Journal of Hazardous Materials 196 (2011) 386–394

Contents lists available at SciVerse ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Biodegradation of chlorobenzoic acids by ligninolytic fungi

Milan Muzikář[a,b], Zdena Křesinová[a,c], Kateřina Svobodová[a], Alena Filipová[a], Monika Čvančarová[a,c], Kamila Cajthamlová[a], Tomáš Cajthaml[a,*]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i., Vídeňská 1083, CZ-142 20 Prague 4, Czech Republic
[b] Institute of Chemical Technology Prague, Faculty of Food and Biochemical Technology, Technická 5, CZ-160 28 Prague 6, Czech Republic
[c] Institute of Environmental Studies, Faculty of Science, Charles University, Benátská 2, CZ-128 01 Prague 2, Czech Republic

ARTICLE INFO

Article history:
Received 27 May 2011
Received in revised form 23 August 2011
Accepted 10 September 2011
Available online 16 September 2011

Keywords:
Chlorobenzoic acid
Polychlorinated biphenyls
Biodegradation
White rot fungi
Irpex lacteus

ABSTRACT

We investigated the abilities of several perspective ligninolytic fungal strains to degrade 12 mono-, di- and trichloro representatives of chlorobenzoic acids (CBAs) under model liquid conditions and in contaminated soil. Attention was also paid to toxicity changes during the degradation, estimated using two luminescent assay variations with *Vibrio fischeri*. The results show that almost all the fungi were able to efficiently degrade CBAs in liquid media, where *Irpex lacteus*, *Pycnoporus cinnabarinus* and *Dichomitus squalens* appeared to be the most effective in the main factors: degradation and toxicity removal. Analysis of the degradation products revealed that methoxy and hydroxy derivatives were produced together with reduced forms of the original acids. The findings suggest that probably more than one mechanism is involved in the process. Generally, the tested fungal strains were able to degrade CBAs in soil in the 85–99% range within 60 days. Analysis of ergosterol showed that active colonization is an important factor for degradation of CBAs by fungi. The most efficient strains in terms of degradation were *I. lacteus*, *Pleurotus ostreatus*, *Bjerkandera adusta* in soil, which were also able to actively colonize the soil. However, in contrast to *P. ostreatus* and *I. lacteus*, *B. adusta* was not able to significantly reduce the measured toxicity.

1. Introduction

Chlorinated organic pollutants are a class of serious environmental contaminants because of their environmental persistence and ecotoxicity. Chlorinated benzoic acids (CBAs) are widespread environmental pollutants resulting primarily from microbial biodegradation of polychlorinated biphenyls (PCBs), reviewed, e.g., in Field and Alvarez [1], and some herbicides [2]. CBAs are significantly more soluble than their parent compounds and can therefore enter into the aqueous phase from the contaminated soil of polluted sites. Some mono-, di-, and tri-CBAs have been shown to cause genomic damage to tobacco plants [3], and to be toxic to aquatic organisms such as ciliate, Daphnia, algae and fish [4–6]. Several mono, di and trichlorinated isomers were also found to possess estrogenic-disrupting activity [7]. CBAs represent crucial recalcitrant metabolites on the biphenyl pathway during bacterial PCB transformation. Although it was found that CBAs are not very toxic toward bacteria, substantial negative effects of their presence on the bacterial transformation of PCBs have been reported [8,9]. Moreover, soil bacteria that co-metabolize PCBs *via* the main biphenyl upper pathway tend to accumulate CBAs as dead-end products because they are generally unable to further transform these substrates [10]. Another great limitation of organopollutant bacterial biodegradation is the fact that bacterial degrading enzymes are usually intracellular and the transfer of the pollutant into the bacterial cell represents an important limiting step.

On the other hand, ligninolytic fungi, with their extracellular low-substrate-specificity enzymes, represent a promising alternative for biodegradation of various aromatic pollutants [11]. The ligninolytic system consists of three major peroxidases: lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidases and laccase, which belong among phenoloxidases. Their degradative abilities have been documented e.g., for chlorophenols, polycyclic aromatic hydrocarbons, PCBs, dioxins, furans, endocrine disrupters and others [12–15]. Moreover, the fungi were shown to be capable of splitting the aromatic rings of various persistent pollutants [14,16]. In contrast to a number of articles dealing with bacterial CBA degradation, only few papers have been published describing potential degradation of these compounds by fungi. Kamei et al. identified 4-CBA acid after degradation of 4,4′-dichlorobiphenyl by *Phanerochaete* sp. MZ 142 and suggested its further transformation *via* a reductive pathway [14]. Other authors showed that *ortho* and *meta* mono-CBAs and benzoic acid (BA)

* Corresponding author. Tel.: +420 241062498; fax: +420 241062384.
E-mail address: cajthaml@biomed.cas.cz (T. Cajthaml).

0304-3894/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2011.09.041

significantly induced the activities of cytochrom P-450 from *Phanerochaete chrysosporium* [17]. BA was then proven to be transformed by microsomes containing P-450 from the same fungus. The role of the monooxygenases of P-450 was clarified earlier by other authors, when the enzyme was heterologically expressed and hydroxyl benzoate and protocatechuic acid were detected as the degradation products of benzoate [18]. Generally, the data published in the literature document was efficiency of fungi to degrade PCBs and suggest possible transformation of PCBs to CBAs. CBAs are critical metabolites on the bacterial degradation pathway mainly, due to high specificity of individual bacterial enzymes. Therefore it is reasonable to investigate also CBA degradation abilities of ligninolytic fungi, especially when these organisms represent a promising alternative to bacterial PCB degradation applications.

The aim of this work was to investigate the abilities of several promising ligninolytic fungal strains to transform 12 representatives of CBAs with various degree of chlorination (mono-, di-, tri-CBAs). The degradation performance was tested in model liquid nutrient media, where the production of CBA degradation products, the activities of ligninolytic enzymes and changes in the acute toxicity were also monitored. Moreover, the applicability of the fungi was also tested in an artificially contaminated soil, where toxicity was also monitored.

2. Materials and methods

2.1. Materials

Standards and chemicals. 2-CBA; 2,3-CBA; 3,4-CBA; 3,5-CBA; 2,3,5-CBA; 2,4,6-CBA and HPLC internal standard 2,3-dichlorophenol were obtained from Sigma–Aldrich (Steinheim, Germany). 3-CBA; 4-CBA; 2,4-CBA; 2,5-CBA and 2,6-CBA were from Merck (Darmstadt, Germany). 2,3,6-CBA was purchased from Supelco (Steinheim, Germany). All the compounds were employed without further purification to prepare stock solutions in dimethyl formamide as described below. All the solvents were purchased from Merck, Germany or Chromservis (Prague, Czech Republic) and were of p.a. quality, trace analysis quality or gradient grade. All the chemicals used for the biochemical studies were from Sigma–Aldrich (Steinheim, Germany).

2.2. Microorganisms, inocula preparation and enzyme activities measurement

Fungal cultures, inocula preparation and degradation experiments. All of the ligninolytic fungal strains used in this study (*Irpex lacteus* 617/93, *Bjerkandera adusta* 606/93, *Phanerochaete chrysosporium* ME 446, *Phanerochaete magnoliae* CCBAS 134/I, *Pleurotus ostreatus* 3004 CCBAS 278, *Trametes versicolor* 167/93, *Pycnoporus cinnabarinus* CCBAS 595, *Dichomitus squalens* CCBAS 750) were obtained from the Culture Collection of Basidiomycetes of the Academy of Science, Prague. Fungal inocula were grown under stationary conditions for 7 d at 28 °C in 250 mL Erlenmeyer flasks containing 20 mL of either complex malt extract-glucose (MEG) medium or low-nitrogen mineral medium (LNMM). MEG medium (pH 5.5) contained 5 g malt extract broth (Oxoid, UK) and 10 g glucose per liter of distillated water and LNMM contained 2.4 mM diammonium tartrate [19]. The cultures were then homogenized with the Ultraturrax-T25 (IKA-Labortechnik, Staufen, Germany) and this suspension was used for inoculation in the degradation experiments.

Enzyme determination. LiP (E.C. 1.11.1.14) was assayed with veratryl alcohol as the substrate [20] and MnP (E.C. 1.11.1.13) was determined with 2,6-dimethoxyphenol [21]. Laccase (Lac, E.C. 1.10.3.2) was estimated with 2,2-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid as the substrate [22]. Manganese-independent peroxidase (MIP) was calculated from the peroxidase activity of MnP assay detected in the absence of Mn^{2+} ions. One unit of enzyme produced 1 μmol of the reaction product per minute under the assay conditions at room temperature.

2.3. Degradation of CBAs in liquid media

The degradation experiments in the liquid media were performed as static cultures, incubated in 250 mL Erlenmeyer flasks in five parallel experiments at 28 °C. Twenty milliliters of the respective medium (MEG or LNMM) was inoculated with a 5% suspension of homogenized pre-inocula (1 mL) of the respective fungal strain. The cultures were immediately spiked with a solution of the CBAs in dimethyl formamide (100 μL). The final amount of each CBA was 200 μg per flask. The heat-killed controls were performed with one-week growth of fungal cultures, which were killed in an autoclave before addition of the CBA solution. All of the cultures were incubated in the darkness at 28 °C and harvested after 7, 14 and 21 days.

2.4. Fungal treatment of contaminated soil

For preparation of the soil degradation experiment, 1.0 mL aliquots of a mycelia suspension of each fungal strain were added to 16 cm × 3.5 cm test-tubes containing 10 g of commercial straw pellets (ATEA Praha, Prague, Czech Republic), the moisture contents of which had been previously adjusted to 70% (w/w) and subsequently sterilized by autoclaving (121 °C, 45 min). After inoculation, the cultures were closed with cotton–wool stoppers and then grown for 14 d at 28 °C [23] The colonized substrate was then covered with a layer of soil (20 g), which had been previously artificially spiked with a mixture of CBAs in acetone. The relevant controls were prepared in the same way, however, without fungal inoculation. Main properties of the used sandy–loamy soil were as follows: total organic carbon 0.8%, total organics 1.4%, pH 5.3, water-holding capacity 31% and the granulometric composition was: sand 50.9%, fine sand 31.2%, silt 10.8%, clay 7.1%. The soil was air-dried and sieved through a 2-mm mesh before contamination and the final concentration of each CBA in the soil after contamination was 10 μg/g. The soil samples were then moistened to 15% humidity. The tubes were incubated at 28 °C and the samples were harvested after 30 and 60 d. All the respective controls and samples were performed in five replicates.

2.5. Extraction and quantitative analyses of CBAs

The whole content of each liquid culture was homogenized with Ultraturrax and acidified to approximately pH 2. It was then extracted with five 10 mL portions of ethyl acetate, the extracts were dried with sodium sulfate and concentrated using a rotary evaporator to a final volume of 10 mL. The extraction recoveries of all the CBAs were better than 95%. To enable HPLC analysis, an aliquot of the ethyl acetate extract was mixed with acetonitrile in a ratio of 1:10 (v/v), and the mixture was used for injection [16].

The soil samples were submitted to extraction using a Dionex 200 ASE extractor (Palaiseau, France). The soil samples (3 g) were mixed with sodium sulfate before extraction (v/v) and the extraction conditions were: 3 cycles; 150 °C; 10.34 MPa; solvent system hexane–acetone, 1% acetic acid [24]. To avoid CBA volatilization, 500 μL of DMSO was added to the extracts as a solvent stopper and the extracts were concentrated using a vacuum rotary evaporator (60 kPa, 40 °C) to approximately 1.5 mL. 50 μL of internal standard (IS, 2,3-dichlorophenol 0.9 mg/mL in ACN) was added to

each sample and the IS was used to calculate the volume extracts. The mixture was then directly injected into the HPLC.

The quantitative analyses were performed using the Alliance Waters system (Prague, Czech Republic) equipped with a PDA detector and Empower software was used for data processing. Separation of the CBA mixture was performed on an XBridge C18 column (250 mm × 4.6 mm I.D., particle size 3.5 μm) from Waters (Prague, Czech Republic). The separation was carried out with a gradient (v/v) of acetonitrile (B) and water solution (A) of 0.1% TFA. The gradient program was as follows (min/%B): 0/17; 30/17; 60/34; 70/50. The applied flow rate was 0.8 mL min^{-1} and temperature was 35 °C [24].

2.6. Qualitative analyses of CBA degradation products

Qualitative analysis of the CBA degradation products was performed in that the degradation intermediates were separated and characterized or identified by gas chromatography–mass spectrometry (GC–MS; 450-GC, 240-MS ion trap detector, Varian, Walnut Creek, CA). The ethyl acetate extracts were injected both directly without any derivatization and also after trimethylsilylation with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Merck, Germany) and methylation with diazomethane [25]. The GC instrument was equipped with a split/splitless injector maintained at 240 °C. DB-5MS column (Agilent, Prague, Czech Republic) was used for the separations (30 m, 0.25 mm I.D., 0.25 mm film thickness). The temperature program was started at 60 °C and was held for 1 min in the splitless mode. Then the splitter was opened with ratio 1:50. The oven was heated to 120 °C at a rate of 25 °C/min with a subsequent temperature ramp up to 240 °C at a rate of 2.5 °C/min, where this temperature was maintained for 20 min. The solvent delay time was set at 5 min and the transfer line temperature was set at 240 °C. The mass spectra were recorded at 3 scans s^{-1} under electron impact at 70 eV and mass range 50–450 amu. The excitation potential for the MS/MS product ion mode employed was 0.2 V and was increased to 0.8 V for more stable ions. Acetonitrile was used as the medium for chemical ionization (CI), where the ionization maximum time was 2000 and 40 μs for the reaction.

2.7. Analyses of ergosterol

The total ergosterol was extracted and analyzed as described previously [23]. Briefly, samples (0.5 g) were sonicated with 3 mL of 10% KOH in methanol at 70 °C for 90 min. Distilled water (1 mL) was added and the samples were extracted three times with 2 mL of cyclohexane, evaporated under nitrogen, redissolved in methanol and analyzed isocratically using a Waters Alliance HPLC system (Waters Milford, MA) equipped with a LiChroCart column filled with LiChrosphere® 100 RP-18e (250 × 4.0 mm; particle size 5 μm; pore size 100 Å) equilibrated with 100% methanol at a flow rate of 1 mL min^{-1}. Ergosterol was detected at 282 nm and quantified with a 5-point calibration curve over a linear range from 0.5 to 50.0 μg/mL.

2.8. Toxicity assay

The luminescent bacteria *Vibrio fischeri* (strain NRRL-B-11177), which was used for all the toxicity tests, were purchased freeze-dried from the supplier Ing. Musial (Czech Republic). The freeze-dried bacteria were rehydrated and stabilized in 2% (w/v) NaCl solution at 15 °C for 1 h according to the standard procedure ISO 2007 [26]. An acute toxicity test of samples after degradation in liquid media was performed using the corresponding ethyl acetate extracts. Aliquots of the extracts (0.5 mL) were evaporated to dryness and dissolved again in dimethyl sulfoxide, which was directly applied to the test (2% of DMSO in the reaction mixture). The amount of dimethyl sulfoxide varied between media, due to different sensitivities of the test toward the media matrix (see below). Three replicates for each sample were used to carry out the ecotoxicity test. The luminescence readings were obtained with a Lumino M90a luminometer (ZD Dolní Újezd, Czech Republic) at a temperature of 15 ± 0.2 °C. The inhibition of bioluminescence was recorded after 15-min exposure.

The toxicity of soil samples was measured by a kinetic Flash assay using the luminescent bacterium [27,28]. The samples were prepared by weighing 1.5 g dried soil and 6 mL 2% (w/v) NaCl solution. The sample suspension was mixed continuously and 0.5 mL was placed into the measuring cuvette. The contents of the measuring cuvette were mixed continuously by adapted luminometer LUMINO M90a and 0.5 mL of the bacterial solution was dispensed into the sample. The signal was recorded permanently for 60 s. The light inhibition was calculated as the difference between the height of the peak that was observed immediately after addition of the bacteria to the sample and the luminescence intensity after a contact time of 60 s.

3. Results and discussion

3.1. Degradation of CBAs in liquid cultures

The representatives of mono, di and tri-CBAs that were tested in this study were employed at a relatively high concentration of 10 μg/mL. The fact that the compounds are partially soluble in water and their acute toxic properties were confirmed by the observation of fungal biomass development. Generally, the fungal strains were partly affected by CBAs and their biomass reached about 50–70% compared to non toxic controls (data not shown). This finding is in agreement with the observation of Dittmann et al. who tested the development of the mycelia of fungal strains in two liquid nutrient media after the addition of various concentrations of 3-CBA [29]. In contrast to this observation, the fungal strains in our study were very efficient in degradation of CBAs in the liquid media. The time course of the individual CBA degradation in both media is presented in Tables 1 and 2. The results clearly demonstrate that all of the tested strains were at least partially able to transform CBAs. *I. lacteus*, *P. cinnabarinus* and *D. squalens* were found to be the most efficient degraders in complex MEG media. *P. cinnabarinus* and *D. squalens* were able to degrade about 78% and 73% of total CBA, respectively, while *I. lacteus* degraded 92% of total CBAs in complex media compared to the heat-killed controls. Particularly *I. lacteus* removed all of the CBAs from the media except 2,6-CBA and 2,3,6-CBA, which were degraded to approximately 50% of original amount, while *P. cinnabarinus* did not significantly transform 2,6-CBA, 2,3,6-CBA, 2,4,6-CBA and *D. squalens* did not significantly degrade 2,3,6-CBA (ANOVA, $P = 0.05$). *B. adusta* exhibited the poorest degradation ability in both the liquid media. The most efficient strains in the LNNM media were once again found to be *I. lacteus*, *P. cinnabarinus* and *D. squalens*. All of the three most efficient strains were able to transform CBAs with percent removals ranging from 76% to 77%. 2,6-CBA, 2,3,6-CBA and 2,4,6-CBA again appeared to be the most recalcitrant compounds where *D. squalens* did not significantly degrade 2,6-CBA and 2,4,6-CBA; *P. cinnabarinus* – 2,6-CBA, 2,3,6-CBA and 2,4,6-CBA; *I. lacteus* – 2,6-CBA and 2,3,6-CBA. These results suggest a possible connection between the substituted *ortho* and *para* positions and persistency toward the fungal degradation mechanism. Only a few publications in journals deal with transformation of CBAs by fungi. The above-mentioned work of Dittmann et al. also included the degradation of 3-CBA by *P. chrysosporium*, *P. ostreatus*, *Heterobasidion annosum* and two other ectomycorrhizal fungi [29]. However, in contrast to our results, the authors observed only limited degradation of the compound in the range of several

Table 1
Residual CBA amounts in heat-killed controls and after incubation of the tested fungal strains in LNNM media (ND: not detected).

Amount of CBA (μg per flask)	2-CBA	3-CBA	4-CBA	2,3-CBA	2,4-CBA	2,5-CBA	2,6-CBA	3,4-CBA	3,5-CBA	2,3,5-CBA	2,3,6-CBA	2.4.6-CBA
LNNM – 7 days												
Control	196 ± 4	207 ± 4	207 ± 2	195 ± 3	207 ± 2	193 ± 3	205 ± 2	182 ± 8	196 ± 1	165 ± 2	199 ± 1	185 ± 0
B. adusta	156 ± 5	53 ± 12	41 ± 34	174 ± 5	156 ± 31	141 ± 11	197 ± 8	64 ± 19	77 ± 28	161 ± 2	185 ± 7	176 ± 8
D. squalens	ND	33 ± 4	ND	ND	ND	ND	211 ± 15	ND	ND	ND	ND	207 ± 6
I. lacteus	ND	19 ± 6	ND	45 ± 7	66 ± 2	85 ± 5	179 ± 18	ND	ND	153 ± 2	176 ± 21	128 ± 11
P. chrysosporium	ND	ND	ND	94 ± 14	ND	92 ± 15	196 ± 5	ND	ND	136 ± 10	177 ± 7	171 ± 6
P. magnoliae	27 ± 2	ND	ND	133 ± 2	74 ± 4	126 ± 6	189 ± 3	ND	165 ± 2	ND	184 ± 8	180 ± 4
P. ostreatus	156 ± 9	28 ± 3	ND	162 ± 8	105 ± 17	167 ± 8	195 ± 5	87 ± 60	55 ± 1	124 ± 46	172 ± 14	163 ± 13
P. cinnabarinus	ND	ND	ND	ND	ND	ND	190 ± 8	ND	ND	ND	176 ± 12	173 ± 7
T. versicolor	73 ± 39	25 ± 1	ND	25 ± 3	104 ± 8	46 ± 3	242 ± 11	ND	ND	ND	206 ± 14	140 ± 4
LNNM – 14 days												
Control	170 ± 15	173 ± 14	184 ± 21	168 ± 19	208 ± 17	167 ± 18	181 ± 16	156 ± 17	165 ± 19	138 ± 19	169 ± 17	159 ± 21
B. adusta	124 ± 5	45 ± 2	ND	157 ± 22	85 ± 5	114 ± 7	198 ± 14	46 ± 0	ND	145 ± 5	187 ± 7	179 ± 10
D. squalens	ND	56 ± 4	ND	126 ± 8	ND	ND	189 ± 11	ND	ND	ND	ND	175 ± 11
I. lacteus	ND	ND	ND	13 ± 2	ND	ND	173 ± 7	ND	ND	103 ± 3	171 ± 9	95 ± 4
P. chrysosporium	ND	ND	ND	25 ± 1	ND	40 ± 1	188 ± 12	ND	ND	92 ± 0	175 ± 12	165 ± 8
P. magnoliae	ND	ND	ND	100 ± 11	60 ± 19	77 ± 13	182 ± 15	ND	136 ± 23	ND	153 ± 32	151 ± 27
P. ostreatus	105 ± 1	ND	ND	132 ± 1	15 ± 2	159 ± 3	193 ± 11	ND	ND	114 ± 6	188 ± 5	161 ± 13
P. cinnabarinus	ND	ND	ND	ND	ND	ND	184 ± 6	ND	ND	ND	163 ± 8	136 ± 41
T. versicolor	66 ± 13	47 ± 4	ND	ND	ND	ND	186 ± 34	ND	ND	ND	183 ± 3	136 ± 3
LNNM – 21 days												
Control	196 ± 5	195 ± 9	204 ± 8	189 ± 3	208 ± 6	191 ± 5	203 ± 6	180 ± 15	188 ± 5	170 ± 7	195 ± 7	183 ± 7
B. adusta	112 ± 10	54 ± 5	ND	167 ± 8	89 ± 2	106 ± 5	201 ± 14	ND	ND	130 ± 8	190 ± 13	182 ± 12
D. squalens	ND	71 ± 1	ND	105 ± 3	ND	ND	186 ± 5	ND	ND	ND	ND	191 ± 4
I. lacteus	ND	ND	ND	19 ± 2	22 ± 0	ND	170 ± 1	ND	ND	67 ± 7	183 ± 1	71 ± 6
P. chrysosporium	ND	ND	ND	20 ± 2	ND	40 ± 6	185 ± 24	ND	ND	98 ± 4	180 ± 18	168 ± 13
P. magnoliae	ND	ND	ND	24 ± 5	35 ± 5	20 ± 2	176 ± 9	ND	ND	129 ± 7	152 ± 12	153 ± 11
P. ostreatus	70 ± 13	ND	ND	101 ± 19	ND	130 ± 25	182 ± 36	ND	ND	105 ± 18	179 ± 39	157 ± 30
P. cinnabarinus	ND	ND	ND	ND	ND	ND	196 ± 7	ND	ND	ND	175 ± 9	176 ± 8
T. versicolor	76 ± 5	32 ± 15	ND	ND	ND	ND	195 ± 13	ND	ND	ND	194 ± 8	134 ± 11

percent, even though, in one case, the authors employed a similar concentration (15.6 mg/L) to that used in our study (20 mg/L).

In order to employ the toxicity test, we diluted the samples from the two media in different ways. The theoretical (original) concentrations of the individual CBAs in the reaction mixture for MEG and LNNM media samples were 0.5 and 0.25 μg/mL, respectively. Since we detected only a decrease in the toxicity in preliminary tests, the dilution of the samples was set to reach about 90% inhibition for the controls. The evaluation of the acute toxicity test with *V. fischeri* was performed by comparison of the inhibition of the sample

Table 2
Residual CBA amounts in heat-killed controls and after incubation of the tested fungal strains in MEG media (ND: not detected).

Amount of CBA (μg per flask)	2-CBA	3-CBA	4-CBA	2,3-CBA	2,4-CBA	2,5-CBA	2,6-CBA	3,4-CBA	3,5-CBA	2,3,5-CBA	2,3,6-CBA	2,4,6-CBA
MEG – 7 days												
Control	211 ± 4	195 ± 4	209 ± 2	197 ± 2	207 ± 6	195 ± 6	206 ± 4	195 ± 5	199 ± 4	175 ± 2	172 ± 6	193 ± 5
B. adusta	201 ± 20	138 ± 64	162 ± 69	200 ± 8	192 ± 25	189 ± 17	200 ± 9	154 ± 39	175 ± 32	166 ± 13	177 ± 8	193 ± 7
D. squalens	ND	97 ± 6	ND	ND	131 ± 11	ND	205 ± 20	ND	ND	ND	151 ± 1	160 ± 13
I. lacteus	ND	ND	ND	ND	46 ± 2	70 ± 27	204 ± 4	ND	ND	86 ± 5	151 ± 4	114 ± 11
P. chrysosporium	ND	ND	ND	52 ± 5	ND	ND	209 ± 7	ND	ND	ND	167 ± 8	185 ± 6
P. magnoliae	ND	44 ± 4	ND	56 ± 7	ND	47 ± 10	213 ± 7	ND	ND	76 ± 6	172 ± 7	188 ± 5
P. ostreatus	71 ± 9	ND	ND	127 ± 7	66 ± 5	145 ± 9	214 ± 14	ND	ND	132 ± 10	156 ± 11	150 ± 14
P. cinnabarinus	ND	ND	ND	ND	ND	ND	201 ± 12	ND	ND	ND	167 ± 18	190 ± 15
T. versicolor	90 ± 22	ND	ND	42 ± 2	89 ± 2	39 ± 1	202 ± 7	ND	ND	ND	154 ± 2	174 ± 3
MEG – 14 days												
Control	210 ± 30	186 ± 32	197 ± 24	199 ± 27	206 ± 26	195 ± 21	212 ± 25	176 ± 26	181 ± 25	164 ± 28	178 ± 26	193 ± 24
B. adusta	208 ± 3	191 ± 2	211 ± 1	162 ± 51	203 ± 7	194 ± 1	202 ± 12	175 ± 3	186 ± 1	151 ± 10	148 ± 28	192 ± 1
D. squalens	ND	ND	213 ± 3	101 ± 6	124 ± 6	ND	ND	ND	ND	ND	145 ± 3	166 ± 1
I. lacteus	ND	ND	ND	ND	ND	ND	98 ± 10	ND	ND	ND	107 ± 12	ND
P. chrysosporium	ND	ND	ND	ND	ND	ND	226 ± 4	ND	ND	ND	161 ± 5	181 ± 2
P. magnoliae	ND	34 ± 1	ND	40 ± 8	39 ± 5	60 ± 15	221 ± 2	ND	ND	86 ± 17	162 ± 4	189 ± 11
P. ostreatus	37 ± 9	ND	ND	91 ± 19	58 ± 3	117 ± 19	231 ± 11	ND	ND	115 ± 15	170 ± 7	150 ± 4
P. cinnabarinus	ND	ND	ND	ND	ND	ND	188 ± 17	ND	ND	ND	151 ± 25	170 ± 23
T. versicolor	84 ± 16	ND	ND	ND	ND	ND	197 ± 5	ND	ND	ND	150 ± 7	169 ± 6
MEG – 21 days												
Control	209 ± 15	175 ± 17	188 ± 11	186 ± 13	193 ± 9	182 ± 8	213 ± 9	172 ± 10	177 ± 9	157 ± 6	166 ± 12	181 ± 8
B. adusta	140 ± 53	47 ± 2	42 ± 9	173 ± 26	133 ± 46	138 ± 54	200 ± 6	80 ± 13	105 ± 10	137 ± 15	170 ± 4	183 ± 7
D. squalens	ND	ND	197 ± 5	ND	74 ± 14	54 ± 9	ND	ND	ND	ND	140 ± 2	151 ± 14
I. lacteus	ND	ND	ND	ND	ND	ND	88 ± 20	ND	ND	ND	96 ± 9	ND
P. chrysosporium	ND	ND	93 ± 19	ND	ND	ND	194 ± 23	59 ± 5	75 ± 4	ND	142 ± 19	167 ± 14
P. magnoliae	ND	ND	29 ± 8	55 ± 9	46 ± 1	52 ± 3	236 ± 3	56 ± 0	ND	82 ± 1	174 ± 3	195 ± 2
P. ostreatus	22 ± 1	ND	ND	86 ± 6	61 ± 4	126 ± 14	200 ± 13	ND	ND	119 ± 12	187 ± 23	171 ± 18=
P. cinnabarinus	ND	ND	ND	ND	ND	ND	187 ± 27	ND	ND	ND	150 ± 25	162 ± 34
T. versicolor	121 ± 30	ND	136 ± 22	ND	ND	ND	206 ± 11	ND	ND	ND	160 ± 5	172 ± 6

Table 3
Luminescence inhibition in heat-killed controls and after incubation of the tested fungal strains in MEG and LNNM media after 21 days of incubation.

Luminescence inhibition (%)	LNMM	MEG
Control	89.4 ± 8.2	94.5 ± 1.0
B. adusta	72.1 ± 4.3	85.9 ± 12.5
D. squalens	11.9 ± 8.5	35.0 ± 9.4
I. lacteus	27.2 ± 13.9	7.4 ± 3.0
P. chrysosporium	44.9 ± 15.4	38.2 ± 30.7
P. magnoliae	41.9 ± 10.1	52.8 ± 17.7
P. ostreatus	27.5 ± 8.3	34 .8 ± 2.8
P. cinnabarinus	11.3 ± 2.7	20.8 ± 5.6
T. versicolor	14.5 ± 4.0	36.3 ± 7.5

luminescence with their respective controls (Table 3). As mentioned above, the toxicity test revealed that the tested fungi were generally able to decrease the measured acute toxicity, suggesting that the degradation products of CBAs were either not accumulated or they were less toxic than the original CBAs.

3.2. Detection of CBA degradation products

The CBA metabolites were analyzed with GC–MS and their structures were suggested by comparing the mass spectra with the data in the NIST 08 library and independently by interpreting the fragmentation pattern. Additionally, unknown structures of metabolites were explored using MS/MS (product ion scan) to clarify the fragmentation sequence. The mass spectral characteristics of the detected CBA degradation products are listed in Table 4. Some of the metabolites were detected after trimethylsilylation (e.g., chlorobenzyl alcohols) and several of them were confirmed by comparison with the available chemical standards. All of the intermediates were detected at only trace levels, suggesting that none of them were accumulated during degradation. The group of the detected intermediates includes chlorobenzaldehydes, chlorobenzyl alcohols, chlorobenzoic acid methyl esters and the methoxy or hydroxy derivatives of these structures. The metabolites were found in various fungal strain cultures when representatives of monochloro and dichloro benzaldehydes and alcohols were found in all cultures, as well as methyl esters of di-CBA. Methyl ester representative of tri-CBA was detected only in the culture of *I. lacteus*, however, methoxy derivatives of tri-CBA and di-CBA methyl esters were found in all fungi. Trichlorinated hydroxybenzyl alcohols were detected in all fungal cultures too. A possible scheme of CBA fungal degradation pathway constructed of the detected metabolites is shown in Fig. 1. The results generally correspond to the results of Kamei et al. [14], who studied the transformation of 4,4′-dichlorobiphenyl by *Phanerochaete* sp. MZ 142, where these authors detected the formation of 4-CBA, the methyl ester of 4-CBA and further reduced transformation products: 4-chlorobenzyl alcohol and 4-chlorobenzaldehyde. Such a reduction mechanism could be explained by the action of an intracellular aryl alcohol oxidase system [30]. Matsuzaki et al. showed that the enzymes that are probably involved in the transformation, i.e. aryl alcohol dehydrogenase, aryl aldehyde dehydrogenase and also cytochrome P-450 of *P. chrysosporium* were up-regulated after the addition of BA to the fungal culture [31].

The other types of transformation products, i.e. hydroxyl and methoxy derivatives, which were found in our study, have already been described by Matsuzaki and Wariishi following transformation of BA by *P. chrysosporium* [18]. The detected metabolites include 4-hydroxy, 2-hydroxy and 4-hydroxy-2-methoxy derivatives. In another work, the authors demonstrated that heterologously expressed P-450 cytochromes from the CYP53 family of *P. chrysosporium*, *Aspergilus niger* and *Rhodotorula minuta* were able to hydroxylate BA at the 4-position [32]. P-450-mediated

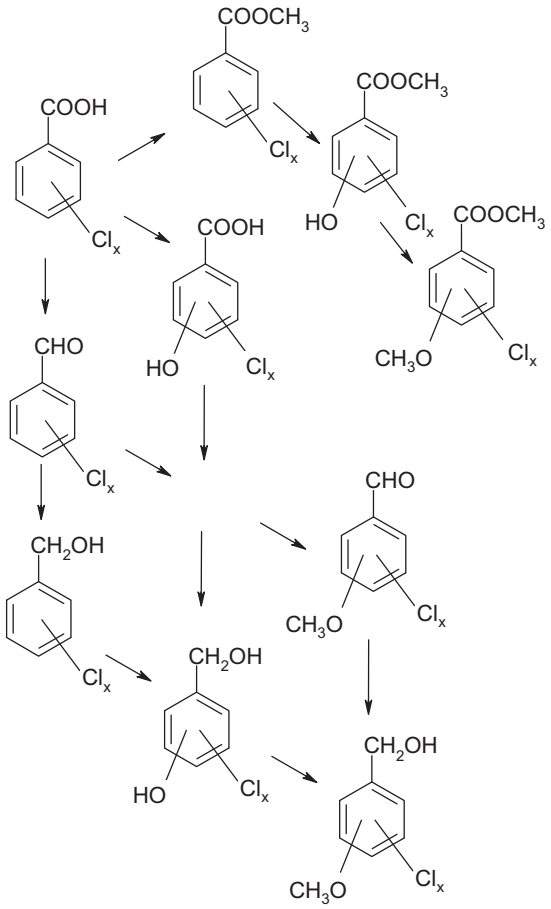

Fig. 1. Proposed pathway of CBA degradation by ligninolytic fungi.

hydroxylation of BA at other positions in fungi has not been reported to date. Moreover, the authors employed quantitative PCR to demonstrate that the expression of the cytochrome is regulated by the presence of BA at the transcription level. The induction of cytochrome P-450 by BA and also by 3 and 4-CBA has been published elsewhere [17].

The measurement of fungal extracellular ligninolytic activities in this study demonstrated that most of the activities were suppressed or the maxima activity peaks were delayed during cultivation by the presence of CBAs. Only rare cases when the situation was different were recorded for the activities of MnP and laccase of *T. versicolor*, which were significantly induced in MEG and LNNM media, respectively. Particularly the laccase activity increased from 20 U/L to 230 U/L. These findings indirectly confirm that ligninolytic enzymes need not play a key role in the degradation of CBAs.

3.3. Degradation of CBAs in soil

The soil degradation experiment was monitored after 30 and 60 days and the residual concentrations after the application of the fungal strains are depicted in Fig. 2. The results show that, except for strains *P. cinnabarinus* and *T. versicolor*, which degraded only 30% and 39% of the total CBA, respectively, within 60 days of incubation, all the other strains under study were able to substantially remove CBAs from soil in the range of 85–99% of total CBA. The results are partially contrary to the experiments in liquid cultures, because *P. cinnabarinus* belonged among the most degrading strains in both the liquid media. On the other hand, *B. adusta* appeared to be effective in soil while this strain belonged among the less degrading in the liquid media. *I. lacteus* was found to be the most efficient

Table 4
Retention data and electron impact mass spectral characteristics of CBA metabolites.

t_R (min)	MW according to CI	*m/z* of fragment ions (relative intensity)	Structural suggestion	Type of derivatization
5.431	140	142 (23.8), 141 (36.6), 140 (73.7), 139 (99.9), 111 (55.0), 75 (32.0), 51 (19.6), 50 (29.8)	o-Chlorobenzaldehyde	
5.494	140	142 (20.6), 141 (36.1), 140 (66.6), 139 (99.9), 113 (18.4), 77 (22.7), 75 (33.5), 74 (19.1)	m-Chlorobenzaldehyde	
5.582	140	142 (16.0), 141 (37.1), 140 (49.4), 139 (99.9), 113 (16.8), 111 (49.6), 77 (15.1), 74 (16.8)	p-Chlorobenzaldehyde	
7.603	174	176 (62.4), 174 (70.3), 173 (99.9), 145 (47.0), 139 (54.2), 111 (50.1), 75 (61.1), 74 (52.6)	3,5-Dichlorobenzaldehyde	
7.724	174	176 (39.5), 175 (70.2), 174 (61.4), 173 (99.9), 147 (16.9), 145 (25.9), 75 (18.0), 74 (15.0)	2,4-Dichlorobenzaldehyde	
7.913	174	176 (38.2), 175 (68.4), 174 (61.3), 173 (99.9), 111 (25.0), 75 (61.0), 74 (45.9), 50 (25.3)	2,5-Dichlorobenzaldehyde	
8.329	174	176 (37.5), 175 (69.0), 174 (62.8), 173 (99.9), 147 (21.9), 145 (37.4), 75 (36.4), 74 (26.3)	2,3-Dichlorobenzaldehyde	
8.532	174	176 (38.9), 175 (69.5), 174 (64.9), 173 (99.9), 147 (28.1), 145 (43.0), 75 (29.1), 74 (24.9)	3,4-Dichlorobenzaldehyde	
8.875	214	201 (34.0), 199 (99.9), 179 (18.5), 163 (30.7), 127 (25.4), 125 (82.5), 89 (25.1), 73 (18.6)	TMS p-chlorobenzyl alcohol	Trimethylsilylation
9.445	214	201 (31.8), 199 (90.9), 179 (33.4), 171 (19.9), 169 (60.1), 127 (30.2), 125 (99.9), 89 (32.7)	TMS m-chlorobenzyl alcohol	Trimethylsilylation
9.685	214	201 (20.8), 199 (58.7), 179 (24.2), 169 (20.5), 127 (31.9), 125 (99.9), 89 (24.2), 73 (12.5)	TMS o-chlorobenzyl alcohol	Trimethylsilylation
10.883	204	206 (32.5), 204 (43.4), 177 (9.9), 175 (63.4), 173 (100), 147 (9.6), 145 (7.9), 109 (7.8), 75 (33.9)	2,6-Dichlorobenzoic acid methyl ester	Trimethylsilylation
11.203	204	208 (2.0), 206 (14.1), 204 (20.5), 177 (8.9), 175 (63.1), 173 (100), 147 (21.1), 145 (33.2), 109 (17.8), 75 (16.0)	3,5-Dichlorobenzoic acid methyl ester	
11.729	204	208 (5.4), 206 (12.9), 204 (20.7), 177 (9.9), 175 (61.1), 173 (100), 147 (15.5), 145 (29.0), 109 (16.2), 75 (95.6)	2,4-Dichlorobenzoic acid methyl ester	
11.894	204	208 (3.7), 206 (18.3), 204 (25.7), 177 (11.9), 175 (62.1), 173 (93.3), 147 (6.4), 145 (19.3), 109 (17.1), 75 (100)	2,5-Dichlorobenzoic acid methyl ester	
12.308	204	208 (2.7), 206 (19.8), 204 (32.4), 177 (10.3), 175 (64.6), 173 (100), 147 (19.7), 145 (35.1), 109 (22.9), 74 (27.6)	3,4-Dichlorobenzoic acid methyl ester	
12.651	204	208 (1.3), 206 (16.3), 204 (19.0), 177 (19.0), 175 (56.9), 173 (100), 149 (29.4), 147 (47.7), 145 (19.0), 109 (14.4), 75 (97.4)	2,3-Dichlorobenzoic acid methyl ester	
12.945	170	172 (19.4), 171 (35.0), 170 (56.3), 169 (100), 141 (6.8), 126 (13.6), 111 (11.7), 77 (15.5)	?-Chloro-?-methoxybenzaldehyde	
13.139	248	235 (67.6), 233 (99.9), 205 (18.5), 203 (25.6), 161 (41.4), 159 (64.2), 123 (18.4), 103 (18.9)	TMS 2,5-dichlorobenzyl alcohol	Trimethylsilylation
13.27	248	235 (58.0), 233 (84.2), 161 (61.1), 159 (99.9), 123 (13.8), 103 (29.1), 73 (12.7)	TMS 2,4-dichlorobenzyl alcohol	Trimethylsilylation
13.604	248	235 (70.9), 233 (99.9), 205 (32.3), 203 (45.8), 161 (58.5), 159 (89.7), 147 (27.7), 123 (21.1)	TMS 3,5-dichlorobenzyl alcohol	Trimethylsilylation
14.091	238	242 (6.4), 240 (19.3), 238 (19.7), 211 (29.3), 209 (95.0), 207 (100), 183 (3.4), 181 (14.1), 179 (14.5), 143 (11.0), 109 (12.8), 74 (12.9)	2,4,6-Trichlorobenzoic acid methyl ester	Trimethylsilylation
14.288	248	235 (69.6), 233 (99.9), 205 (17.8), 203 (25.3), 161 (46.3), 159 (71.1), 123 (17.5), 103 (22.8)	TMS 2,3-dichlorobenzyl alcohol	Trimethylsilylation
15	248	235 (68.5), 233 (94.6), 203 (16.7), 161 (67.3), 159 (99.9), 75 (27.5), 73 (28.5), 59 (34.2)	TMS 3,4-dichlorobenzyl alcohol	Trimethylsilylation
15.801	238	242 (7.1), 240 (25.7), 238 (25.1), 211 (31.5), 209 (98.1), 207 (100), 183 (5.7), 181 (12.6), 179 (14.5), 143 (10.5), 109 (11.7), 74 (14.1)	2,3,6-Trichlorobenzoic acid methyl ester	Trimethylsilylation
16.391	238	242 (9.3), 240 (29.4), 238 (29.5), 211 (29.7), 209 (95.4), 207 (100), 183 (6.8), 181 (21.6), 179 (20.7), 143 (14.2), 109 (15.6), 74 (14.5)	2,3,5-Trichlorobenzoic acid methyl ester	
18.625	310	271 (32.4), 269 (100), 267 (76.8), 241 (15.8), 239 (45.5), 237 (42.8), 197 (13.7), 195 (46.6), 193 (44.2), 157 (14.2), 125 (5.5), 123 (15.8), 93 (15.9)	TMS ?,?,?-trichlorobenzyl alcohol	Trimethylsilylation
18.699	298	285 (33.2), 283 (100), 281 (99.5),239 (49.1), 237 (50.6), 209 (80.6), 207 (83.3), 205 (2.8), 165 (13.3), 167 (33.3)	TMS ?,?,?-trichloro-?-hydroxybenzyl alcohol	Trimethylsilylation
18.741	310	271 (32.8), 269 (100), 267 (87.2), 241 (13.0), 239 (38.3), 237 (37.2), 197 (22.0), 195 (68.5), 193 (65.9), 157 (14.6), 125 (5.6), 123 (15.7), 93 (28.1)	TMS ?,?,?-trichlorobenzyl alcohol	Trimethylsilylation
19.695	234	238 (4.3), 236 (41.1), 234 (67.2), 207 (15.4), 205 (84.6), 203 (100), 162 (14.1), 160 (18.6), 111 (15.0), 97 (31.9)	?,?-Dichloro-?-methoxybenzoic acid methyl ester	
19.82	186	188 (7.2), 186 (25.8), 157 (31.6), 155 (100), 127 (17.8), 99 (13.7)	?-Chloro-?-hydroxybenzoic acid methyl ester	Methylation
20.46	298	285 (31.5), 283 (100), 281 (98.0), 239 (73.8), 237 (36.1), 209 (68.2), 207 (68.5), 167 (12.6),165 (33.8)	TMS ?,?,?-trichloro-?-hydroxybenzyl alcohol	Trimethylsilylation
20.592	310	271 (35.5), 269 (100), 267 (99.1), 241 (10.0), 239 (31.1), 237 (31.1), 197 (26.8), 195 (81.5), 193 (82.1), 157 (16.7), 125 (6.4), 123 (18.0), 93 (39.2)	TMS ?,?,?-trichlorobenzyl alcohol	Trimethylsilylation
20.688	298	285 (31.6), 283 (100), 281 (95.3), 239 (73.8), 237 (34.6), 209 (47.7), 207 (43.3), 167 (14.8),165 (33.0)	TMS ?,?,?-trichloro-?-hydroxybenzyl alcohol	Trimethylsilylation
21.057	298	285 (34.4), 283 (100), 281 (98.5), 239 (36.8), 237 (32.4), 209 (51.5), 207 (54.4), 167 (13.2),165 (35.6)	TMS ?,?,?-trichloro-?-hydroxybenzyl alcohol	Trimethylsilylation
21.526	234	238 (3.0), 236 (22.3), 234 (30.4), 207 (10.2), 205 (68.4), 203 (100), 162 (5.3), 160 (8.3), 111 (11.2), 97 (17.0)	?,?-Dichloro-?-methoxybenzoic acid methyl ester	
22.693	298	285 (26.1), 283 (100), 281 (90.9), 239 (52.1), 237 (49.7), 209 (74.2), 207 (64.2), 167 (19.7),165 (48.1)	TMS?,?,?-trichloro-?-hydroxybenzyl alcohol	Trimethylsilylation
23.481	234	238 (5.1), 236 (21.3), 234 (29.5), 207 (11.5), 205 (70.5), 203 (100), 162 (10.9), 160 (11.9), 111 (15.4), 97 (19.9)	?,?-Dichloro-?-methoxybenzoic acid methyl ester	
27.313	268	270 (15.1), 268 (15.3), 241 (28.3), 239 (95.8), 237 (100)	?,?,?-Trichloro-?-methoxybenzoic acid methyl ester	
29.207	312	312 (12.3), 314 (13.6), 301 (42.6), 299 (100), 297 (97,3), 271 (24.3), 269 (76.7), 267 (73.4), 227 (31.8), 225 (75.6), 223 (88.5)	TMS ?,?,?-trichloro-?-methoxybenzyl alcohol	Trimethylsilylation

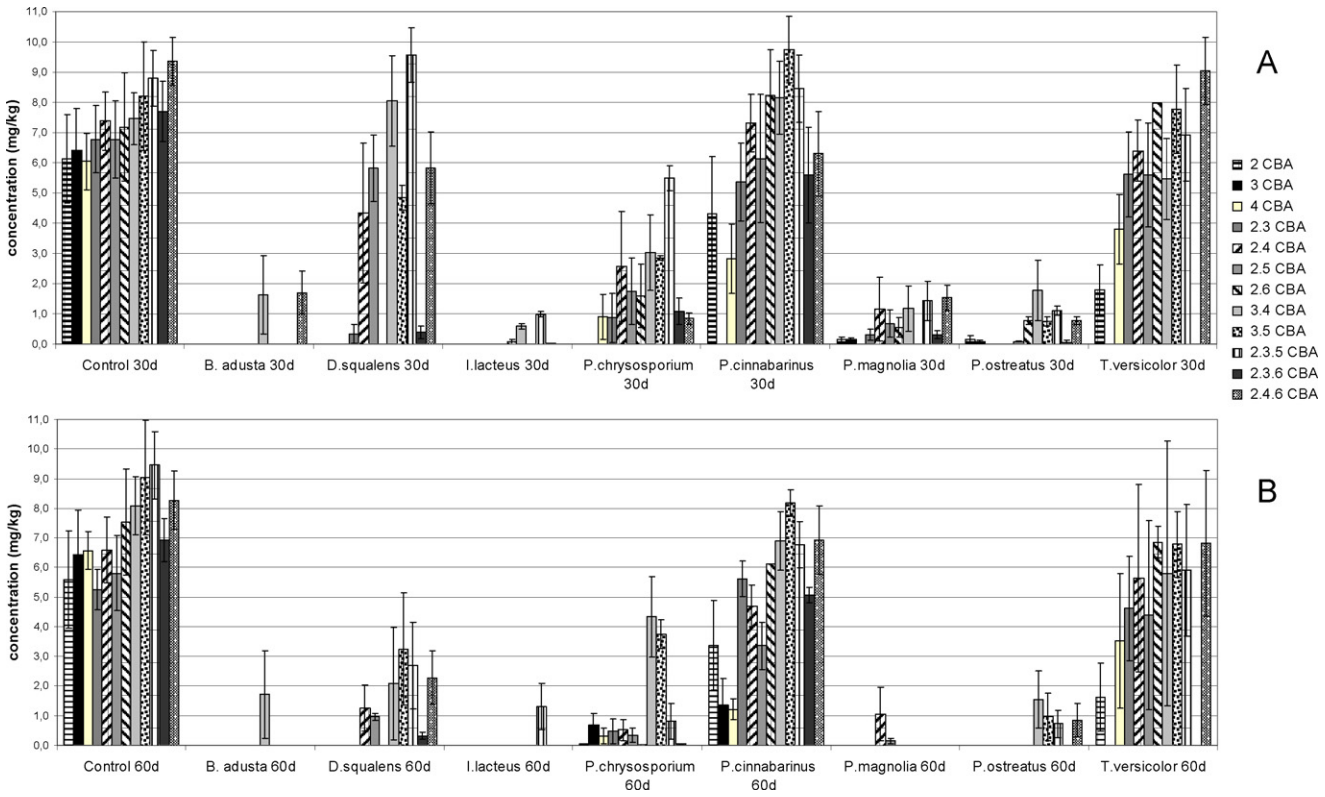

Fig. 2. Residual concentrations of CBAs in contaminated soil after incubation with the tested fungal strains: A – 30 days; B – 60 days.

strain in soil, where this fungus had already depleted 98% of the CBA within 30 days. One probable explanation for the discrepancy between these results and model conditions in the liquid media and the soil degradation experiment lies in the different abilities of fungi to penetrate into contaminated soil [33]. Therefore, we tried to estimate the relative amount of fungal biomass using the analysis of ergosterol in soil samples with CBAs and also without the addition of pollutants (Fig. 3). Despite the high variability of the data, the results indicate that the fungi that showed the highest CBA depletion (*I. lacteus*, *P. ostreatus*, and *B. adusta*), were also the strains that were able to significantly colonize the contaminated soil. The only exception was *P. magnolia*, where we detected a significantly lower amount of ergosterol despite the high removal of CBAs (99% within 60 days).

The parameters of kinetic Flash toxicity assay were adjusted to also include recording of a possible increase in toxicity. The data obtained from this test are depicted in Fig. 4. The best results, in terms of inhibition reduction, were obtained with strains *I. lacteus* and *P. ostreatus*, corresponding to their CBA degradation efficiencies. On the other hand, unexpected results were observed with strains *B. adusta* and *P. magnolia* where, in spite of their high degradation rate, the detected residual toxicity was not significantly different from the controls (*t*-test, $P < 0.05$). These results of Flash assay are in agreement with the results from toxicity estimation in

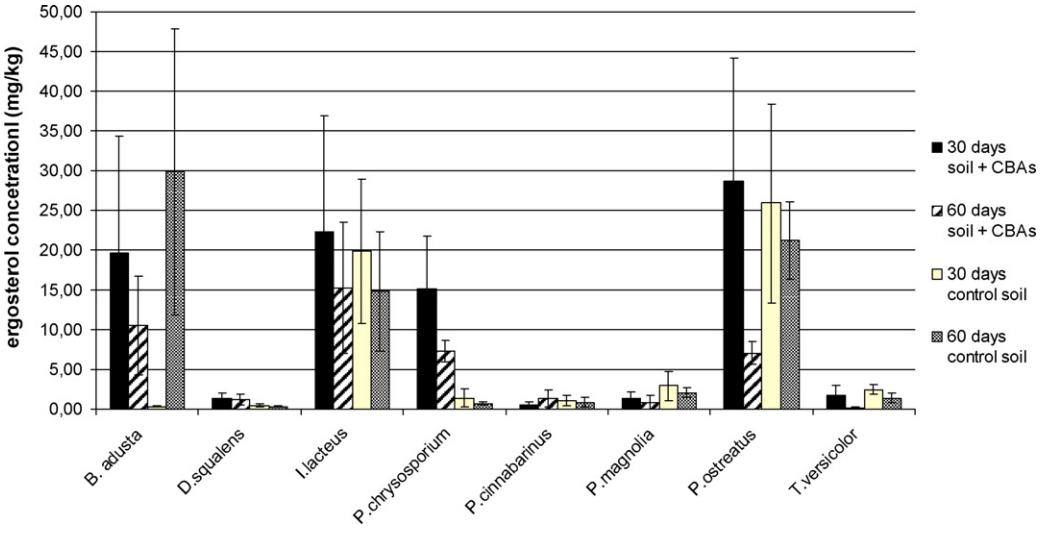

Fig. 3. Ergosterol concentrations in non-contaminated soil and in soil contaminated by CBAs after 30 and 60 days of incubation.

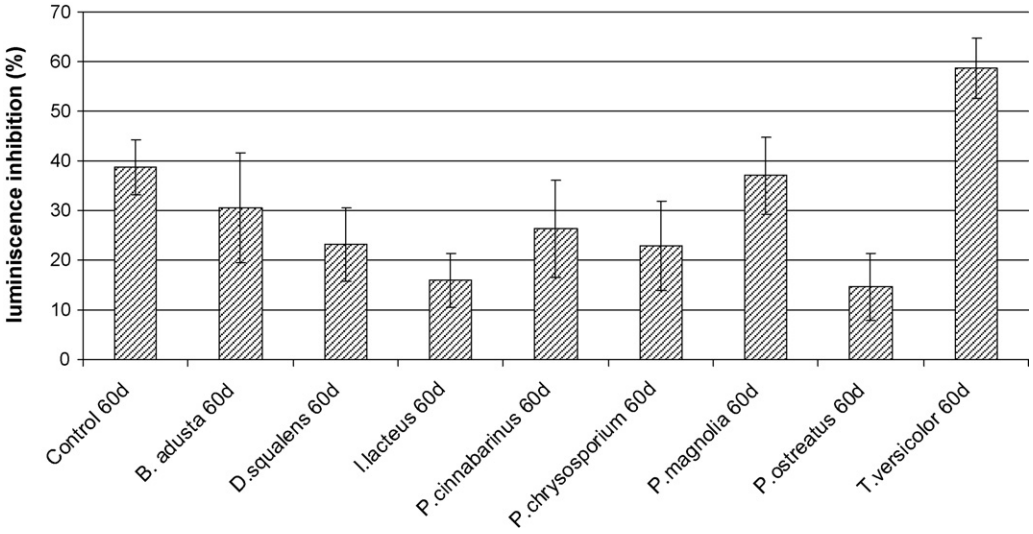

Fig. 4. Luminescence inhibition of the Flash assay in contaminated soil (control) and in soil with the tested fungal strains after 60 days of incubation.

the liquid cultures, where a residual toxicity in these fungal cultures was also detected. This could possibly be explained by the formation and accumulation of toxic metabolites and, probably for the same reason, significantly elevated toxicity was observed for *T. versicolor*.

4. Conclusion

To the best of our knowledge, this is the first paper providing a general description of the ability of ligninolytic fungi to biodegrade CBAs that represent crucial toxic and highly persistent metabolites on bacterial biodegradation pathways of polychlorinated biphenyls. The ability of the fungi has been examined under liquid conditions and also verified in contaminated soil. The tested fungal strains were able to degrade CBAs in soil in the 85–99% range within 60 days when *I. lacteus* was found to be the most efficient degrading strain under both of the tested conditions. Several new degradation products have been identified when mainly methoxy and hydroxy derivatives were produced together with reduced forms of the original acids. The results show that the fungi are probably able to transform CBAs *via* several pathways with significant reduction of toxicity during the process. The promising degradation results from this study emphasize the need for further research, especially to identify the participation of different enzymatic machineries, in order to improve the understanding of the degradation mechanisms. The results for the liquid media and from the consequent soil experiment show that the presence of a bioremediative organism is of key importance; however, in soil, i.e. under conditions with limited pollutant bioavailability, active colonization of the soil is of equal importance.

Acknowledgments

This work was supported by grants no. 2B06156 of the Ministry of Education, Youth and Sports of the Czech Republic and no. 525/09/1058 of the Science Foundation of the Czech Republic and by Institutional Research Concept No. AV0Z50200510.

References

[1] J.A. Field, R.S. Alvarez, Microbial transformation and degradation of polychlorinated biphenyls, Environ. Pollut. 155 (2008) 1–12.
[2] S.R. Sorensen, M.S. Holtze, A. Simonsen, J. Aamand, Degradation and mineralization of nanomolar concentrations of the herbicide dichlobenil and its persistent metabolite 2,6-dichlorobenzamide by *Aminobacter* spp. isolated from dichlobenil-treated soils, Appl. Environ. Microb. 73 (2007) 399–406.
[3] T. Gichner, P. Lovecká, B. Vrchotová, Genomic damage induced in tobacco plants by chlorobenzoic acids—metabolic products of polychlorinated biphenyls, Mutat. Res. 657 (2008) 140–145.
[4] M. Muccini, A.C. Layton, G.S. Sayler, T.W. Schultz, Aquatic toxicities of halogenated benzoic acids to *Tetrahymena pyriformis*, B. Environ. Contam. Tox. 62 (1999) 616–622.
[5] Y.H. Zhao, G.D. Ji, M.T.D. Cronin, J.C. Dearden, QSAR study of the toxicity of benzoic acids to *Vibrio fischeri*, *Daphnia magna* and carp, Sci. Total Environ. 216 (1998) 205–215.
[6] P.Y. Lee, C.Y. Chen, Toxicity and quantitative structure–activity relationships of benzoic acids to *Pseudokirchneriella subcapitata*, J. Hazard. Mater. 165 (2009) 156–161.
[7] K. Svobodová, M. Plačková, V. Novotná, T. Cajthaml, Estrogenic and androgenic activity of PCBs, their chlorinated metabolites and other endocrine disruptors estimated with two in vitro yeast assays, Sci. Total. Environ. 407 (2009) 5921–5925.
[8] D.H. Pieper, M. Seeger, Bacterial metabolism of polychlorinated biphenyls, J. Mol. Microbial. Biotech. 15 (2008) 121–138.
[9] S.A. Adebusoye, F.W. Picardal, M.O. Ilori, O.O. Amund, Influence of chlorobenzoic acids on the growth and degradation potentials of PCB-degrading microorganisms, World J. Microb. Biot. 24 (2008) 1203–1208.
[10] K. Furukawa, H. Fujihara, Microbial degradation of polychlorinated biphenyls: biochemical and molecular features, J. Biosci. Bioeng. 105 (2008) 433–449.
[11] D.P. Barr, S.D. Aust, Mechanisms white rot fungi use to degrade pollutants, Environ. Sci. Technol. 28 (1994) 78A–87A.
[12] C. Pinedo-Rivilla, J. Aleu, I.G. Collado, Pollutants biodegradation by fungi, Curr. Org. Chem. 13 (2009) 1194–1214.
[13] N. Kasai, S. Ikushiro, R. Shinkyo, K. Yasuda, S. Hirosue, Metabolism of mono- and dichloro-dibenzo-p-dioxins by *Phanerochaete chrysosporium* cytochromes P450, Appl. Microbiol. Biot. 86 (2010) 773–780.
[14] L. Kamei, R. Kogura, R. Kondo, Metabolism of 4,4′-dichlorobiphenyl by white-rot fungi *Phanerochaete chrysosporium* and *Phanerochaete* sp. MZ142, Appl. Microbiol. Biot. 72 (2006) 566–575.
[15] T. Cajthaml, Z. Křesinová, K. Svobodová, M. Möder, Biodegradation of endocrine-disrupting compounds and suppression of estrogenic activity by ligninolytic fungi, Chemosphere 7 (2009) 745–750.
[16] T. Cajthaml, P. Erbanová, V. Šašek, M. Möder, Breakdown products on metabolic pathway of degradation of benz[a]anthracene by a ligninolytic fungus, Chemosphere 64 (2006) 560–564.
[17] D. Ning, H. Wang, Y. Zhuang, Induction of functional cytochrome P450 and its involvement in degradation of benzoic acid by *Phanerochaete chrysosporium*, Biodegradation 21 (2010) 297–308.
[18] F. Matsuzaki, H. Wariishi, Functional diversity of cytochrome P450s of the white-rot fungus *Phanerochaete chrysosporium*, Biochem. Bioph. Res. Co. 324 (2004) 387–393.
[19] T. Cajthaml, P. Erbanová, A. Kollmann, Č. Novotný, V. Šašek, Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*, Folia Microbiol. 53 (2008) 289–294.
[20] B.R.M. Vyas, S. Bakowski, V. Šašek, M. Matucha, Degradation of anthracene by selected white rot fungi, FEMS Microbiol. Ecol. 14 (1994) 65–70.
[21] E. De Jong, A.E. Cazemier, J.A. Field, J.A. De Bont, Physiological role of chlorinated aryl alcohols biosynthesized de novo by the white rot fungus *Bjerkandera* sp. strain BOS55, Appl. Environ. Microbiol. 60 (1994) 271–277.

[22] E. Matsumura, E. Yamamoto, A. Numata, T. Kawano, T. Shin, S. Murao, Structure of the laccase-catalyzed oxidation products of hydroxybenzoic acids in the presence of ABTS [2,2-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid)], Agric. Biol. Chem. 50 (1986) 1355–1357.
[23] S. Covino, M. Čvančarová, M. Muzikář, K. Svobodová, A. D'annibale, M. Petruccioli, F. Federici, Z. Křesinová, T. Cajthaml, An efficient PAH-degrading *Lentinus* (*Panus*) *tigrinus* strain: effect of inoculum formulation and pollutant bioavailability in solid matrices, J. Hazard. Mater. 183 (2010) 669–676.
[24] Z. Křesinová, M. Muzikář, J. Olšovská, T. Cajthaml, Determination of 15 isomers of chlorobenzoic acid in soil samples using accelerated sample extraction followed by liquid chromatography, Talanta 84 (2011) 1141–1147.
[25] T. Cajthaml, M. Möder, P. Kačer, V. Šašek, P. Popp, Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection, J. Chromatogr. A 974 (2002) 213–222.
[26] ISO 11348-3: 2007 Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test) – Part 3: Method using freeze-dried bacteria.
[27] J. Lappalainen, R. Juvonen, K. Vaajasaari, M. Karp, A new flash method for measuring the toxicity of solid and colored samples, Chemosphere 38 (1999) 1069–1083.
[28] J. Lappalainen, R. Juvonen, J. Nurmi, M. Karp, Automated color correction method for *Vibrio fischeri* toxicity test. Comparison of standard and kinetic assays, Chemosphere 45 (2001) 635–641.
[29] J. Dittmann, W. Heyser, H. Bücking, Biodegradation of aromatic compounds by white rot and ectomycorrhizal fungal species and the accumulation of chlorinated benzoic acid in ectomycorrhizal pine seedlings, Chemosphere 49 (2002) 297–306.
[30] F. Guillen, A.T. Martinez, M.J. Martinez, C.S. Evans, Hydrogen-peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase, Appl. Microbiol. Biot. 41 (1994) 465–470.
[31] F. Matsuzaki, M. Shimizu, H. Wariishi, Proteomic and metabolomic analyses of the white-rot fungus *Phanerochaete chrysosporium* exposed to exogenous benzoic acid, J. Proteome Res. 7 (2008) 2342–2350.
[32] F. Matsuzaki, H. Wariishi, Molecular characterization of cytochrome P450 catalyzing hydroxylation of benzoates from the white-rot fungus *Phanerochaete chrysosporium*, Biochem. Biophys. Res. Commun. 334 (2005) 1184–1190.
[33] P. Baldrian, Wood-inhabiting ligninolytic basidiomycetes in soils: ecology and constraints for applicability in bioremediation, Fungal Ecol. 1 (2008) 4–12.