Journal of Environmental Management 111 (2012) 115–119

Contents lists available at SciVerse ScienceDirect

Journal of Environmental Management

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

Identification of metabolites from benzo[*a*]pyrene oxidation by ligninolytic enzymes of *Polyporus* sp. S133

Tony Hadibarata [a,*], Risky Ayu Kristanti [b]

[a] *Institute of Environmental and Water Resources Management, Faculty of Civil Engineering, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor Bahru, Malaysia*
[b] *Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, 4-3-11, Takeda, Kofu-city, Yamanashi 400-8511, Japan*

ARTICLE INFO

Article history:
Received 9 March 2009
Received in revised form
21 June 2012
Accepted 27 June 2012
Available online 24 July 2012

Keywords:
Benzo[*a*]pyrene
Biotransformation
Dioxygenase
Metabolites
Phenoloxidase
Polyporus sp. S133

ABSTRACT

The biodegradation of benzo[*a*]pyrene (B*a*P) by using *Polyporus* sp. S133, a white-rot fungus isolated from oil-contaminated soil was investigated. Approximately 73% of the initial concentration of B*a*P was degraded within 30 d of incubation. The isolation and characterization of 3 metabolites by thin layer chromatography, column chromatography, and UV–vis spectrophotometry in combination with gas chromatography–mass spectrometry, indicated that *Polyporus* sp. S133 transformed B*a*P to B*a*P-1,6-quinone. This quinone was further degraded in 2 ways. First, B*a*P-1,6-quinone was decarboxylated and oxidized to form coumarin, which was then hydroxylated to hydroxycoumarin, and finally to hydroxyphenyl acetic acid by addition of an epoxide group. Second, *Polyporus* sp. S133 converted B*a*P-1,6-quinone into a major product, 1-hydroxy-2-naphthoic acid. During degradation, free extracellular laccase was detected with reduced activity of lignin peroxidase, manganese-dependent peroxidase and 2,3-dioxygenase, suggesting that laccase and 1,2-dioxygenase might play an important role in the transformation of PAHs compounds.

1. Introduction

Environment pollution is a worldwide problem that can result in the uptake and accumulation of toxic chemicals in food chains, as well as cause harm to the flora and fauna in the affected habitats. Although substantial progress has been made over the recent decade to eliminate industrial pollution, major instances of chemical release still occur. In addition, a considerable number of polluted sites have been identified, and new ones are continually being discovered (Makkar and Rockne, 2003). Polycyclic aromatic hydrocarbons (PAHs) are one of the main kinds of widely distributed environmental pollutants that are byproducts of fuel combustion. Adverse health effects of these compounds are concerning because of their tendency to persist in the environment, and their toxic and carcinogenic properties. B*a*P, a symmetrical PAH consisting of 5 condensed benzene rings, is produced by incomplete combustion of organic materials such as fossil fuels, other industrial processes, and natural occurring forest fires. Microbial biodegradation is one of the most important natural processes that can influence the fate of pollutants in both terrestrial and aquatic environments. Bacteria, fungi, and algae such as *Rhodococcus* sp., *Pleurotus ostreatus*, *Trichoderma* sp., *Ochrobactrum* sp., and *Selenastrum capricornutum* play important roles in aromatic hydrocarbons biotransformation (Dean-Ross et al., 2001; Andersson et al., 2003; Chan et al., 2006; Hadibarata et al., 2007; Arulazhagan and Vasudevan, 2011).

B*a*P degradation has been extensively studied in many microorganisms, in particular in bacteria (Su et al., 2006). However, pathways for B*a*P catabolism by the white-rot fungi *Polyporus* species are not well explored. White-rot fungi metabolize B*a*P at different sites of the molecule under mesophilic conditions, presumably via the action of ligninolytic enzymes and dioxygenase on the aromatic nucleus. Meanwhile, degradation pathways have not been studied under thermophilic conditions although thermophilic hydrocarbon degraders are needed for the bioremediation of oil-polluted soil and the composting or mitigation of paraffin deposition-related problems (Sorkoh et al., 1993; Ma et al., 2003; Sood and Lal, 2008). Hence, the current study represents an effort to characterize the structures of B*a*P metabolites produced by this *Polyporus* species under thermophilic condition. Using thin layer chromatography (TLC), UV–vis Spectrophotometry, gas chromatography–mass spectrometry (GC–MS), and enzymatic assay, the ring cleavage pathway of B*a*P transformation was studied along with identification of key metabolites.

* Corresponding author. Tel./fax: +60 7 5531760.
E-mail addresses: hadibarata@utm.my, hadibarata@yahoo.com (T. Hadibarata).

0301-4797/$ – see front matter
http://dx.doi.org/10.1016/j.jenvman.2012.06.044

2. Materials and methods

2.1. Chemicals

B*a*P and the reference compounds (≥98% pure) for the identification of metabolites were purchased from Sigma–Aldrich. Malt extract and polypeptone were purchased from Difco (Detroit, USA). Thin layer chromatography (TLC) aluminum sheets (Silica gel 60 F_{254}, 20 × 20 cm) were obtained from Merck (Darmstadt, Germany). B*a*P-1,6-quinone was prepared from B*a*P by a modified method using dichromate oxidation (Vogel, 1989). B*a*P in glacial acetic acid (at ~90 °C) was placed in tightly capped flasks and stirred at 110 °C for 23 h. After cooling, ethyl acetate and H_2O were added to the mixtures, followed by shaking. The ethyl acetate fraction was collected, and the H_2O fraction was more 5 times extracted with ethyl acetate. Then, the 35% concentrated crude B*a*P-1,6-quinone was purified by column chromatography.

2.2. Microorganism and growth conditions

The fungus was maintained at 4 °C prior to use, in a plastic petri dish containing malt extract agar (2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) polypeptone, and 1.5% (w/v) agar). *Polyporus* sp. S133 was selected on the basis of its ability to degrade B*a*P in 20 ml of malt extract agar supplemented with B*a*P dissolved in dimethylformamide (DMF) and 300 mg/l benomill for bacterial growth inhibition, and then incubated at room temperature for 2 weeks with daily observation. A single colony of B*a*P-degrading fungus was transferred to a mineral medium containing B*a*P. Modified mineral salt broth medium was used for degradation studies (Hadibarata et al., 2011). The fungal inoculum was prepared by growing fungus on malt extract agar at 25 °C for 7 d. The inoculum was added to a flask containing the mineral medium. The cultures were incubated at 35 °C on a shaker (120 rpm) for 7 d and 14 d. Moreover, cells grown in the presence of B*a*P were washed twice in liquid medium and used as an inoculum for metabolite degradation studies. Control experiments were performed with autoclaved *Polyporus* sp. S133 cells.

2.3. Biomass determination

The culture broth was centrifuged at 1000 rpm for 45 min. The pellet was then removed, washed, and filtered through a pre-dried, pre-weighed filter paper. The filter paper was dried until a constant weight was reached. The dry weight of the biomass was determined in miligram per liter (mg/l).

2.4. Preparation of cell-free extract and enzyme assays

Cells grown in the presence of B*a*P were harvested at different time intervals (15 and 30 d). The cultures were centrifuged (10,000 rpm, 15 min) at 25 °C and rapidly washed 3 times with 50 mM phosphate buffer (pH 7). Cells were disintegrated with a probe-type sonic oscillator for 10 min. The extract was centrifuged at 15,000 rpm at 4 °C for 20 min to remove whole cells and large debris. The supernatant was used immediately for the enzymatic assays-phenoloxidases and dioxygenases activity. Manganese peroxidase activity was assayed using 50 mmol/l malonate buffer and dimethoxyphenol in 20 mmol/l $MnSO_4$ (Wariishi et al., 1992). Laccase activity was assayed using syringaldazine in 100 mmol/l sodium acetate buffer (Tien and Kirk, 1984). Lignin peroxidase activity was determined using veratryl alcohol as a substrate (Kuwabara et al., 1984). A modified previously applied method was used to measure the activities of 1,2-dioxygenase and 2,3-dioxygenase, the associated activities were assayed using catechol as the substrate (Nakazawa and Nakazawa, 1970). One unit of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute and the activity was expressed in U/l.

2.5. Extraction and analysis of metabolites

After growth on B*a*P, the contents of the flasks were blended with ethyl acetate and acidified with 1 N HCl. The filtrate (liquid medium) and residue (fungal body) were separated by filtration and the filtrate was extracted with 3 equal volumes of ethyl acetate. The residual extracts were dried using anhydrous sodium sulfate and concentrated under reduced pressure at room temperature by using a vacuum concentrator. The B*a*P's degradation products present in the culture extracts were analyzed by TLC on silica gel 60 F_{254} plates (20 × 20 cm, thickness 0.25 mm), by using hexane–dichloromethane (30:15, v/v) as the solvent system for short-term incubation, and dichloromethane–ethyl acetate (10:30 v/v/v), for long-term incubation. The UV light was used to detect the position of the compounds on the TLC plates. The R_f values of the detected spots were compared with those of authentic compounds known or suspected to be B*a*P degradation products. B*a*P and its metabolites were detected under UV illumination at 254 nm. UV–vis absorption spectra were recorded using a UV–vis spectrophotometer.

Derivatization of carboxylic acids and aromatic hydroxyl groups for GC–MS analysis was performed using a mixture of N,O-bis-trimethylsilyl acetamide, pyridine, and trimethylchlorosilane (40:40:20 v/v/v) at 80 °C for 10 min. A portion of the resulting products was injected into a TC-1 capillary column (30 m × 0.25 mm; ID 0.25 μm) using a gradient at 60 °C for 2 min, raised to 150 °C at 15 °C/min, then raised to 300 °C at 25 °C/min, and maintained at 300 °C for 6 min. The injector and interface temperatures were 260 °C. We used GC–MS analysis to confirm the metabolites of B*a*P degradation and to determine the degradation pathway. The conditions for GC–MS consisted of the use of a detector at 1.3 eV, scan intervals of 1 s, and a mass range of 50–500. Mass profiles were also compared with the spectra of similarly analyzed authentic standards. Mass spectra of individual total ion peaks were identified by comparison with authentic standards and the Wiley 275 L mass spectra database. The metabolites were identified by comparing their TLC analyses, UV absorption spectra, retention times, and mass spectra with those of the corresponding authentic standards.

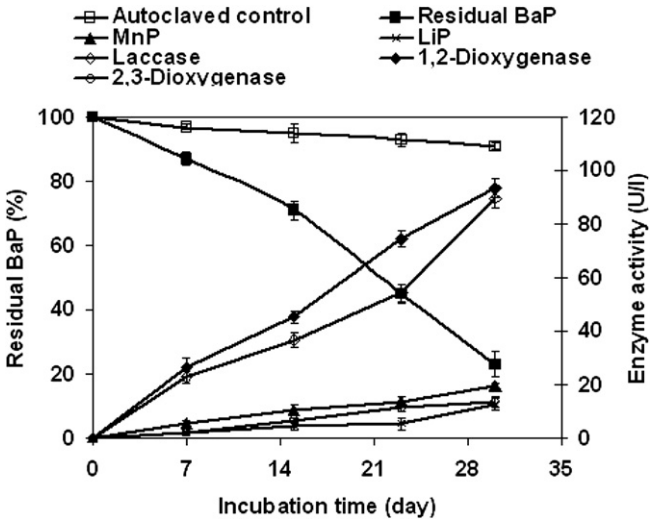

Fig. 1. Residual B*a*P and enzyme activity of *Polyporus* sp. S133.