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Degradation of bisphenol A and acute toxicity reduction by different thermo-tolerant ascomycete strains isolated from arid soils

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ABSTRACT

Four different laccase-producing strains were isolated from arid soils and used for bisphenol A (BPA) degradation. These strains were identified as *Chaetomium strumarium* G5I, *Thielavia arenaria* CH9, *Thielavia arenaria* HJ22 and *Thielavia arenaria* SM1(III) by internal transcribed spacer 5.8 S rDNA analysis. Residual BPA was evaluated by HPLC analysis during 48 h of incubation. A complete removal of BPA was observed by the whole cell fungal cultures within different times, depending on each strain. *C. strumarium* G5I was the most efficient degrader, showing 100% of removal within 8 h of incubation. The degradation of BPA was accompanied by the production of laccase and dye decolorizing peroxidase (DyP) under degradation conditions. The presence of aminobenzotriazole (ABT) as an inhibitor of cytochrome P450s monooxygenases (CYP) demonstrated a slight decrease in BPA removal rate, suggesting the effective contribution of CYP in the conversion. The great involvement of laccase in BPA transformation together with cell-associated enzymes, such as CYP, was supported by the identification of hydroxylated metabolites by ultra-high performance liquid chromatography-mass spectroscopy (UHPLC-MS). The metabolic pathway of BPA transformation was proposed based on the detected metabolites. The acute toxicity of BPA and its products was investigated and showed a significant reduction, except for *T. arenaria* SM1(III) that did not caused reduction of toxicity (IC_{50} < 8%), possibly due to the presence of toxic metabolites. The results of the present study point out the potential application of the isolated ascomycetes in pollutant removal processes, especially *C. strumarium* G5I as an efficient degrader of BPA.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a wide group of synthetic compounds of anthropogenic origin (Erkurt, 2015). Some of them are known for their capacity to mimic and interfere with the action of endogenous hormones and thus adversely affect wildlife and human health by disrupting development, reproduction, growth, and neurological and immune systems (Daâssi et al., 2016; de Freitas et al., 2017). Bisphenol A (BPA; 2,2-bis (4-hydroxyphenyl) propane) is an organic compound with two phenolic rings connected by a bridge with two methyl functional groups (Kamaraj et al., 2014). This compound is widely used as a monomer in the production of a great variety of consumer products, including food plastic packing materials, adhesives, powder paints, thermal paper, polycarbonate plastics, epoxy resins and polyesters (Telke et al., 2009; Zhang et al., 2013; Kamaraj et al., 2014). This has led to the widespread and continuous increase in the environmental contamination by BPA detected in soils, surface water, sediments (fresh and marine) groundwater, drinking water and wastewater (Eio et al., 2014). The United States Environmental Protection Agency (USEPA) has identified bisphenol A as an environmental endocrine disruptor due to its estrogenic and genotoxic activities and continues to assess the safety of BPA in cooperation with international regulatory and public health counterparts (Zhang et al., 2013).

Due to the bioactivity, toxicity and stability structure of BPA even at low but environmentally high concentrations, the conventional physicochemical technologies, such as Fenton reaction (Mohapatra et al., 2011), ozonation (Wang et al., 2014), photodegradation (Zhang et al., 2013) and ultrasonic oxidation (Yu et al., 2014), are not effective to remove these compounds, they only transfer the pollutant from one phase to another. For this reason, biodegradation involving microorganisms has been proven to be an advanced biological approach to remove the toxic BPA compounds from the environment in a safe and economic way (Zhang et al., 2013; Eio et al., 2014; Agrawal and Shahi, 2017). Ligninolytic fungi, belonging mostly to basidiomycetes, have

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received considerable attention for their biodegradation potential (Lee et al., 2005; Harms et al., 2011; Fouda et al., 2015). The ability of these fungi to transform BPA has been related to the production of non-specific extracellular oxidative enzymes, i.e. lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), versatile peroxidase (VP, EC 1.11.1.16) and laccase (or polyphenol oxidase, Lac EC 1.10.3.2) (Daâssi et al., 2016; Kabiersch et al., 2011; Taboada-Puig et al., 2011). Their non-specific nature facilitates the oxidation and mineralization of numerous substrates (Marco-Urrea and Reddy, 2012). Furthermore, ascomycete fungi represent an extremely diverse phylum (Whiteway et al., 2015) and they have demonstrated an effective role in the removal of organic pollutants (Reyes-Cesar et al., 2014; Zafra et al., 2014; Marco-Urrea et al., 2015; Yao et al., 2015). In fact, it has been demonstrated that a broad range of xenobiotics can be more effectively removed by whole-cell fungal treatment compared to enzymatic treatment, due to the combined effects of intracellular, extracellular and mycelium-bound enzymes and of sorption to the fungal biomass (Nguyen et al., 2014a; Nguyen et al., 2014b; Hofmann and Schlosser, 2016). Additionally, some Ascomycota fungi have oxidative pathways catalyzed by extracellular oxidative enzymes, including laccases and peroxidases (such as dye-decolorizing peroxidases (DyPs) and heme-containing peroxidases), which have been identified in the last decade and are widespread among several ascomycete fungi, according to the genome database (Büttner et al., 2017).

Most reports addressing the ascomycete fungal biotransformation and degradation of xenobiotics showed that the detoxification metabolism is catalyzed by phase I and phase II enzymes, mainly cytochrome P450 (CYP) proteins (Morel et al., 2013). This intracellular detoxification system constitutes the predominant pathway in the initial oxidation step of micropollutants (Cerniglia, 1997). Cytochrome P450 monooxygenases (CYP) and epoxide hydrolases (EHs) catalyze phase I oxidation reactions. The phase II conjugation reactions are mediated by transferases, including glutathione S-transferase, UDP-glucuronosyltransferase and NAD(P)H quinone oxidoreductase. This is followed by the phase III reactions which involve the transportation of the conjugated metabolites to vacuoles for the excretion of their respective derivatives, catalyzed by membrane transporters (Cerniglia, 1997). Cerniglia (1997) reported that conjugation pathways lead to detoxification whereas the oxidation products, like dihydrodiol epoxides, may be bioactive and toxic. Despite the attractive role of these intra- and extracellular enzymes in the detoxification pathways, their metabolisms have not been investigated in depth due mainly to the various enzymatic profiles. These profiles differ widely among different strains even of the same family. To our knowledge, the degradation of BPA and the enzyme mediated systems of *Chaetomium* and *Thielavia* species, isolated from soils, have not previously been described. In the present study, we investigated the removal of BPA by novel laccase-producing ascomycete strains. The degradation of BPA was monitored by high performance liquid chromatography (HPLC) and the resulting metabolites were studied by ultra high performance liquid chromatography-mass spectroscopy (UHPLC-MS). A metabolic pathway of BPA degradation was then suggested. Finally, Microtox® bioassays were performed to evaluate the acute toxicity of the fungal culture media at different assay times.

2. Material and methods

2.1. Chemicals

Bisphenol A (BPA: 2,2-bis (4-hydroxyphenyl) propane) (99% purity)), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (96% purity) and 1-aminobenzotriazole (98% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents, HPLC gradient grade acetonitrile and water were purchased from VWR (Barcelona, Spain). All other chemicals were of analytical grade.

2.2. Isolation, screening and fungal identification

Fungal strains were isolated from soil samples collected from arid regions in the south of Tunisia using the method of Waksman (1992). They were screened for laccase production on Malt Extract Agar (MEA) plates amended by 2,6-dimethoxyphenol (2,6-DMP) as described by Mtibaà et al. (2017). Among the isolated laccase-producing fungi, a total of four strains were selected for the BPA degradation study on the basis of their notable laccase production. They were genetically identified by amplifying, cloning and further sequencing fragments corresponding to the internal transcribed spacer (ITS) region in their total genomic DNA. The PCR amplification was performed using universal primers ITS1 (5′–TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCG CTTATTGATATGC) (White et al., 1990). PCR reaction mixtures, amplification conditions, electrophoresis and products purification were performed as described by Godoy et al. (2016). The resulted PCR products were analyzed using an ABI 3130xl automatic capillary sequencer (Applied Biosystems). A homology search was performed by using the BLAST software tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare nucleotide acid sequence similarities against the GenBank database. The obtained homologous sequences were used for ClustalW multiple sequence alignments (Thompson et al., 1994) using BioEdit software (Hall, 1999). The phylogenetic tree was inferred by using the Maximum Likelihood estimation method based on the Tamura-Nei model (Tamura et al., 2004). The evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). Neighbor-Joining and BIONJ algorithms were applied to a matrix to generate the phylogenetic tree and the one showing the highest log likelihood was selected.

2.3. Fungal culture conditions

Cultures were carried out in 250 mL Erlenmeyer flasks containing 50 mL of synthetic medium composed of 2.5% glucose, 0.35% soya peptone and 0.4% yeast extract at pH 6.0. In order to induce extracellular laccase, fungi were cultured in an orbital shaking incubator at 120 rpm and 35 °C for 4 days in presence of 0.25 mM $CuSO_4$ as inducer (Farnet et al. (1999) according to a previous optimization process (data not shown). Inoculation was carried out using 0.5 mL of mycelial suspension prepared as described by Aranda et al. (2009). BPA diluted in acetonitrile was aseptically added to the cultures at a final concentration of 0.1 mM after 2 days of incubation. Heat-inactivated mycelium and non-inoculated cultures were used as adsorption and abiotic controls, respectively, as reported by Aranda et al. (2017). In order to study the role of CYP on BPA degradation, additional cultures included 1 mM of the CYP inhibitor 1-aminobenzotriazole (ABT). Experiments were performed in triplicate for 2 days. Samples of each individual flask were taken every 2 h to analyze the extracellular enzymatic activities (laccase and dye decolorizing peroxidase (DyP)), sugar content and pH. BPA concentrations in the remaining liquid culture were determined by HPLC as described previously by Aranda et al. (2010). The reported data represent the mean values with standard deviation of triplicate measurements.

2.4. Enzyme assays

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 0.3 mM ABTS at 420 nm ($\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$) (Eggert et al., 1995) in 50 mM sodium citrate buffer (pH 4.5). Dye decolorizing peroxidase activity (DyP) was measured as previously described by Liers et al. (2010). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the oxidation of one micromole of substrate per minute and per milliliter (Telke et al., 2009).