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Degradation of polycyclic aromatic hydrocarbons (2–7 rings) under microaerobic and very-low-oxygen conditions by soil fungi

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ABSTRACT

Soil fungi were studied regarding their ability to degrade polycyclic aromatic hydrocarbons (PAHs) and produce ligninolytic enzymes under microaerobic and very-low-oxygen conditions. Several PAHs were used as substrates for soil fungi under microaerobic and very-low-oxygen conditions. Activities of lignin-peroxidase, manganese-peroxidase, and laccase were monitored over a 30-day period. PAH degradations were analyzed using C_{18} reversed-phase HPLC. Low-molecular-weight PAHs (LMW-PAHs, 2–3 rings) were degraded most extensively by *Aspergillus* sp., *Trichocladium canadense*, and *Fusarium oxysporum*. When growing on high-molecular-weight PAHs (HMW-PAHS, 4–7 rings), the highest degradations were reached by *T. canadense*, *Aspergillus* sp., *Verticillium* sp., and *Achremonium* sp. In this study, these fungi revealed a great capability to degrade a broad range of PAHs under low-oxygen conditions. In addition, lignolytic enzyme activities were observed during fungal growth on these compounds. These results suggest fungi have the ability to bioremediate PAH-contaminated soils and that they use these compounds as carbon sources for growth.

1. Introduction

The ability to catabolize PAHs is commonly found in aerobic organisms, with the presence of oxygen being required for the initial breakdown of the aromatic rings (Cerniglia and Heitkamp, 1989). Fungi, including saprophytic, plant root-parasitic ground fungi, and ectomycorrhizal and mitosporic fungi, as well as wood-decaying fungi, have been shown to degrade PAHs, indicating their potential as environmental bioremediators of these compounds. To the wood-decaying fungi, the average rates of PAH conversion have been correlated with average activities of ligninolytic enzymes (Gramss et al., 1999; Bamforth and Singleton, 2005; Eibes et al., 2006). White-rot ligninolytic basidiomycete fungi, such as *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Trametes* spp., *Bjerkandera* spp., and *Coriolopsis* spp. are considered good producers of complex ligninolytic extracellular enzymes (peroxidases and phenoloxidases) that are effective not only for the degradation of lignin but also for the degradation of several aromatic compounds due to the nonspecificity of these enzymatic systems (Tekere et al., 2005; Tortella et al., 2005). Non-basidiomycete filamentous fungi are also involved in the degradation of PAHs: *Penicillium* spp. (Saraswathy and Hallberg, 2005), *Aspergillus* ssp. (Cortes-Espinosa et al., 2006), *Fusarium* sp. (Potin et al., 2004), *Trichoderma* ssp. (Mollea et al., 2005), *Mucor* sp. and *Cladosporium* sp. (Salvo et al., 2005), have been shown to efficiently degrade PAHs under aerobic conditions with high potential to degrade HMW-PAHs.

In contrast, PAHs accumulate in areas where oxygen levels are low or absent, indicating that under these conditions their degradation is significantly reduced in comparison to aerobic conditions where oxygen is used for the initial cleavage of these compounds. Microorganisms able to degrade PAHs in areas with low-oxygen levels use terminal electron acceptors other than oxygen. These microorganisms can be divided into facultative anaerobes (nitrate, iron, and manganese-reducing) and obligate anaerobes (sulphate-reducing) (McRae and Hall, 1998).

Fungi have been isolated from habitats with low-oxygen levels, and these organisms have a great capability of degrading lignocellulosic wastes by producing ligninolytic enzymes under microaerobic and anaerobic conditions (Durrant et al., 1995; Durrant, 1996; Pavarina and Durrant, 2002). However, we know of no reports on the degradation of these compounds by fungi under microaerobic conditions. The present work provides data demonstrating the ability of fungi to degrade PAHs under low-oxygen conditions and suggesting that fungi may be a useful tool in bioremediation of PAH-contaminated environments with low-oxygen levels.

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2. Materials and methods

2.1. Chemicals

Naphthalene, phenanthrene, chrysene, perylene, naphthol[2,3-a]pyrene and decacyclene were purchased from Sigma (USA), at a 97% purity level or higher. Substrates used in the enzymatic activity assays (syringaldazyne, veratryl alcohol, and phenol red) were acquired from Sigma–Aldrich. Organic solvents used in PAH extractions and HPLC (ethyl acetate and acetonitrile, respectively) were obtained from Mallinckrodt Baker (USA), at 99.5% purity or higher.

2.2. Microorganisms

Seven soil fungi were used in this study. Four of the seven were a gift from the Tropical Culture Collection – Fundação André Tosello (http://www.fat.org.br), Campinas-SP, Brazil, and were isolated from soil samples collected at the Juréia-Itatins Ecological Reserve (located in the southern coastal area of São Paulo State, between latitudes 24°17′ and 24°40′S and longitudes 47°00′ and 47°30′W), Brazil, and classified as Deuteromycetes and identified as *Achremonium* sp., *Aspergillus* sp., *Trichoderma* sp., and *Verticillium* sp. Two strains, *Trichocladium canadense* and the basidiomycete strain H2, were isolated under microaerobic conditions from soil samples and identified as described by Durrant et al. (1995) and Durrant (1996). *Fusarium oxysporum* was a gift from Dr. S. Leschine (Dept. of Microbiology, UMASS, MA, USA). The procedure for strain identification involved detailed observation of fungal morphology using phase-contrast microscopy. For visualization lactophenol cotton blue (Dring, 1971) was used to stain fungal structures. The strains were identified with the help of a specific manual (Barnett and Hunter, 1972). These strains have previously been described as having ligninolytic activity and the ability to grow on and degrade PAHs under aerobic conditions (Clemente et al., 2001; Clemente and Durrant, 2003, 2005). All fungi were maintained on PDA (potato dextrose agar—DIFCO) and GYP (0.2% glucose, 0.5% yeast extract, 1.0% peptone, and 1.0% agar) slants. Agar plates containing these media were inoculated with a mycelium portion of each fungus and incubated at 30 °C for 7–10 days. To inoculate liquid medium containing PAHs, a 3 × 1 cm^2 sample of mycelia was removed aseptically from the agar plates and transferred to Erlenmeyer flasks containing 30 ml of salt basal medium, trace elements, vitamins, and PAHs as described below.

2.3. Culture conditions

Fungal strains were grown under microaerobic (5–15% of O_2 and 10% of CO_2) or very-low-oxygen (<1% O_2) conditions for 30 days in 125-ml Erlenmeyer flasks containing 30 ml of salt basal medium containing, per liter: 1 g KH_2PO_4; 0.5 g $(NH_4)_2HPO_4$; 0.3 g $CaCl_2$; 1 g cysteine chloridrate as reducing agent to remove oxygen; 1 ml 0.1% resazurine as redox indicator dye (pH 5.5–6.0); 0.5 ml of vitamin solution; 1 ml of trace elements solution; and one of the following PAHs: (0.5% w/v naphthalene or phenanthrene, or 0.05% w/v chrysene, perylene, naphthol [2,3-a]pyrene or decacyclene). Stock solutions (100×) for all PAHs were prepared by dissolving them in a mixture of hexane and acetone, 3:1 (v/v), before use. The solutions were added to the flasks to give the desired final concentrations, which were then left for 3–4 h in a sterile cabinet until all the solvents had evaporated (Anderson and Henrysson, 1996). The trace elements solution contained, per liter, 0.22 g ammonium tartarate, 0.66 g manganese sulphate, 0.15 g iron sulphate, 0.10 g cobalt sulphate, 0.10 g zinc sulphate, 0.64 g copper sulphate, and 10 mg aluminium potassium sulphate. The vitamins solution contained, per liter: 5 mg thiamine, 2 mg biotine, 5 mg nicotinic acid, 0.1 mg cianocobalamine, 2 mg folic acid, 5 mg riboflavin, 10 mg pyridoxine, 5 mg DL-pantetonate Ca^{+2}, and 5 mg thiotic acid. Microaerobic and very-low-oxygen conditions were achieved using L-cysteine hydrochloride as the oxygen scavenger and resazurine as the redox indicator in the medium, by placing culture flasks in sealed jars containing microaerobic or anaerobic gas generator envelopes (Probac-Brazil), reducing the oxygen level to 5–15% or less than 1%, respectively and generating an enriched carbon dioxide environment within the incubator jars after the system was properly activated according to the manufacturer's instructions.

Samples were incubated for 30 days at 30 °C in sealed jars. Abiotic controls were kept in flasks containing the same amount of liquid medium and PAH, without mycelial inoculum. Inoculated controls containing liquid medium but no added PAH or liquid medium plus solvent used for dissolving PAHs but no added PAH were also run.

The culture supernatants were obtained by centrifugation for subsequent HPLC analyses, and enzymatic assays were carried out immediately after sampling. All experiments, including controls, were performed in duplicate.

2.4. Enzymatic assays

Lignin-peroxidase (LiP, EC1.11.1.14) activity was assayed by determination of veratryl alcohol oxidation to veratryl aldehyde (Tien and Kirk, 1983), by mixing in a 1-ml cuvette the supernatant (0.6 ml), 2 mmol l^{-1} of veratryl alcohol in 0.4 mol l^{-1} tartaric buffer pH 3 (0.2 ml), and 2 mmol l^{-1} of H_2O_2 (0.2 ml). After a 10-min incubation period at room temperature, absorbances were measured at 310 nm. Manganese-peroxidase (MnP, EC1.11.1.13) activity was determined by the phenol red oxidation (Kuwahara et al., 1984): 0.5 ml of culture supernatant was added to a 1-l cuvette containing 0.25 mol l^{-1} of sodium lactate (0.1 ml), 2 mmol l^{-1} $MgSO_4$ (0.05 ml), 5% bovine serum albumin (0.2 ml), 2 mmol l^{-1} H_2O_2 in 0.2 mol l^{-1} of succinate buffer pH 4.5 (0.05 ml), and 1% phenol red (0.1 ml). After 5 min of incubation at 30 °C, 2 M NaOH (40 μl) was added to cease the reaction, and absorbances were measured at 610 nm. Laccase (Lac, EC1.10.3.2) was measured via the H_2O_2-independent oxidation of syringaldazine to its quinone form as described by Szklarz et al. (1989): a volume of 0.6 ml of culture supernatant was added to a cuvette, followed by 0.05 mol l^{-1} citrate-phosphate buffer (0.2 ml), distilled water (0.1 ml), and 0.1% seringaldazine in absolute ethanol (0.1 ml). After 10 min of reaction, absorbances were measured at 525 nm (Singh et al., 2007).

All the activities are expressed in μmol of substrate oxidized per liter per minute, in the following equation: U l^{-1} min^{-1} = ΔAbs (10^6) $(\varepsilon RT)^{-1}$, where ΔAbs is the difference between final and initial absorbances, ε is the extinction coefficient of each enzyme product (LiP – ε_{310} = 9300 mol^{-1} cm^{-1}; MnP – ε_{610} = 4460 mol^{-1} cm^{-1}; Lac – ε_{525} = 65 000 mol^{-1} cm^{-1}), R is the volume in milliliters of supernatant, and T is the reaction time in minutes.

2.5. HPLC analyses of the extracted PAHS for the determination of degradation

Extraction of PAHs and HPLC analyses were carried out as described by Anderson and Henrysson (1996): naphtalene, phenanthrene, crysene, perylene, naphthol[2,3-a]pyrene, and decacyclene were extracted from each flask containing liquid medium plus fungal growth with ethyl acetate (v/v) in a separatory funnel and concentrated using rotatory evaporation as follows. The pH of the media plus mycelia was adjusted to 2–3 by addition of 0.5 M HCl (Launen et al., 1995), followed by addition of an equal volume of ethyl acetate with vigorous shaking. Samples were then sonicated in an ice bath for 3 min, using 30-s bursts with a 1-min interval