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Marine-derived filamentous fungi and their potential application for polycyclic aromatic hydrocarbon bioremediation

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ABSTRACT

Eight marine-derived fungi that were previously selected for their abilities to decolorize RBBR dye were subjected to pyrene and benzo[a]pyrene degradation. The fungus *Aspergillus sclerotiorum* CBMAI 849 showed the best performance with regard to pyrene (99.7%) and benzo[a]pyrene (76.6%) depletion after 8 and 16 days, respectively. Substantial amounts of benzo[a]pyrene (>50.0%) depletion were also achieved by *Mucor racemosus* CBMAI 847. Therefore, these two fungal strains were subjected to metabolism evaluation using the HPLC-DAD-MS technique. The results showed that *A. sclerotiorum* CBMAI 849 and *M. racemosus* CBMAI 847 were able to metabolize pyrene to the corresponding pyrenylsulfate and were able to metabolize benzo[a]pyrene to benzo[a]pyrenylsulfate, suggesting that the mechanism of hydroxylation is mediated by a cytochrome P-450 monooxygenase, followed by conjugation with sulfate ions. Because these fungi were adapted to the marine environment, the strains that were used in the present study are considered to be attractive targets for the bioremediation of saline environments, such as ocean and marine sediments that are contaminated by PAHs.

1. Introduction

The marine ecosystem represents a largely unexplored niche for unidentified fungi that could potentially be used in biotechnological processes. Marine-derived fungi that are able of degrading pollutants such as polycyclic aromatic hydrocarbons (PAHs) are largely uncharacterized.

PAHs, especially those with four or more rings, and their metabolites pose a potential health risk because of their detrimental biological effects, which include acute and chronic toxicity, mutagenicity and carcinogenicity. PAHs are commonly released into air, soil, sediments and water (Cerniglia, 1992; Sutherland, 1992; Zang et al., 2007). The PAHs, including naphthalene, anthracene, phenanthrene, pyrene and benzo[a]pyrene, are considered to be one of the largest groups of environmental pollutant compounds, due to their impact on health and the environment (Baborová et al., 2006).

Pyrene and benzo[a]pyrene are high molecular weight compounds that accumulate in sediments due to their limited water solubility and high affinity for particulate matter (Rothermich et al., 2002). Pyrene and benzo[a]pyrene are recalcitrant molecules that have a long half-life in soil, ranging from 270 days to 5.2 years and 269 days to 8.2 years, respectively (Juhasz and Naidu, 2000). Both are classified by the Environmental Protection Agency of the United States (USEPA) as priority pollutants.

PAHs can be found in petroleum, and they are released during oil industry activities such as oil transport. These compounds may be discharged by accidental oil release into the marine environment, causing serious environmental, social and economic damages. For this reason, petroleum-producing countries should encourage the development of effective and low-cost technologies to deal with these problems. In this context, the use of microorganisms may be a promising alternative for the environmental pollutant remediation process because some microorganisms are able to mineralize PAH compounds to water-soluble products. Despite the identification of the basic mechanism involved in PAH bioremediation, the process remains poorly understood (Verdin et al., 2006).

Ligninolytic fungi can oxidize PAHs by producing a non-specific enzymatic extracellular complex that is normally used for lignin depolymerization. The enzymes that are responsible for lignin degradation are lignin-peroxidase (LiP), manganese peroxidase (MnP) and laccases (Hamman, 2004; Steffen et al., 2007; Peng et al., 2008). A wide variety of microorganisms have been implicated in lignin biodegradation. Among them, white rot fungi (WRF) have received extensive attention due to their powerful extracellular lignin-degrading enzymatic systems, which degrade a broad variety of different pollutants (Junghanns et al., 2005; Sette et al., 2008).

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Another known PAH metabolic pathway that is present in fungi involves hydroxylation by cytochrome P-450 monooxygenase through a sequence of reactions that is similar to the reactions that are involved in mammalian metabolism (Capotorti et al., 2004). This pathway is shared by many non-ligninolytic fungi that could effectively degrade PAHs (Krivobok et al., 1998; Ravelet et al., 2000).

The use of marine-derived fungi in the bioremediation of polluted saline environments is facilitated by their tolerance to saline conditions. Therefore, the aim of the present study was to assess eight fungal strains that were isolated from marine cnidarian samples with regard to the potential to degrade the PAH compounds pyrene and benzo[a]pyrene. All fungi used in this work were previously selected due to their abilities to decolorize RBBR dye, an anthracene derivative (da Silva et al., 2008).

2. Materials and methods

2.1. Marine-derived filamentous fungi

The eight filamentous fungi that were tested in the present investigation were isolated from different Brazilian cnidarian samples. *Mussismilia hispida*, *Palythoa caribaeorum*, *Palythoa variabilis* and *Zoanthus solanderi* (Table 1) were collected in the town of São Sebastião on the northern coast of the state of São Paulo, Brazil. These fungi were previously selected due to their capacities to decolorize/tolerate RBBR dye, and they were identified by our research group using conventional (morphologic analyses) and molecular (sequencing and phylogenetic analyses) approaches (da Silva et al., 2008; Bonugli-Santos et al., 2010). These fungi were deposited in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI) under the accession number shown in Table 1.

2.2. Culture conditions

The marine-derived fungi were cultured in 2% (w/v) malt extract agar plus 3% (w/v) NaCl for 7 days at 28 °C. Following the incubation, three fungal culture plugs (0.5 cm diameter) that were taken from the edge of the colony, were transferred to a 125-mL Erlenmeyer flasks containing 30 mL of Sabouraud Dextrose Broth (SDB: 10 g L^{-1} of neopeptone and 20 g L^{-1} of bacto-dextrose). The experiments were performed in duplicate. The flasks were incubated for 48 h at 30 °C and 150 rpm. Then, 2 mg of pyrene and 1 mg of benzo[a]pyrene (dissolved in 0.5 mL of dimethylsulfoxide) were added separately and the flasks were incubated in the dark for 4–8 days in pyrene or 8–16 days in benzo[a]pyrene. The controls were incubated following the same culture conditions either without PAHs or without the microbial inoculum (da Silva et al., 2004).

Table 1
Marine-derived fungal identification and data from sampling, isolation and culture collection accession numbers.

Filamentous fungi	CBMAI accession number	Cnidarian source	Sampling site
Mucor racemosus	847	*M. hispida*	Praia Portinho[a]
Aspergillus sclerotiorum	849	*P. variabilis*	Praia Portinho[a]
Khuskia oryzae	850	*P. variabilis*	Praia Portinho[a]
Trichoderma sp.	852	*P. variabilis*	Praia Portinho[a]
Penicillium citrinun	851	*Z. solanderi*	Praia Preta[b]
Fusarium oxysporum	854	*M. hispida*	Praia Preta[a]
Microsphaeropsis sp.	856	*P. caribaeorum*	Praia Preta[a]
Cladosporium cladosporioides	857	*P. caribaeorum*	Praia Preta[a]

[a] Ilha Bela, São Sebastião (south 23°50′25″ and west 45°24′22″).
[b] Ilha Bela, São Sebastião (south 23°49′10″ and west 45°24′37″), Brazil.

2.3. Pyrene and benzo[a]pyrene degradation analyses

2.3.1. Sample preparation

The extraction of the PAHs and their degradation products in fermentative medium was accomplished using an Ultra-Turrax system. Ethyl acetate was used as solvent to rupture the fungal cellular walls and remove adsorbed compounds. To each Erlenmeyer flask containing the fungus in fermentative medium, 50 mL of ethyl acetate was added, and the fungus was subjected to cellular disintegration at 14,000 rpm for 1 min. The material was then filtered, transferred to a 250-mL separating funnel and subjected to vigorous shaking for 1 min. The organic phase was filtered by anhydrous sodium sulfate saturated with ethyl acetate directly into a 250-mL, round-bottom flask; the aqueous phase was re-extracted with an additional 50 mL of ethyl acetate. The organic phases were combined and evaporated using a vacuum at 40–45 °C until the volume was reduced to approximately 2 mL. The contents of the flask were analytically transferred into a 10-mL volumetric flask and diluted to the mark with ethyl acetate. An aliquot of 1 mL of this solution was then diluted in 10 mL of ethyl acetate containing 1 mL of the internal standard solution prior to GC–MS analysis.

2.3.2. GC–MS analysis

For the GC–MS analyses, an Agilent (Palo Alto, CA) 6890 N GC that was equipped with an Agilent 7683B autosampler coupled to an Agilent 5975 mass-selective detector was used. Data acquisition and analyses were performed using the standard software supplied by the manufacturer. The compounds were separated on a fused-silica capillary column (HP-5MS, 30 m × 0.25 mm i.d., 0.25-μm film thickness) (J&W Scientific, Cologne, Germany). The GC temperature program was as follows: 180 °C, 5 °C min^{-1} up to 310 °C, held for 10 min. The temperatures for the injection port and detector were set at 290 °C and 300 °C, respectively. The splitless injection mode was used with the split outlet opened after 3 min, and helium, with a flow rate of 1.0 mL min^{-1}, was used as the carrier gas. The retention times and characteristic mass fragments were recorded, and the chosen diagnostic mass fragments were monitored in the selected ion monitoring (SIM) mode. The characteristic ions that were used for quantification were as follows: benzo[a]pyrene (*m*/*z* 252, 126, 250), pyrene (*m*/*z* 202, 101, 200) and dibutyl phthalate (*m*/*z* 149, 205, 223) as an internal standard. For quantification, the peak area ratios of the analytes to the internal standard were calculated as a function of the compound concentration.

2.4. Metabolism of pyrene and benzo[a]pyrene by marine-derived fungi

The identification of metabolites that were produced pyrene and benzo[a]pyrene metabolism was carried out by HPLC-DAD-MS analysis using the fungal strains *Aspergillus sclerotiorum* CBAMI 849 and *Mucor racemosus* CBMAI 847, after growth in the same conditions described in Section 2.2.

2.4.1. Sample preparation

The extractions of pyrene and benzo[a]pyrene and their degradation products were performed separately using an Ultra-Turrax system. The homogenized material (mycelium and medium) was purified on C-18 cartridges by elution with H_2O/MeOH (3:7 v/v).