; Xiao *et al*., 2011b, 2011c; Xiao and Kondo, 2019). For example, the results offered by Mori *et al*. (2009) suggest that the formation of hydroxylated products from the degradation of polychlorinated naphthalenes by *P. lindtneri* was mostly performed by Cyt P450 monooxygenase. Additionally, Nakamura *et al*. (2012) isolated three Cyt P450 monooxygenase genes from *P. brevispora* using reverse transcription PCR and further found that one of these genes was upregulated upon exposure to PCBs and polychlorinated dioxin. Furthermore, the involvement of P450 in lindane degradation was found not only in *Phlebia* species but also in other white rot fungi. As reported by Mougin *et al*. (1996), 1-aminobenzotriazole, another Cyt P450 inhibitor, drastically reduced lindane degradation by *P. chrysosporium*.

In conclusion, *P. brevispora* and *P. lindtneri* were able to tolerate high concentrations of lindane and transform lindane into dechlorinated and hydroxylated metabolites under aerobic conditions. A novel metabolic pathway of lindane in two *Phlebia* strains was proposed in this study. It has also been demonstrated that two fungi can mineralize lindane into carbon dioxide, and Cyt P450 is a critical enzyme responsible for lindane transformation. Our preliminary results indicate that two *Phlebia* strains are promising tools for degrading lindane as well as other insecticides, which is beneficial to the development of bioremediation methods.

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Conflict of Interest

The authors declare no competing financial interests to this work.

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