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Transformation of diphenyl ethers by *Trametes versicolor* and characterization of ring cleavage products

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Abstract

The white-rot fungi *Trametes versicolor* SBUG 1050, DSM 11269 and DSM 11309 are able to oxidize diphenyl ether and its halogenated derivatives 4-bromo- and 4-chlorodiphenyl ether. The products formed from diphenyl ether were 2- and 4-hydroxydiphenyl ether. Both 4-bromo- and 4-chlorodiphenyl ether were transformed to the corresponding products hydroxylated at the non-halogenated ring. Additionally, ring-cleavage products were detected by high perfomance liquid chromatography and characterized by gas chromatography/mass spectrometry and proton nuclear magnetic resonance spectroscopy. Unhalogenated diphenyl ether was degraded to 2-hydroxy-4-phenoxymuconic acid and 6-carboxy-4-phenoxy-2-pyrone. Brominated derivatives of both these compounds were formed from 4-bromodiphenyl ether, and 4-chlorodiphenyl ether was transformed in the same way to the analogous chlorinated ring cleavage products. Additionally, 4-bromo- and 4-chlorophenol were detected as intermediates from 4-bromo- and 4-chlorodiphenyl ether, respectively. In the presence of the cytochrome-P450 inhibitor 1-aminobenzotriazole, no metabolites were formed by cells of *Trametes versicolor* from the diphenyl ethers investigated. Cell-free supernatants of whole cultures with high laccase and manganese peroxidase activities were not able to transform the unhydroxylated diphenyl ethers used.

Introduction

In recent years, halogenated diphenyl ethers have become increasingly important environmental pollutants. Chlorinated diphenyl ethers are formed as by-products during the synthesis of chlorinated phenols and phenoxy acids (Ahlborg & Thunberg 1980). Furthermore, brominated diphenyl ethers are used extensively as flame retardants in electronic equipment. Since their first commercial introduction in the 1970s, the concentrations of brominated diphenyl ethers in the environment have risen sharply. High concentrations were detected in sea sediments, sewage sludge and animal tissue (Pijnenburg et al. 1995). Up to now, most research has dealt with degradation of diphenyl ethers by bacteria (Takase et al. 1986, Pfeifer et al. 1989, 1993; Schmidt et al. 1992, 1993). Only a few reports exist on the transformation of these compounds by fungi (Seiglé-Murandi et al. 1991; Schauer et al. 1995). There are also naturally occuring halogenated diphenyl ether derivatives, which are synthesized mainly by fungi and marine organisms (Elyakov et al. 1991; Unson et al. 1994, Takahashi et al. 1992; Takahashi et al. 1993).

White-rot fungi have been investigated extensively for their ability to transform xenobiotics. In this study the biotransformation of diphenyl ether and its halogenated derivatives 4-bromo- and 4-chlorodiphenyl ether as model compounds for polyhalogenated diphenyl ethers was investigated using strains of the white-rot fungus *Trametes versicolor.* The abilities of the ligninolytic enzyme system to degrade these compounds were considered.

In the investigation of pollutant degradation by white-rot fungi the role of ligninolytic enzymes including manganese peroxidases, lignin peroxidases and laccases is a question of considerable interest. In contrast to what is known about some polycyclic aro-

matic hydrocarbons (Bumpus 1989; Hammel 1992; Bezalel et al. 1996; Collins et al. 1996; Kotterman et al. 1998) little or no information exists concerning their abilities for the degradation of biarylic compounds like polychlorinated biphenyls, diphenyl ethers or dibenzo-*p*-dioxins (Bumpus et al. 1985, Valli et al. 1992). Obviously, in addition to the action of extracellular ligninolytic enzymes, intracellular processes also seem to be of importance for the biotransformation of these compounds by white rot fungi (Dietrich et al. 1995, Takada et al. 1996).

Material and methods

Microorganisms, growth and culture conditions

The strain *Trametes versicolor* SBUG-M 1050 was from the Culture Collection of the University of Greifswald, Germany. The strains *Trametes versicolor* DSM 11269 and *Trametes versicolor* DSM 11309 used were purchased from the German Culture Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig).

For cultivation, a nitrogen-rich (8mM) medium (NRM) was used. Its composition was: glucose 5 g, KH_2PO_4 0.5 g, L-asparagine 0.52 g, yeast extract 0.5 g, KCl 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $FeSO_4$ 0.01 g, deionized water 950 ml. 50 ml of a mineral salt solution were added containing $Mn(CH_3COO)_2{\cdot}4H_2O$ 8 mg $ZnSO_4{\cdot}7H_2O$ 2 mg, $Ca(NO_3)_2{\cdot}4H_2O$ 50 mg, $CuSO_4{\cdot}5H_2O$ 3mg. The pH of the NRM-medium was 4.5. The diphenyl ethers to be investigated were pipetted as a solution in acetone (0.25 mM w/v) into 100 ml Erlenmeyer flasks. The solvent was evaporated at room temperature for 12 h. Afterwards, the flasks were filled with 40 ml NRM containing 0.05% (w/v) Tween 80 and inoculated with 3 ml of homogenized mycelium (IKA Ultra-Turrax, Janke & Kunkel, Staufen, Germany) of a pre-culture which had been grown in NRM for 10 days under static conditions. After inoculation the cultures were incubated at 30 °C on a rotary shaker at 160 rpm.

Laccase activity of the pre-culture was induced by adding 20 mM xylidine. For studying the ability of laccase to transform diphenyl ethers, 2 ml samples of culture supernatants with a laccase activity of 2,5 units ml^{-1} and no detectable manganese peroxidase activity were put into vials and 3 ml sodium acetate buffer (0.1 M, pH 5) was added. Supernatants with high manganese peroxidase activities were obtained from cultures without addition of xylidine. Samples of 2 ml of supernatant with a manganese peroxidase activity of 0.1 units ml^{-1} and a laccase acitivity of 0.05 units ml^{-1} was put in vials together with 3 ml sodium tartrate buffer (0.05 M, pH 5), 10 mM $MnSO_4$ and 1 mM H_2O_2. The vials were incubated at 30 °C for 72 hours and then treated in the same way as in the experiments done with whole cells.

For investigating the degradation of diphenyl ether by laccase in the presence of the mediator ABTS (2,2-azino-bi-(3-ethyl-benzthiazolin-sulfonate)) partially purified laccase, obtained as described by Jonas et al. (1998), was used. Diphenyl ether diluted in dimethylformamide was added to a final concentration of 3 mM in sodium acetate buffer (0.1 M) containing 0.1 units ml^{-1} of laccase activity. The assay was incubated for 72 h. Afterwards, the aqueous supernatants were analyzed by HPLC as described in the text.

The cytochrome-P450 inhibitor 1-aminobenzotriazole was added to a final concentration of 0.25 mM directly to the cultures at the beginning of the incubation.

Enzyme assays

Laccase activity was determined spectrophotometrically at 420 nm with ABTS as substrate (Bourbonnais & Paice 1990) using the method described by Jonas et al. (1998). One unit was defined as 1 μmol of ABTS oxidized per min.

Manganese peroxidase activity was measured according to the method of Akamatsu & Shimada (1996) with a reaction mixture containing $MnSO_4$ (3.3 mM), H_2O_2 (0.25 mM), sodium tartrate buffer (50 mM, pH 4.5) and a sufficient amount of supernatant. The increase in absorbance at 264 nm due to the formation of Mn(III)-ions was measured (ϵ_{264} = 6 mM^{-1} cm^{-1}) and one unit of manganese peroxidase was defined as 1 μmol of Mn^{3+} formed per min.

Lignin peroxidase was measured by following the oxidation of veratryl alcohol to veratrylaldehyde as described by Kirk et al. (1990).

Analytical methods

Analysis of metabolites was done by HPLC for aqueous culture supernatants. RP (Reverse phase)-Analytical HPLC was carried out onto a Hewlett-Packard 1050 Series pump system and a 1040 M Series I Diode array detector. Data analysis was done with a Hewlett Packard HPLC Chemstation (Hewlett Packard, Waldbronn, Germany). A solvent system

of methanol and phosphoric acid (0.1% w/v) was used with a linear gradient from 30% methanol and 70% phosphoric acid to 100% methanol (flow rate: 1 ml/min) within 16 min.

Purification of metabolites was performed on a Merck-Hitachi HPLC system equipped with a Model L 6200 A Intelligent Pump, a Rheodyne 7161 injection valve with a 100μl-loop and a Model L-4250 absorbance detector operating at 254 nm. A solvent system of methanol and acetic acid (0.1%) was used with the same conditions described for the analytical HPLC system. For both HPLC systems a RP 18 column (Lichro Cart 125-4, 5 μm, endcapped, Merck, Darmstadt, Germany) was used.

For gas chromatographic analysis whole cultures were extracted without a previous centrifugation step. It was necessary to extract the cells also because of the adsorption of the metabolites investigated to the fungal mycelia. After 7 days of incubation, the cultures were extracted with ethyl acetate at pH 7 and once again after acidification of the aqueous residue to pH 2. The resulting organic phases were dried over anhydrous sodium sulfate and evaporated with a vacuum evaporator at 50 °C. Finally, the extracts were evaporated to dryness under a stream of nitrogen.

Gas chromatography/mass spectrometry (GC/MS) was done using a Fisons GC 8000 gaschromatograph (Fisons, Mainz-Kastel, Germany) equipped with a DB-5-MS fused silica capillary column (J & W Scientific, Folsom, California) and coupled with a Fisons MD 800 EI mass selective detector. Measurements were performed under the following conditions using helium as carrier gas: injector temperature 240 °C, temperature program: 80 °C to 300 °C at 8 °C/min, 10 min 300 °C.

Non-volatile compounds were converted to their methylated derivatives (De Boer & Backer 1956) using a methylation apparatus with diazomethane as methylating agent (Aldrich, Steinheim, Germany).

Proton nuclear magnetic resonance (^{1}H-NMR-) spectroscopy was carried out using a Bruker ARX 300 MHz spectrometer (Bruker, Karlsruhe, Germany), deuterated methanol as solvent and tetramethyl silane as reference.

Chemicals

Diphenyl ether (>98%), 4-chloro- (99%) and 4-bromodiphenyl ether (>99%) as well as 1-aminobenzotriazole were purchased from Aldrich. ABTS (2,2-azino-bi-(3-ethyl-benzthiazolin-sulfonate)) was obtained from Boehringer (Ingelheim, Germany). Tween 80 was obtained from Serva Company Ltd. (Heidelberg, Germany). All solvents were of a purity suitable for chromatography.

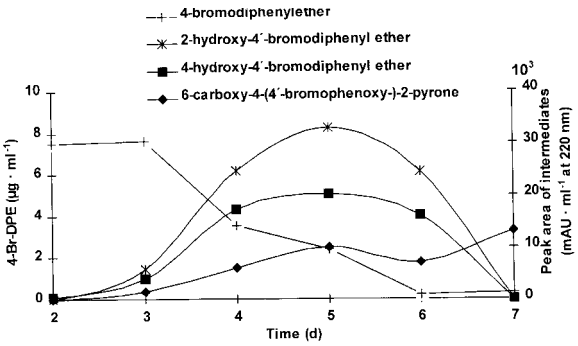

Figure 1. Formation of products from 4-bromodiphenyl ether in cultures of *Trametes versicolor* SBUG-M 1050.

Results

All strains of *Trametes versicolor* investigated formed the same pattern of metabolites from diphenyl ether, 4-chloro- and 4-bromodiphenyl ether. Thus, the ability of *Trametes versicolor* to transform diphenyl ethers is not strain-specific. For more detailed studies of the transformation products the strain SBUG-M 1050 was used.

By comparing their HPLC and GC-MS-data with those of authentic standards, two products formed from diphenyl ether were identified as 2- and 4-hydroxydiphenyl ether in supernatants as well as in neutral extracts. It was calculated that at least 20% of the concentration of diphenyl ether added to the cultures was converted to the monohydroxylated products 2- and 4-hydroxydiphenyl ether. The halogenated diphenyl ethers investigated were metabolized to the corresponding monohydroxylated products 2-hydroxy-4′-bromo- and 4-hydroxy-4′-bromo-diphenyl ether or 2-hydroxy-4′-chloro- and 4-hydroxy-4′-chlorodiphenyl ether. During further incubation, the monohydroxylated diphenyl ethers disappeared again from the supernatants (Figure 1) and were not detectable by HPLC after seven days, but low quantities could be identified in the ethylacetate extracts.

Furthermore, low pH ethylacetate extracts of cultures exposed to the investigated diphenyl ethers were analyzed by GC-MS. Two additional metabolites A and B from each diphenyl ether used were found in

the low pH extracts. For these intermediates, GC-MS was successful only after methylation. After methylation, product A, which was formed from diphenyl ether, had a molecular ion peak at m/z 246 and remarkable fragments at m/z 218 (M^+-CO), 187 (M^+-$COOCH_3$), 159 (M^+-$COOCH_3$-CO), 93 (C_6H_5O), 77 (C_6H_5), and 59 ($COOCH_3$). These data are consistent with 6-carboxy-4-phenoxy-2-pyrone, the lactone of 2-hydroxy-4-phenoxymuconic acid. This product was already characterized as metabolite formed from diphenyl ether by *Trichosporon* spec. SBUG 752 (Schauer et al. 1995).

Product B formed from nonhalogenated diphenyl ether had after methylation a molecular ion peak at m/z 292 (Figure 2A). The base peak of m/z 233 resulted in the loss of $COOCH_3$ from the molecular ion. This and the occurrence of a m/z 59 fragment indicated the formation of a carboxylic acid as an intermediate. The ^{1}H-NMR spectrum (Figure 2B) of the non-methylated product B showed 7 proton signals, five of them being quite similar to those of the non-substituted parent compound diphenyl ether or 6-carboxy-4-phenoxy-2-pyrone (Schauer et al. 1995). The spin-spin coupled signals at δ = 5.31 (d, 1) and δ = 6.90 (d, 1) indicated two aliphatic methine protons. The low coupling constant $J_{3,5}$ of 2.3 Hz point to a long-range coupling of these protons. Because of the mass, ^{1}H-NMR spectral data and the fact that both products A and B can be chemically transformed to each other, product B can be assumed to be the ring cleavage product 2-hydroxy-4-phenoxy muconic acid.

Two further intermediates (C and D) resulting from aromatic ring cleavage were also obtained from 4-bromodiphenyl ether. Like for diphenyl ether, they could be detected by GC-MS only after methylation. Due to their characteristic mass spectra resulting from bromine and chlorine substituents, products from 4-bromo- and 4-chlorodiphenyl ether were easy to detect by GC-MS.

Product C formed from 4-bromodiphenyl ether had after methylation a molecular ion peak at m/z 324 and remarkable fragment ions at 296 (M^+-CO) 265 (M^+-$COOCH_3$), 172 (C_6H_5OBr) 155 (C_6H_4Br) and 59 ($COOCH_3$, Figure 3). Because of the similarities in the behaviour of the compound in the analytical procedures and the fragmentation pattern of the mass spectrum to that of the lactone formed from unsubstituted diphenyl ether it was concluded that it was 6-carboxy-4-(4′-bromophenoxy)-2-pyrone, the lactone of 4-(4′-bromophenoxy)-2-hydroxymuconic acid.

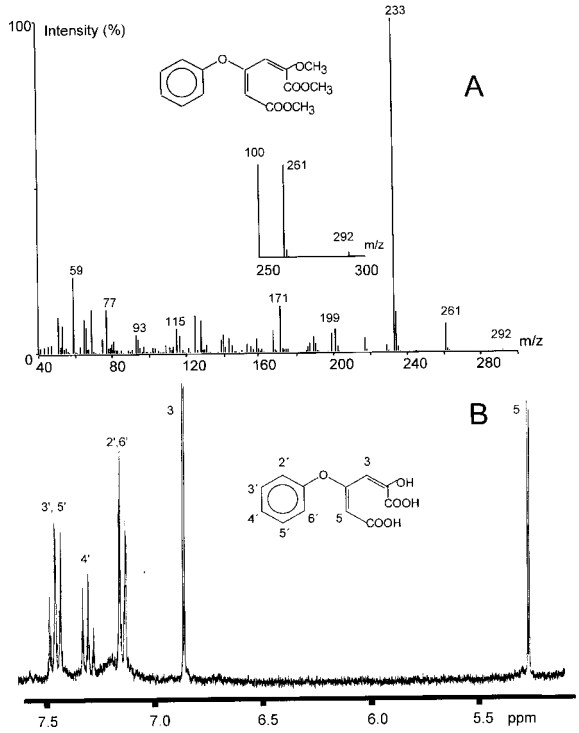

Figure 2. Mass spectrum of methylated 2-hydroxy-4-phenoxymuconic acid (A) and ^{1}H-NMR spectrum of the non-methylated derivative (B) formed from diphenyl ether by *Trametes versicolor.*

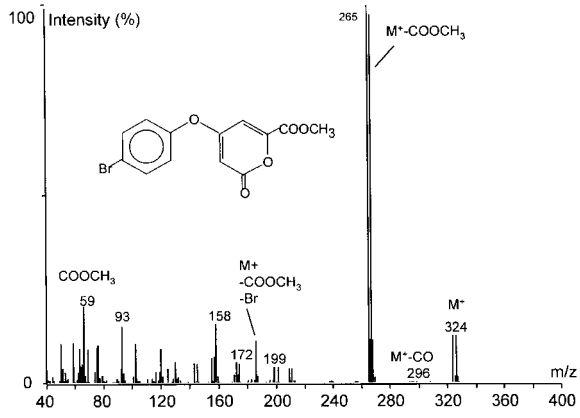

Figure 3. Mass spectrum of methylated 6-carboxy-4-(4′-bromophenoxy)-2-pyrone, the lactone of 4-(4′-bromophenoxy)-2-hydroxymuconic acid formed from 4-bromodiphenyl ether by *Trametes versicolor.*

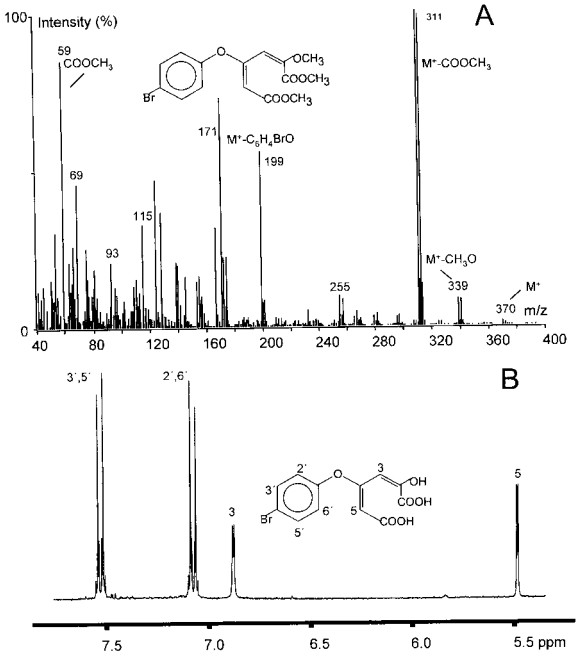

Figure 4. Mass spectrum of methylated 4-(4′-bromophenoxy)-2-hydroxymuconic acid (A) and ^{1}H-NMR spectrum of the non-methylated derivative (B) formed from 4-bromodiphenyl ether by *Trametes versicolor.*

The mass spectrum of product D showed a molecular ion at m/z 370 and a base peak of m/z 311 indicating the loss of $COOCH_3$. Further important fragment ions were detected at m/z 339 (M^+-CH_3O), 255 (M^+-$C_3O_3HCH_3$), 199 (M^+-C_6H_5O) and 172 (C_6H_5OBr, Figure 4A). The signals in the ^{1}H-NMR spectrum (Figure 4B) were like those measured for 2-hydroxy-4-phenoxymuconic acid, except that the signals of aromatic protons were those of a para-substituted aromatic ring (Figure 4B). From these results it was concluded that product D was 4-(4′-bromophenoxy)-2-hydroxymuconic acid, the brominated ring-cleavage product analogous to that formed from unbrominated diphenyl ether. This product was stable to acidic conditions (0.5 mg of product in 1 ml of 25% HCl was shaken at room temperature for 24 h) but unstable on alkaline hydrolysis (0.5 mg of product in 1 ml of 40% KOH was shaken at room temperature for 24 h), which resulted in the formation of 4-bromophenol. These findings are consistent with those obtained from 2-phenoxymuconic acid as a similar bacterial metabolite from diphenyl ether by Takase et al. (1986).

From 4-chlorodiphenyl ether the products 4-(4′-chlorophenoxy)-2-hydroxymuconic acid and 6-carboxy-4-(4′-chlorophenoxy)-2-pyrone were formed by cleavage of the non-halogenated ring. After GC-MS analysis of the extracts also 4-bromophenol was found as metabolite from 4-bromodiphenyl ether as well as 4-chlorophenol from 4-chlorodiphenyl ether.

During incubation with 4-bromodiphenyl ether in the presence of the cytochrome-P450-inhibitor 1-aminobenzotriazole no metabolites were formed although the fungus was able to grow in the presence of 0.25 mM of 1-aminobenzotriazole.

Trametes versicolor secreted laccase and manganese peroxidase but no lignin peroxidase into the supernatant. Laccase activity was enhanced distinctly in the presence of diphenyl ether, 4-bromo- and 4-chlorodiphenyl ether. Nevertheless, supernatants of cultures of *Trametes versicolor* showing a manganese peroxidase activitity (0.1 units ml^{-1}) and a laccase activity (2,5 units ml^{-1}) were not able to transform any of the diphenyl ethers used. No products were formed during an incubation period of 72 hours. Even in the presence of the laccase mediator ABTS no products were formed from diphenyl ether by laccase as described for anthracene by Johannes et al. (1996).

Discussion

The white-rot fungus *Trametes versicolor* is able to transform diphenyl ether and its monohalogenated derivatives 4-bromo- and 4-chlorodiphenyl ether. After hydroxylation reactions a ring-cleavage step occurs which leads to a phenoxymuconic acid derivative and its corresponding lactone (Figure 5). The structure of the degradation products suggests that *Trametes versicolor* metabolizes diphenyl ethers in the same way as the yeast *Trichosporon* spec. SBUG 752 (Schauer et al. 1995). This yeast strain, which does not express any ligninolytic enzymes, oxidizes diphenyl ether via hydroxylation up to the dihydroxylated and presumably to the trihydroxylated product (Figure 5). The latter is finally cleaved by an *ortho*-fission of the aromatic ring. Like *Trichosporon* spec. SBUG 752 *Trametes versicolor* degrades diphenyl ether to 6-carboxy-4-phenoxy-2-pyrone, the lactone of 2-hydroxy-4-phenoxymuconic acid. In the experiments presented here additionally 2-hydroxy-4-phenoxymuconic acid, which was hitherto not detected as an intermediate, was also found in extracts of cultures incubated with diphenyl ether.

Diphenyl ethers halogenated in the *para*-position were transformed to the corresponding ring cleavage products by cleavage of the non-halogenated ring. The

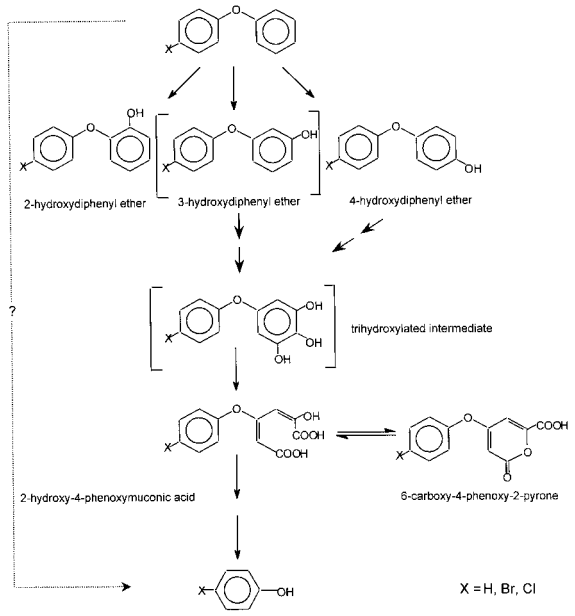

Figure 5. Proposed pathway for the degradation of diphenyl ethers by *Trametes versicolor*.

fact that only the non-halogenated ring of 4-bromo- and 4-chlorodiphenyl ether is cleaved by *Trametes versicolor* may support the hypothesis that a trihydroxylated compound with hydroxyl groups lying next to each other is required as precursor of the ring cleavage product. If the aromatic ring of the diphenyl ether is halogenated in the *para*-(4-)-position, three hydroxyl groups cannot be placed next to each other without dehalogenation. However, for more detailed studies concerning this problem, investigations with diphenyl ethers halogenated in *ortho*-position are necessary. The route of diphenyl ether degradation as found for a yeast (Schauer et al. 1995) and presented in this paper seems to be similar both in nonligninolytic as well as in white-rot fungi and may be therefore performed by nonligninolytic enzymes.

Trichosporon spec. SBUG 752 oxidizes non-halogenated diphenyl ether efficiently. However, from 4-chlorodiphenyl ether only much smaller amounts of monohydroxylated products were formed (Henning 1993). In contrast to these results, cells of the investigated strains of *Trametes versicolor* formed ring-cleavage products both from diphenyl ether, 4-bromo and 4-chlorodiphenyl ether. No products were formed from 4-bromodiphenyl ether in the presence of the strong cytochrome P-450 inhibitor 1-aminobenzotriazole. Supernatants containing high manganese peroxidase and laccase activities were not able to transform 4-bromodiphenyl ether either. These results may suggest that at least the initial hydroxylation steps of diphenyl ether transformation may be performed by intracellular, nonligninolytic enzyme systems under the given conditions. This notion is supported by the fact that biarylic ether compounds like diphenyl ethers in general have high oxidation potentials. Hammel et al. (1986) investigated the oxidation abilities of lignin peroxidase for polycyclic aromatic hydrocarbons and found that only PAHs with an ionization potential lower than 7.55 eV were modified. The ionization potential for diphenyl ether was determined to be 8.09 eV (Lide 1995) and the values for its halogenated derivatives might be even higher. By the Mn(II)/Mn(III)-reaction catalyzed by manganese peroxidase PAHs with a ionization potential of 7.7 eV or lower are oxidized (Cavalieri & Rogan 1985). Laccases degrade only compounds with even lower ionization potentials (De Jong et al. 1994).

In some cases authors could show that in the presence of specific mediators, aromatic compounds with higher oxidation potentials can be oxidized by laccases (Collins et al. 1996; Johannes et al. 1996) and manganese peroxidases (Scheibner et al. 1997), too. Collins et al. (1996) incubated PAHs with laccases from *Trametes versicolor* and the laccase mediator ABTS. However, they found that even in the presence of this mediator compound only PAH with an ionization potential lower than 7.45 eV were oxidized. Thus, it seems understandable that also diphenyl ether with its relatively high ionization potenial is not attacked under the conditions we used.

Yadav et al. (1995) observed extensive degradation of Arochlor 1242 and 1254 by *Phanerochaete chrysosporium* both in non-ligninolytic as well as in ligninolytic defined medium. Krĉmar & Ulrich (1998) observed no degradation of lower-chlorinated and higher-chlorinated biphenyl (PCB) congeners by *Phanerochaete chrysosporium* when incubated in a nitrogen-limited medium. Nevertheless, they observed a significant PCB degradation in a non-limited medium. All the results we obtained point to a transformation catalyzed by nonligninolytic enzymes.

Phenol and its halogenated derivatives 4-bromo- and 4-chlorophenol are formed from the diphenyl ethers used by *Trametes versicolor*. This fact may suggest that further degradation of the diphenyl ethers happens after ring cleavage. However, from the results presented it is not yet clear whether the formation of the halophenols occurs by further degradation of the detected cleavage products or by direct enzymatic

scission of the ether bridge between the two intact aromatic rings.

The results suggest that in *Trametes versicolor* not only a polymerization of hydroxylated biarylic ether compounds by laccases (Jonas et al. 1998) occurs, but also further transformation up to ring cleavage.

Acknowledgments

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