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ORIGINAL ARTICLE

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Degradation of endosulfan and endosulfan sulfate by white-rot fungus *Trametes hirsuta*

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Abstract Endosulfan, an organochlorine insecticide, and its metabolite endosulfan sulfate are persistent in environments and are considered toxic. We investigate the possible nontoxic bioremediation of endosulfan. An endosulfan-degrading fungus that does not produce endosulfan sulfate was selected from eight species of white-rot fungi. High degradation of endosulfan and low accumulation of endosulfan sulfate were found in cultures of *Trametes hirsuta*. A degradation experiment using endosulfan sulfate as the substrate revealed that *T. hirsuta* is able to further degrade endosulfan sulfate following the oxidative conversion of endosulfan to endosulfan sulfate. Endosulfan and endosulfan sulfate were converted to several metabolites via hydrolytic pathways. In addition, endosulfan dimethylene, previously reported as a metabolite of the soil bacterium *Arthrobacter* sp., was detected in *T. hirsuta* culture containing endosulfan sulfate. Our results suggest that *T. hirsuta* has multiple pathways for the degradation of endosulfan and endosulfan sulfate and thus has great potential for use as a biocatalyst in endosulfan bioremediation.

Key words Endosulfan · Endosulfan sulfate · Biodegradation · White-rot fungi

Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzodioxyanthiepin-3,-oxide) is a cyclodiene insecticide that exhibits a relatively broad spectrum of activity. This insecticide has been used extensively for over 30 years on a variety of vegetables, fruit, cereals, and cotton, as well as on shrubs, trees, vines, and ornamentals.[1] Endosulfan is extremely toxic to fish and other aquatic organisms and affects the central nervous system, kidney, liver, blood chemistry, and parathyroid gland; it has reproductive, teratogenic, and mutagenic effects.[2] Endosulfan is applied as a technical mixture of α and β isomers at a ratio of 7:3.[1] Upon entering the environment α- and β-endosulfan are primarily converted to the sulfate form in the soil, and remain as significant residues.[3] Endosulfan sulfate is the only breakdown product considered toxic.[3]

White-rot fungi can degrade lignin, a complex high-molecular-weight aromatic polymer, and a wide spectrum of recalcitrant organopollutants, including polychlorinated biphenyls (PCBs),[4–6] polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs).[7–10] Kullman and Matsumura[11] studied the metabolism of endosulfan by the model white-rot fungus *Phanerochaete chrysosporium*; in this study, the rapid disappearance of endosulfan was observed, as well as detection of several endosulfan metabolites: endosulfan sulfate, endosulfan diol, endosulfan hydroxyether, and endosulfan dialdehyde. Kullman and Matsumura suggested that the metabolism of endosulfan is mediated by two divergent pathways: one hydrolytic and the other oxidative. The end product of the oxidative pathway is endosulfan sulfate; because endosulfan sulfate is more toxic and more persistent than its parent endosulfan, its accumulation presents a bioremediation challenge. Several fungi are reported to degrade endosulfan; however, there is currently no evidence of fungal degradation of endosulfan sulfate.[12–14]

Our aim was to select a white-rot fungus able to degrade endosulfan without accumulation of the toxic endosulfan sulfate. We measured the selected fungus for its ability to degrade endosulfan sulfate, and proposed several metabolic pathways by which endosulfan and endosulfan sulfate may be metabolized.

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Materials and methods

Chemicals

α-Endosulfan, β-endosulfan, and endosulfan sulfate (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3,3-dioxide) were purchased from Wako Pure Chemical Industries (Osaka, Japan), and endosulfan diol (1,4,5,6,7,7-hexachlorobicyclo[2.2.1]hept-5-ene-2,3-dimethanol), endosulfan ether (4,5,6,7,8,8-hexachloro-1,3,3a,4,7,7a-hexahydro-4,7-methanoisobenzofuran), and endosulfan lactone (4,5,6,7,8,8-hexachloro-4,7-methano1,3,3a,4,7,7a-hexahydroisobenzofuran-1-one) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Microorganisms and culture conditions

We selected fungi that are frequently used for the degradation of organic pollutants: *Phanerochaete chrysosporium* ATCC34541 (American Type Culture Collection, Manassas, VA, USA), *Trametes versicolor* IFO6482, *Trametes hirsuta* IFO4917 (Institute for Fermentation, Osaka, Japan), *Phlebia lindtneri* GB1027 (United States Department of Agriculture, Washington, DC, USA), *Phlebia brevispora* TMIC33929 (Tottori Mycological Institute, Tottori, Japan), *Ceriporia lacerata* MZ340, *Pycnoporus coccineus*, and *Phlebia* sp. MG-60 (Laboratory of Systematic Forest and Forest Products Sciences, Kyushu University, Fukuoka, Japan). These fungi were maintained on potato dextrose agar (PDA; Difco, Detroit, MI, USA).

Degradation experiments

Degradation experiments were carried out as described previously.[15] *P. chrysosporium*, *Ph. lindtneri*, *Ph. brevispora,* and *Phlebia* sp. MG-60 were incubated on potato dextrose agar plates at 30°C, and other fungi were incubated on malt extract agar plates at 25°C. Six-millimeter-diameter disks were punched from the edge of each mycelium. Five disks of each fungus were placed in a 100-ml Erlenmeyer flask containing 10 ml of nitrogen-limited medium.[16] Cultures were incubated statically at 30°C under ambient atmospheric conditions. After 5 days, 10 µl of 10 mM substrate *N*,*N*-dimethylformamide solution plus an endosulfan chemical were added to each inoculated flask (for screening: α-endosulfan and β-endosulfan mixture; for metabolic experiments: α-endosulfan, endosulfan sulfate, endosulfan diol, endosulfan ether, or endosulfan lactone; final concentrations 10 µM). The headspace of each flask was flushed with oxygen, sealed with a glass stopper and sealing tape, and then incubated statically at 30°C. After additional incubation, cultures were killed by adding about 0.2 g of sodium azide. As a control, cultures were killed by adding about 0.2 g of sodium azide after an initial 5 days of incubation before addition of substrate, and then incubated under the same condition as for the treatment culture. To determine the concentration of each substrate, an internal standard (pentachloronitrobenzene) was added to the sodium azide-killed cultures and the cultures were homogenized with 15 ml of acetone using a polytron PT2100 homogenizer (Kinematica, Switzerland). The biomass was removed by centrifugation at 3000 *g* for 10 min at room temperature. One milliliter of supernatant was extracted by 1 ml of *n*-hexane and analyzed by gas chromatography using an electron capture detector (GC-ECD) (Hewlett-Packard HP 6890 GC system). A HP-50+ column (Hewlett-Packard, 0.53 mm inside diameter, 15 m length, 1 µm film thickness) was used and the oven temperature was programmed to increase from 100°C to 290°C at 20°C min^{-1}. The recovery rates of endosulfan and endosulfan sulfate from the culture without mycelium were measured and were compared with those from control cultures containing azide-killed mycelium that were pre-incubated for 5 days. The recovery of each substrate from both sets of cultures was found to be greater than 90%, which indicated that the extraction was efficient, the analysis deviation was small, and endosulfan and endosulfan sulfate absorption on the fungal cells was negligible.

Metabolite detection

The whole homogenate was acidified to pH 2.0 with 0.1 N HCl and then shaken vigorously with 10 ml of acetone–hexane (20:80 vol/vol) in a glass centrifuge tube. After the water and organic fraction were separated by centrifugation at 3000 *g* for 10 min at room temperature, the organic fraction was collected in another glass tube. This extraction procedure was repeated three times. The residual water-soluble fraction was re-extracted by ethyl acetate three times. The collected organic fractions were combined and were concentrated by a N_2 stream and analyzed by gas chromatography-mass spectrometry (GC-MS) performed on an Agilent HP 6890 GC system linked to an Agilent HP 5973 MSD mass selective detector spectrometer and a 30-m fused DB-5ms GC column (Agilent JW Scientific, Folson, CA, USA). The oven temperature was programmed to increase from 120°C to 320°C at 10°C min^{-1}. Mass spectra were recorded at 70 eV using the full scan mode.

Results and discussion

Selection of white-rot fungus able to degrade endosulfan without accumulation of toxic endosulfan sulfate

Figure 1 shows results of degradation of the mixture of α- and β-endosulfan and the accumulation of endosulfan sulfate. After 14 days of incubation, the initial endosulfans were degraded by all the fungal species tested. Over 90% of the endosulfan initially present was degraded by *T. hirsuta*, *Ph. lindtneri*, *Ph. brevispora*, and *C. lacerata*. This indicates that many fungi in addition to *P. chrysosporium* have the ability to degrade endosulfan. However, the accumulation of toxic endosulfan sulfate was observed for

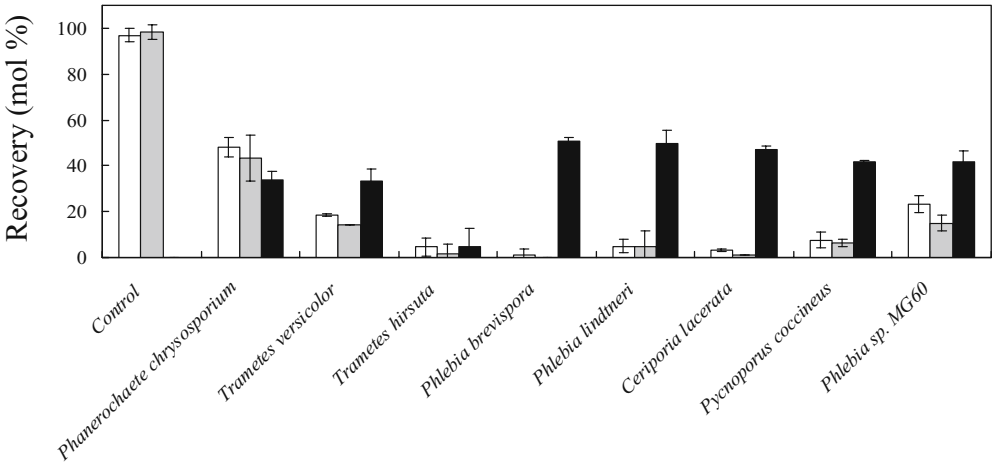

Fig. 1. Degradation of endosulfan isomers and accumulation of toxic metabolite endosulfan sulfate by white-rot fungi after a 10-day incubation period. Recovery rate of α-endosulfan (*open columns*), β-endosulfan (*gray columns*), and metabolite endosulfan sulfate (*closed columns*) from cultures of selected white-rot fungi. Values are means ± SD of triplicate samples

almost all the tested fungi, including *P. chrysosporium* (20–30 mol% of parent endosulfans). In contrast, endosulfan sulfate was found at relatively low levels in the culture of *T. hirsuta* (approximately 2% of parent endosulfans). This suggests that *T. hirsuta* uses a different mechanism to degrade endosulfan, without the accumulation of endosulfan sulfate. Endosulfan has been shown to be microbially degraded by two separate pathways: hydrolytic and oxidative.[11] Endosulfan sulfate is produced via the oxidative pathway. Therefore, for *T. hirsuta,* either the hydrolytic pathway is quantitatively dominant compared with the oxidative pathway, or this fungus is able to further degrade endosulfan sulfate. The time course of the degradation of α-endosulfan and the production of endosulfan sulfate is shown in Fig. 2A. Endosulfan sulfate accumulated during the first 7 days of incubation; however, after 7 days, the parent endosulfan had almost disappeared and endosulfan sulfate levels had also decreased (Fig. 2A). Figure 2B compares the ability of *P. chrysosporium* and *T. hirsuta* to degrade endosulfan sulfate. When *P. chrysosporium* was incubated with endosulfan sulfate, there was no degradation, just as in the case of incubation with an azide-killed mycelium. Our result confirms the previous research that showed that endosulfan sulfate is the terminal end product in endosulfan degradation by *P. chrysosporium*.[11] However, in the *T. hirsuta* culture, endosulfan sulfate was drastically degraded, decreasing approximately 70% within a 10 day incubation period (Fig. 2B). This indicates that *T. hirsuta* further degrades endosulfan sulfate following the initial oxidative conversion of endosulfan.

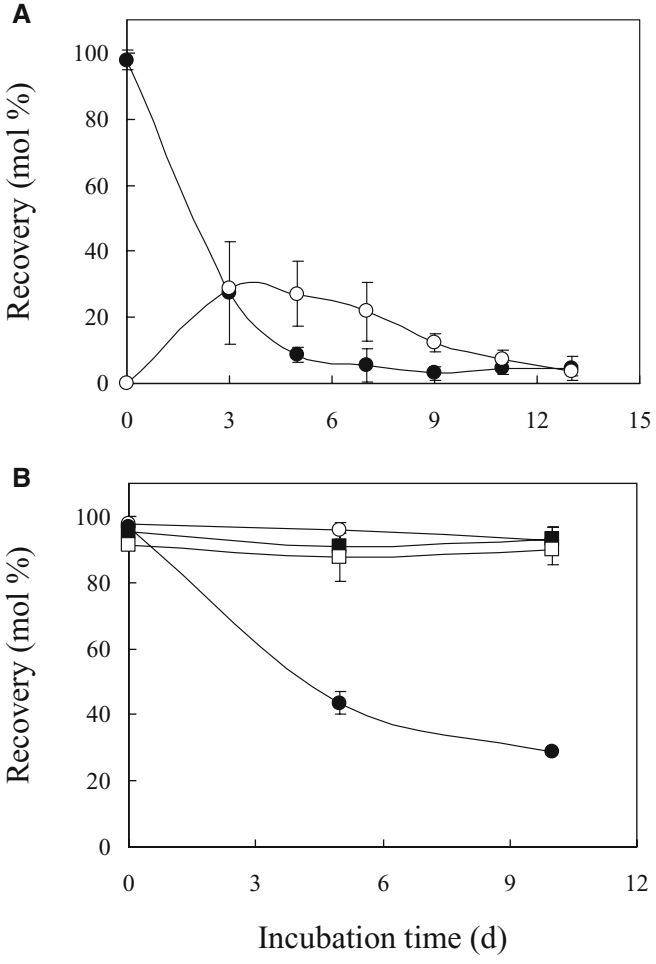

Fig. 2. Time course of degradation of α-endosulfan and endosulfan sulfate in *Trametes hirsuta* cultures. **A** Recoveries of α-endosulfan (*closed circles*) and endosulfan sulfate (*open circles*) are shown. **B** Recovery of endosulfan sulfate in the culture with *Phanerochaete chrysosporium* (*closed squares*), *T. hirsuta* (*closed circles*), and their azide-killed mycelia (*open symbols*). Values are means ± SD of triplicate samples

Metabolites of endosulfan and endosulfan sulfate in *T. hirsuta* culture

Several other metabolites were detected by GC-ECD and GC-MS analysis. From *T. hirsuta* culture with α-endosulfan, the known metabolites endosulfan diol, endosulfan ether, and endosulfan lactone were detected (Table 1). These metabolites have previously been observed during endosulfan degradation by *P. chrysosporium*.[11] Thus, both hydrolytic and oxidative pathways exist in *T. hirsuta* also. When each

Table 1. Metabolism of endosulfan and individual endosulfan metabolites

Original substrate	Formation of metabolic products					
	Diol	Ether	Lactone	Putative hydroxyether	Sulfate	Putative dimethylene
Endosulfan	+	+	+	–	+	+
Endosulfan diol		+	+	–	–	–
Endosulfan ether	–		+	+	–	–
Endosulfan sulfate	+	+	+	–		+

Each individual substrate was purchased from a commercial source and incubated in nitrogen-deficient cultures of *T. hirsuta* at 30°C for 2–3 days
+, formation of product; –, no product formation

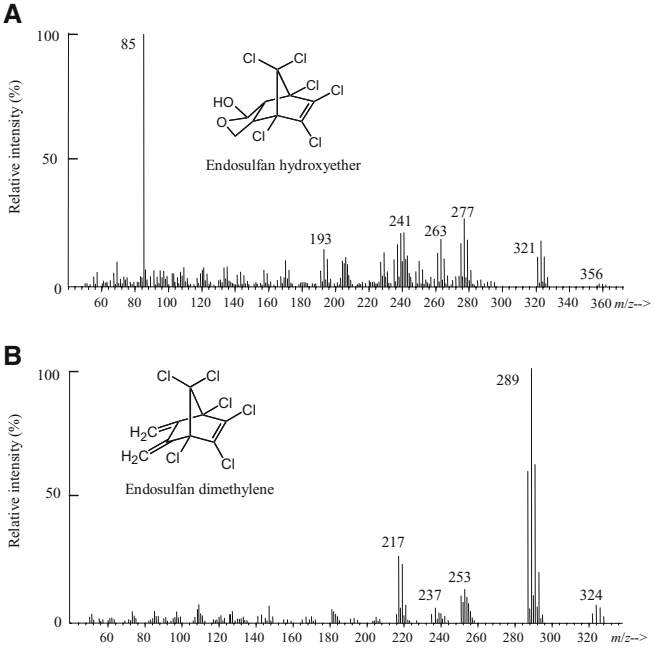

Fig. 3. Metabolites of endosulfan sulfate in *T. hirsuta* cultures. Mass spectra of the metabolites and their corresponding structure are shown

metabolite was used as a parent substrate, each was degraded effectively, and some intermediates were detected. The metabolites detected are summarized in Table 1. In the culture containing endosulfan diol as a parent substrate, two compounds, endosulfan ether and endosulfan lactone, were detected as metabolites. In the culture containing endosulfan ether, endosulfan lactone and putative endosulfan hydroxyether (1,3,3a,4,7,7a-hexahydro-4,5,6,7,8,8-hexachloro-4,7-methanoisobenzofuran-1-ol) were detected. The mass spectrum of the putative endosulfan hydroxyether is shown in Fig. 3A. The mass spectrum of this compound has molecular ion peaks at *m*/*z* 356 (molecular mass of endosulfan ether [340]+16 mass), and 321 (M^+–Cl), suggesting the introduction of a hydroxyl group into the endosulfan ether. The longer retention time of this compound (13.47 min) compared with the retention time of endosulfan ether (11.91 min) also supports the introduction of a polar group into the endosulfan ether. These properties agree with the chemical information given for endosulfan hydroxyether previously.[11] Again this indicates that *T. hirsuta* possesses similar metabolic pathways to *P. chrysosporium*. The greatest difference in degradation abilities between *P. chrysosporium* and *T. hirsuta* is in the ability to degrade endosulfan sulfate. While *P. chrysosporium* appears not to metabolize endosulfan sulfate, *T. hirsuta* cultures show rapid degradation of endosulfan sulfate and production of endosulfan diol, endosulfan ether, and endosulfan lactone (Table 1). This indicates that *T. hirsuta* degrades endosulfan sulfate by hydrolytic pathways. Production of endosulfan diol via hydrolysis may be an important detoxification pathway of endosulfan and endosulfan sulfate. Endosulfan diol is nontoxic to fish and other organisms, and can be further degraded to nontoxic endosulfan ether, endosulfan hydroxyether, and endosulfan lactone. Thus, bioconversion of endosulfan sulfate to endosulfan diol has definite advantages in the development of bioremediation techniques. Few reports document microbial hydrolysis of endosulfan sulfate to endosulfan diol. Transformation of endosulfan sulfate to endosulfan diol has been observed in mixed cultures of soil microorganisms;[17] the microorganisms, however, were not specified. *Klebsiella oxytoca* KE-8 has the ability to degrade endosulfan sulfate, and a trace amount of endosulfan diol was detected as a metabolite.[18] A later article reported the isolation of a soil bacterium, *Pseudomonas* sp. strain IITR01, capable of degrading α-endosulfan and endosulfan sulfate.[19] These are the only two reports of identified bacterial strains that are capable of transforming endosulfan sulfate to endosulfan diol. Endosulfan degradation by several fungi, namely, *Trichoderma harzianum*,[12] *Aspergillus sydoni*,[13] *Aspergillus niger*,[20] *Mucor thermohyalospora*,[21] and *Mortierella* species,[14] has also been reported. However, as with *P. chrysosporium*, the metabolic pathways described did not include the degradation of endosulfan sulfate. Our research is the first reported simultaneous conversion and detoxification of endosulfan and endosulfan sulfate by a fungus.

In addition, we detected a metabolite that was not reported for *P. chrysosporium*.[11] The mass spectrum of this compound is shown in Fig. 3B. The mass spectrum has a base peak of *m*/*z* 289 and a molecular ion peak of *m*/*z* 322, identical to that described for 1,2,3,4,7,7-hexachloro-5,6-bis(methylene) bicyclo[2.2.1]-2-heptane (endosulfan dimethylene).[22] Endosulfan dimethylene was detected in the culture containing endosulfan and endosulfan sulfate (Table 1). This metabolite was not detected in the culture containing endosulfan diol and endosulfan ether, which are

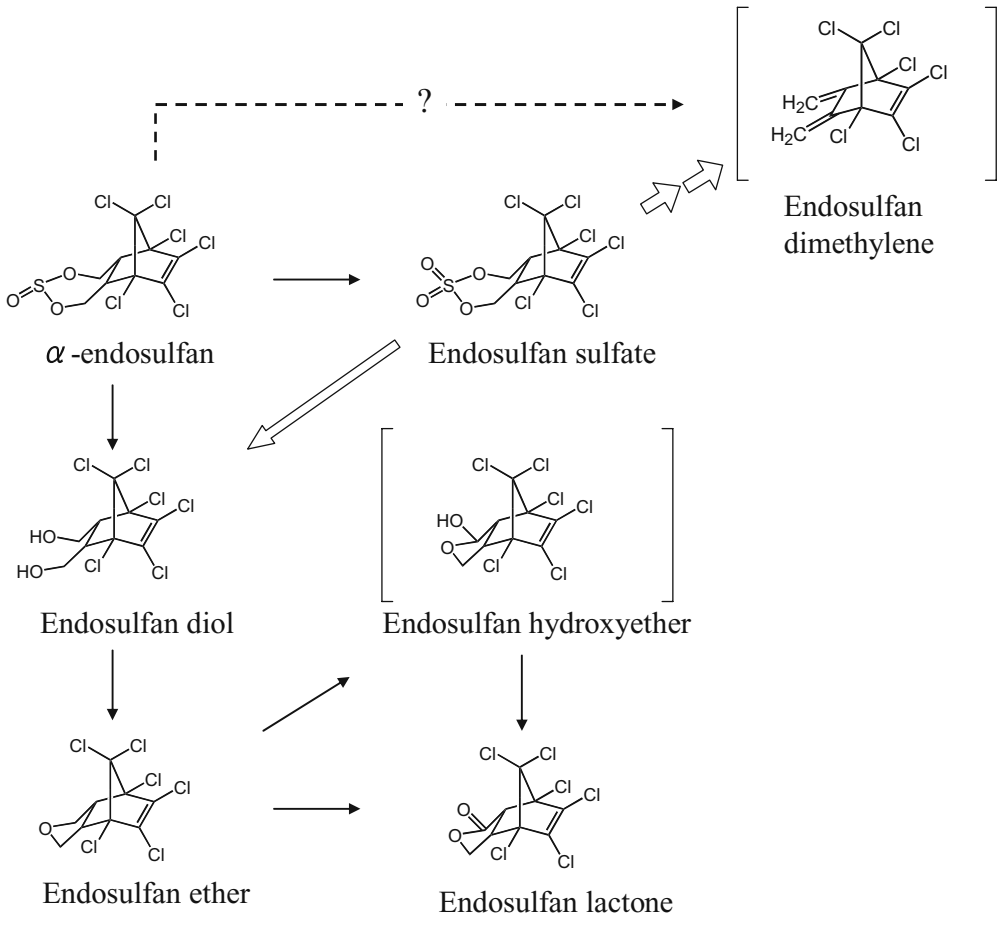

Fig. 4. Proposed pathways for the degradation of α-endosulfan by *T. hirsuta*. *Black arrows* indicate pathways identical to those of *P. chrysosporium,* and *open arrows* indicate the newly clarified pathways for *T. hirsuta*. The *dotted arrow* indicates a pathway that remains uncertain. *Brackets* indicate suggested structures by mass spectra and information from the literature

intermediates in the hydrolytic pathway (Table 1). This suggests that the endosulfan dimethylene-producing pathway is independent of the hydrolytic pathway that produces endosulfan diol. Sutherland and co-workers[22] reported the detection of endosulfan dimethylene from a mixed microbial culture capable of degrading endosulfan sulfate. The isolation of esd and ese monooxygenases has been reported from *Mycobacterium* sp. strain ESD and *Arthrobacter* sp. strain KW; esd and ese are two-component flavin-dependent monooxygenases involved in the degradation of endosulfan.[23,24] Ese monooxygenase can degrade endosulfan sulfate to endosulfan monoalcohol and endosulfan dimethylene, via endosulfan hemisulfate.[24] This oxidative degradation pathway is separate from the oxidative formation of endosulfan sulfate from endosulfan.[24] Because we detected endosulfan dimethylene not only from the culture containing endosulfan sulfate but also from that containing α-endosulfan, we do not know whether endosulfan dimethylene is produced directly from α-endosulfan without endosulfan sulfate production. For *Arthrobacter* sp. strain KW, endosulfan hemisulfate and endosulfan monoalcohol were detected as well as endosulfan dimethylene.[24] However, these metabolites were not detected in our *T. hirsuta* cultures.

These results suggest that the white-rot fungus *T. hirsuta* has three independent pathways for the degradation of endosulfan. Putative metabolic pathways are shown in Fig. 4. One of the degradation pathways is the hydrolytic production of endosulfan diol from endosulfan. Second is the hydrolytic production of endosulfan diol followed the oxidative production of endosulfan sulfate. Third, *T. hirsuta* has another mechanism producing endosulfan dimethylene from endosulfan sulfate. Although the enzyme(s) involved in the formation of endosulfan dimethylene are unknown, we strongly suggest that *T. hirsuta* has an additional degradation pathway resembling the oxygenation of endosulfan sulfate by *Arthrobacter* sp. strain KW.[24] The evidence for this is that endosulfan dimethylene was not detected in the culture with the hydrolytic metabolite endosulfan diol.

We thus selected *T. hirsuta* as a good degrader of endosulfan and endosulfan sulfate. Metabolic pathways include the hydrolytic degradation of endosulfan sulfate following the oxidation of endosulfan. We detected a metabolite, endosulfan dimethylene, from the culture containing endosulfan sulfate. This metabolite has not previously been described in fungal metabolism. Our results suggest that *T. hirsuta* has multiple pathways for the degradation of endosulfan and endosulfan sulfate (Fig. 4), and that it has potential for use as a biocatalyst in endosulfan bioremediation.

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