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Biodegradation of tetrabromobisphenol A by oxidases in basidiomycetous fungi and estrogenic activity of the biotransformation products

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ABSTRACT

Tetrabromobisphenol A (TBBPA) degradation was investigated using white rot fungi and their oxidative enzymes. Strains of the *Trametes, Pleurotus, Bjerkandera and Dichomitus* genera eliminated almost 1 mM TBBPA within 4 days. Laccase, whose role in TBBPA degradation was demonstrated in fungal cultures, was applied to TBBPA degradation alone and in combination with cellobiose dehydrogenase from *Sclerotium rolfsii.* Purified laccase from *Trametes versicolor* degraded approximately 2 mM TBBPA within 5 h, while the addition of cellobiose dehydrogenase increased the degradation rate to almost 2.5 mM within 3 h. Laccase was used to prepare TBBPA metabolites 2,6-dibromo-4-(2-hydroxypropane-2-yl) phenol (**1**), 2,6-dibromo-4-(2-methoxypropane-2-yl) phenol (**2**) and 1-(3,5-dibromo-4-hydroxyphen-1-yl)-2,2′,6,6′-tetrabromo-4,4′-isopropylidene diphenol (**3**). As compounds **1** and **3** were identical to the TBBPA metabolites prepared by using rat and human liver fractions (Zalko et al., 2006), laccase can provide a simple means of preparing these metabolites for toxicity studies. Products **1** and **2** exhibited estrogenic effects, unlike TBBPA, but lower cell toxicity.

1. Introduction

Brominated flame retardants (BFRs) improve the fire safety of various materials such as plastics and textiles. However, the high volumes of BFRs used, of which TBBPA is produced in the largest volumes, may be a cause for concern, since their biological properties, e.g. the potential interference of TBBPA with thyroid hormone pathways (Legler, 2008), are still not fully understood. The biological pathways by which TBBPA is degraded in the natural environment or which can be applied to its controlled biodegradation are of great interest, but very little is known about its potential microbial degraders. A recent study reported that TBBPA debromination under anaerobic conditions was achieved by using a bacterial and archaeal consortium, in which strains of *Pelobacter carbinolicus* and *Sphaerochaeta* sp. seemed to play an important role (Iasur-Kruh et al., 2010).

Degradation of TBBPA by *Trametes versicolor* CCBAS 612 recently reported by ourselves was probably the first example of using a white rot fungus for this purpose (Uhnáková et al., 2009). This prompted us to also examine other basidiomycetes and their extracellular enzymes such as laccase (EC 1.10.3.2) and cellobiose dehydrogenase (CDH; EC 1.1.99.18) as potential TBBPA degraders. The utility of laccase in TBBPA degradation was recently indicated in our study, which also reported a preliminary LC–MS analysis of the products as dibrominated monoaromatic compounds (Uhnáková et al., 2009). CDH has not been used for TBBPA degradation as far as we know. However, this enzyme seems to be an emerging biocatalyst with a remarkable biodegradation potential (Nyanhongo et al., 2007). To study the degradation mechanism of TBBPA in more detail, pure enzyme(s) – laccase plus CDH or either alone – were employed as catalysts. TBBPA and its degradation products were also examined with respect to their estrogenic effects by studying the proliferation and viability of MCF-7 human estrogen receptor-positive breast cancer cells – a commonly used model for the *in vitro* testing of compounds suspected of disrupting the endocrine system (Zava et al., 1997; Dorosh et al., 2011).

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2. Methods

2.1. Materials

Laccase from *T. versicolor* was purchased from Fluka. CDH from *Sclerotium rolfsii* was produced and purified as described previously (Baminger et al., 2001; Ludwig and Haltrich, 2003). All other chemicals were of analytical grade and purchased from commercial suppliers.

2.2. Microorganisms and maintenance

Bjerkandera adusta CCBAS 930, *Dichomitus squalens* CCBAS 750, *Lentinus edodes* CCBAS 389, *Phanerochaete chrysosporium* CCBAS 571, *Pleurotus ostreatus* CCBAS 477, *T. versicolor* CCBAS 612, *T. versicolor* CCBAS 613, *Trametes hirsuta* CCBAS 610, *Trametes gibbosa* CCBAS 806 (from the Culture Collection of Basidiomycetes, Institute of Microbiology AS CR, Prague, Czech Republic) and *Trametes villosa* CBS 678.70 (from the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) were maintained on wort agar slants at 4 °C. *Trametes pubescens* MB 89 (from the culture collection of the Institute of Applied Microbiology, University of Natural Resources and Life Sciences, Vienna, Austria) was maintained on glucose–maltose Sabouraud agar at 4 °C.

2.3. MCF-7 cell cultures

A human breast cancer estrogen-sensitive MCF-7 cell line (American Type Culture Collection, ATCC HTB-22) was maintained in Dulbecco's Minimal Essential Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in T-75 cm^2 flasks at 37 °C, in an atmosphere of 5% CO_2/95% air at saturating humidity. The stock culture was passaged every 4 days using a 0.25% trypsin-0.02% EDTA solution. Cells were inoculated into 24-well plates (NUNC) and estrogen-exhausted for three days in phenol-red-free DMEM supplemented with 10% dextran–charcoal-stripped FBS (prepared according to Jorgensen et al. (2000)) before treatment. Plasticware was chosen carefully to minimize medium contamination with xenoestrogenic compounds (Ishikawa et al., 2001). Cells were then exposed to the reaction products, TBBPA or 17-β-estradiol (positive control) for 3 days. The test chemicals were dissolved in ethanol (the final ethanol concentration in the culture medium did not exceed 0.2%, a concentration that did not affect cell growth).

2.4. Enzyme assays

Enzyme activities were determined in the filtrates of culture fluids. Laccase activity was assayed as described previously (Bourbonnais and Paice, 1990; modified by Uhnáková et al. (2009)). The activity of CDH was determined by 2,6-dichloroindophenol assay as described by Ludwig et al. (2004).

2.5. Biodegradation

TBBPA was degraded with cultures of basidiomycetous fungi grown under laccase- and/or CDH-inducing conditions, and with purified enzymes (laccase and/or CDH). Laccase was used in preparative-scale biodegradations; the reaction products were isolated, purified and characterized by NMR and MS. The experimental scheme is shown in Fig. 1.

2.5.1. Biodegradations of TBBPA in fungal cultures

The fungal strains were grown as described previously (Uhnáková et al., 2009) in a mineral medium with 3,4-dimethoxybenzyl alcohol (veratryl alcohol; a laccase inducer) added to a final concentration of 13 mM (if not otherwise stated). *Trametes* strains were also grown in the same medium (with or without veratryl alcohol), in which sucrose was replaced with α-cellulose (30 g L^{-1} – for CDH induction). From the day TBBPA (1 mM) was added (from 50 mM stock solutions in methanol; 4 days after inoculation), samples were withdrawn at 1-day intervals and their extracellular laccase activity (see below) and TBBPA concentration (quantified by reversed-phase HPLC; Uhnáková et al., 2009) determined. TBBPA concentration was also determined in mycelial extracts. The mycelium from each flask was extracted with 10 mL of methanol three times, the extracts were combined and their TBBPA concentration determined using the same method. In

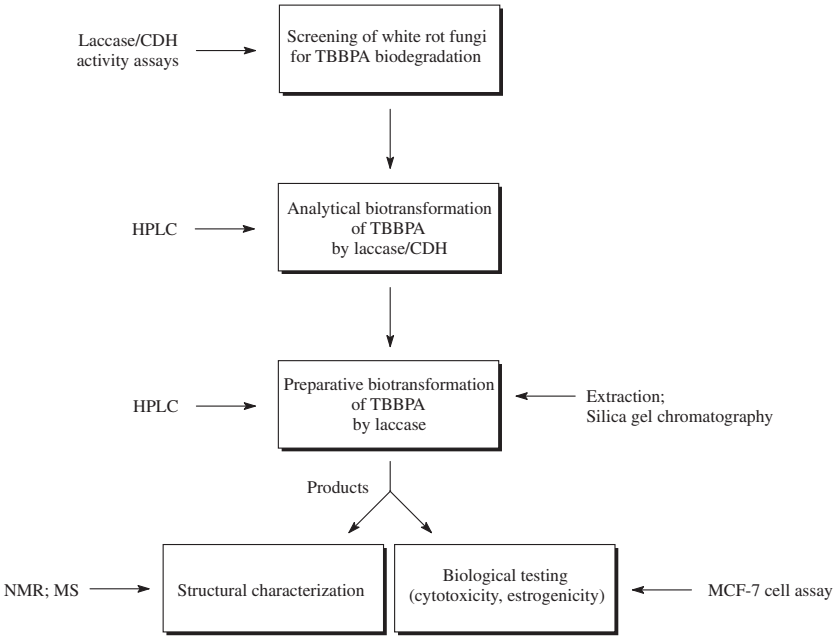

Fig. 1. Scheme of biodegradation experiments.