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Filamentous Fungi Isolates of Contaminated Sediment in the Amazon Region with the Potential for Benzo(a)pyrene Degradation

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Abstract Filamentous fungi were isolated from contaminated sediment samples in the Amazon region of Brazil to select species with potential for benzo(a)pyrene (BaP) degradation, a polycyclic aromatic hydrocarbon (PAH) with high molecular weight and known for its mutagenic and carcinogenic properties. The isolates were submitted to biodegradability test using 2.6-dichlorophenol indophenol (DCPIP), gallic acid reaction, and evaluation of BaP influence in the enzymatic (ligninolytic) activity. The selected fungi were submitted to the taxonomic identification and used in biodegradation assays, which were carried out using gas chromatography coupled to mass spectrometry (GC-MS). A qualitative analysis of the presence of BaP metabolites that have recently been reported in literature was also performed by GC-MS. A total of 146 fungal isolates were recovered. Among them, 63.7 % were positive for the redox indicator DCPIP. From these isolates, 22.6 % showed positive responses to the gallic acid reaction. In enzymatic tests, the fungi *Megasporoporia* sp. S47 and unidentified Sordariales S69 presented highest activities of laccase and manganese peroxidase in the presence of BaP. Additionally, the white-rot fungus *Megasporoporia* sp. S47 showed better performance in BaP degradation (54 %). Therefore, *Megasporoporia* sp. S47, obtained from an environment with considerable PAH contamination, was selected as a promising genetic resource for application in new studies related to enzyme production and characterization and BaP degradation optimization.

Keywords Fungi · Ligninolytic enzymes · Biodegradation · Polycyclic aromatic hydrocarbons · Benzo(a)pyrene

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1 Introduction

The polycyclic aromatic hydrocarbons (PAHs) are organic pollutants derived mainly from the burning of

fossil fuels and waste from industrial activities (Almeida et al. 2007; Netto et al. 2000). Through pyrolysis, the molecules are disintegrated and their subsequent recombination can promote the formation of high molecular weight PAHs (HMW) consisting of four to eight aromatic rings fused together and known to be highly toxic (Haritash and Kaushik 2009).

The US Environmental Protection Agency (US-EPA) has classified a group of 16 individual PAHs as priority pollutants in environmental studies. Among these, benzo(a)pyrene (BaP), a HMW HPA consists of five aromatic rings, is known for its mutagenic, carcinogenic, and teratogenic properties in mammals and aquatic organisms (IARC 1983; Juhasz and Naidu 2000; Jennings 2012). Because of its hydrophobicity, BaP tends to be associated with the particulate material, becoming one of the main xenobiotics that remaining deposited in soils and sediments for long periods. In the aquatic environment, they can be absorbed into the skin or by ingestion of animals, causing bioaccumulation and biomagnification by feeding along the trophic chain (Netto et al. 2000; Mackay and Fraser 2000; Perelo 2010). Thus, the presence of this contaminant can be a threat to different environments and their biota.

Due to the high chemical stability in BaP molecule, processes such as volatilization and photolysis are not efficent in the disintegration of this compound, as occurs naturally with low molecular weight PAHs. However, microbial degradation of PAHs is considered as an attractive biotechnological alternative (Cerniglia and Sutherland 2010; Perelo 2010). Filamentous fungi present some advantages in degradation process in comparison to bacteria and yeast, since they present mycelial growth, rapid branching and colonization of insoluble substrates, ability to tolerate high concentrations of toxic compounds and to grow under stressful conditions. The white-rot basidiomycetes (ligninolytic fungi) are considered the best group of fungi for environmental pollutants degradation, since they are able to produce a range of extracellular oxidoreductases with nonspecific activities associated with intracellular monooxygenases that can mineralize the pollutant and/or transform it into less toxic compounds (Lemos et al. 2008; Haritash and Kaushik 2009; Harms et al. 2011). Due to the various attributes mentioned, filamentous fungi can be considered as important components for biotechnological models of bioremediation of environments contaminated with PAHs.

Several species of fungi with abilities to degrade PAH were reported in the literature, including ligninolytic and non-ligninolytic strains. Among the non-ligninolytic fungi, *Aspergillus ochraceus*, *Cunninghamella elegans*, and *Cunninghamella echinulata* were mentioned as able to degrade phenanthrene, fluoranthene, pyrene, chrysene, and benzo(a)pyrene (Cerniglia 1992; Muncnerová and Augustin 1994), *Aspergillus sclerotiorum* and *Mucor racemosus* as able to degrade pyrene and benzo(a)pyrene (Ravelet et al. 2000; Passarini et al. 2011a), and *Penicillium* sp. and *Trichoderma harzianum* as efficient in benzo(a)pyrene degradation (Machín-Ramírez et al. 2010). Among the ligninolytic fungi, particularly white-rot fungi, *Phanerochaete chrysosporium*, *Bjerkandera* sp., and *Trametes versicolor* are cited as excellent degraders of low and high molecular weight PAHs, and there are information related to PAHs mineralization by *P. chrysosporium* (Muncnerová and Augustin 1994; Lemos et al. 2008). Other genera of basidiomycetes such as *Anthracophyllum*, *Armillaria*, and *Polyporus* are also cited as promising in benzo(a)pyrene degradation (Acevedo et al. 2011; Hadibarata and Kristanti 2012a, b).

One of the strategies used to select fungi with potential in bioremediation is isolation that comes from sites contaminated with petroleum products and PAHs. Soils and sediments are an excellent source of microorganisms that retain high tolerance, since these environments are selective for the pollutant adapted fungi (Volkering and Breure 2003; Colla et al. 2008; Zafra et al. 2014). Environmental contamination with PAHs has been reported by several researchers in Brazil revealing high concentrations of these compounds in river, dam, and estuary sediment (Brito et al. 2005; Bícego et al. 2006; Meire et al. 2008; Leite et al. 2011). Recently, a research study conducted with sediment samples from the Negro River in the Amazon showed that 70 % of the total abundance of priority PAHs were classified as high molecular weight, such as chrysene, BaP, benzo(a)anthracene, and dibenzo(a,h)anthracene (Souza et al. 2015).

After the isolation of fungal strains from the contaminated environment, the use of phenolic oxidation (gallic acid, guaiacol) and hydrocarbon oxidoreduction indicators, Remazol Brilliant Blue R discoloration, PAHs tolerance tests, ligninolytic activities, and biodegradation experiments is some of the major methods employed

successfully to obtain fungi of interest in bioremediation (Hanson et al. 1993; Conceição et al. 2005; Passarini et al. 2011a, b; Hadibarata and Kristanti, 2012a, b; Argumedo-Delira et al. 2012; Bonugli-Santos et al. 2012; Lee et al. 2014). In the Amazon region, the existence of research studies that involve the isolation and selection of microorganisms in contaminated areas with a view to bioprospecting and bioremediation is still largely incipient. Thus, taking into consideration the presented context, this paper aims to select filamentous fungi isolated from sediments from an Amazon region that is contaminated with PAHs, and with potential for benzo(a)pyrene degradation.

2 Material and Methods

2.1 Isolation of Filamentous Fungi from Contaminated Sediments

The fungal isolates were obtained from surface sediment samples from the Negro River, Amazonas State, Brazil, in six different locations: close to the Tupé Sustainable Development Reserve (TR), the mouth of the São Raimundo basin (SR), Modern Manaus Port (MM), Panair Port (PA), Iranduba (IR) on the right bank of the river, and Ceasa Port region (CE). All locations were characterized by having different levels of PAHs contamination, as described by Souza et al. (2015) (Table 1). Fungal isolation was performed by serial suspension technique, based on Gomes et al. (2011) in which 25 g of sediment samples were diluted in 225 mL of sterile distilled water and further dilutions (1:1000) were performed seeding 1 mL in petri plates containing Sabouraud Dextrose Agar (SDA; 40 g L^{-1} dextrose, 10 g L^{-1} mycological peptone, and 15 g L^{-1} agar) supplemented with chloramphenicol (100 mg L^{-1}) in quadruplicate. The inoculated plates were incubated at 28 °C for 5 days and purified isolates were preserved in vials containing sterile distilled water (Castellani 1939) and cryopreservation tubes at −20 °C.

2.2 Biodegradability Test Using the 2.6-Dichlorophenol Indophenol

The use of redox indicator 2.6-dichlorophenol indophenol (DCPIP) has enabled us to understand bacteria's ability to degrade diesel hydrocarbons (substrate) through redox reactions. In this study, the method of Hanson et al. (1993) was adapted to evaluate the ability of fungal cultures to oxidize hydrocarbons and reduce the DCPIP indicator. Therefore, we used the liquid medium Bushnell-Hass (BH), consisting of KH_2PO_4 (1.0 g/L); K_2HPO_4 (1.0 g/L); $NH_4H_2PO_4$ (1.0 g/L); $MgSO_4.7H_2O$ (0.010 g/L); $FeCl_2.4H_2O$ (0.05 g/L); $CaCl_2.2H_2O$ (0.020 g/L), distilled water (1000 mL), pH 7.0. After sterilization at 121 °C for 15 min, 0.010 g/L of the redox DCPIP indicator was added. In test tubes (triplicate) 3000 μL of the liquid medium was added, 30 μL of diesel oil (filtered in Millipore membrane 0.22 μm) and culture plugs (7 mm diameter) from the edge of the colony to each fungi after 7 days of culture. The tubes were put into an incubator under agitation at 180 rpm and 30 °C for 48 h. The color change of the (DCPIP) indicator from blue (oxidized form) to transparent (reduced form) was monitored at three different times: within 2 h, after 24 and 48 h.

2.3 Gallic Acid Reaction

The fungal isolates that showed positive responses to the redox DCPIP indicator were subjected to the 3,4,5-trihydroxybenzoic acid oxidation test (gallic acid), which is characterized by the presence of a brown halo around the mycelium, indicating the formation of quinones. This technique has been applied to select fungi able to produce phenoloxidase enzymes that may be directly involved in xenobiotic degradation, such as PAHs (Conceição et al. 2005; Erden et al. 2009). Therefore, the isolates were inoculated on Petri plates containing Potato Dextrose Agar (PDA; 4 g L^{-1} potato broth, 20 g L^{-1} Dextrose 15 g L^{-1} agar) and incubated for 7 days at 28 °C. After this period, three fungal culture plugs (0.5 cm diameter) from the edge of the colonies were transferred to plates containing Extract Malt 2 % (MEA 2 %; 20 g L^{-1} of malt extract, 15 g L^{-1} agar), supplemented with gallic acid 0.5 % and incubated for 5 days at 28 °C. The experiments were conducted in triplicate. In cultures with positive responses, the intensity of the halo color was recorded and classified as described by Lee et al. (2014): DB (dark brown), BR (brown), and YB (yellowish brown).

2.4 Influence of the Presence of Benzo(a)pyrene in Ligninolytic Enzyme Production

The isolates of filamentous fungi that were able to oxidize gallic acid were submitted to in vitro assays to

Table 1 Sampling sites of surface sediments with different levels of contamination by polycyclic aromatic hydrocarbons (PAHs) and in different locations of the Negro River, Amazon, Brazil (available in Souza et al. 2015)

Locations	Sampling sites	Geographical position	Granulometry	ΣPAH(ng g^{-1})	Σ16PAH (ng g^{-1})
Tupé Sustainable Development Reserve	TR1	S 03° 03′ 02.6″ W 060° 15′ 24.0″	Sand	22.6	9.1
	TR2	S 03° 03′ 04.7″ W 060° 15′ 23.9″	Sand	nd	nd
	TR3	S 03° 03′ 06.9″ W 060° 15′ 25.1″	Sand	6.5	6.5
Bacia do São Raimundo	SR1	S 03° 07′ 55.2″ W 060° 02′ 05.3″	Mud	5273.8	1187
	SR2	S 03° 08′ 05.45″ W 060° 01′ 53.02″	Sand	138.4	56.4
	SR3	S 03° 08′ 01.55″ W 060° 01′ 58.30″	Sand	2460.5	1354
Manaus Moderna Port	MM1	S 03° 08′ 34.88″ W 060° 01′ 20.30″	Sand	299.5	133
	MM2	S 03° 08′ 35.5″ W 060° 01′ 21.4″	Sand	272.9	182
	MM3	S 03° 08′ 28.1″ W 060° 01′ 28.9″	Sand	1265.8	601
Panair Port	PA1	S 03° 08′ 49.2″ W 060° 00′ 42.4″	Mud	892.7	250
	PA2	S 03° 08′ 46.64″ W 060° 00′ 40.50″	Mud	995.5	232
	PA3	S 03° 08′ 43.65″ W 060° 00′ 44.49″	Sand	358.2	72.1
Iranduba	IR1	S 03° 09′ 36.2″ W 060° 02′ 10.8″	Mud	22.8	5.6
	IR2	S 03° 09′ 14.2″ W 060° 02′ 59.8″	Mud	142.5	116
	IR3	S 03° 08′ 49.9″ W 060° 04′ 10.8″	Mud	50.2	35.2
Ceasa Port	CE1	S 03° 08′ 06.7″ W 059° 56′ 17.4″	Mud	2375.3	407
	CE2	S 03° 08′ 08.15″ W 059° 56′ 16.04″	Sand	437.6	163
	CE3	S 03° 08′ 04.0″ W 059° 56′ 11.2″	Mud	1113.8	529

nd not detected

evaluate the influence of BaP on the production of ligninolytic enzymes. The fungal isolates were grown in petri dishes containing 2 % MEA and were incubated for 7 days at 28 °C. After this period, three fungal culture plugs (7 mm diameter) from the edge of the colonies were transferred to Erlenmeyer flasks of 125 mL containing 50 mL of broth Extract Malt 2 % (MEB2; 20 g L^{-1} of malt extract, 1000 mL distilled water) in quadruplicate. The flasks were incubated with agitation for 72 h at 150 rpm and 30 °C for each isolate. Then, a duplicate of each isolate was supplemented with 2 mg of BaP (Sigma-Aldrich, 99 %) (St. Louis, MO, USA) dissolved in 0.5 ml of dimethylformamide and the other flasks remained without BaP supplementation. The flasks were again incubated for 7 days under the same conditions described above. The enzymatic broth was obtained by vacuum filtered and centrifugation at 7830 rpm for 45 min (Eppendorf Centrifuge 5430R). The resulting supernatant was used as enzyme source.

The activity of laccase, lignin peroxidase (LIP), and manganese peroxidase (MnP) was measured spectrophotometrically (Biochrom Libra S60, Cambridge, England) in triplicate, through the absorbance difference calculation (ΔAbs = Initial Abs − Final Abs). One enzyme unit was defined as 1.0 μmol of product formed per minute under the assay conditions. Laccase activity was determined by the oxidation of 2.2-azino-bis-ethylbenzthiazoline (ABTS) through monitoring the increase in absorbance at 420 nm as described by Buswell et al. (1995). The mixture was composed of 300 μL sodium acetate buffer (0.1 M, pH 5.0), 600 μL of enzyme solution and 100 μL ABTS solution (0.03 % *w/v*). The mixture was incubated at 37 °C for 10 min. LIP activity was determined by the oxidation of veratryl alcohol through monitoring the increase in absorbance at 310 nm as described by Arora and Gill (2001). The mixture was composed of 600 μL enzyme solution, 200 μL of veratryl alcohol (2 mM) in sodium tartrate buffer (0.4 M, pH 4.5) and 200 μL of hydrogen peroxide (2 mM). The reaction was initiated with hydrogen peroxide and the mixture was incubated for 10 min. The MnP activity was determined by oxidation of Mn^{2+} to Mn^{3+} and formation of the malonate complex Mn^{3+}, which was accompanied by an increase in absorbance at 270 nm, according to Giardina et al. (2000). The reaction mixture contained 800 μL of

sodium malonate buffer (50 mM, pH 4.5), 50 μL $MnSO_4$ (10 mM), 100 μL of enzyme solution and 50 μL of hydrogen peroxide (2 mM). The reaction was initiated with hydrogen peroxide, acting as a cofactor for enzymes. The mixture was incubated at room temperature for 5 min.

2.5 Molecular Identification of Selected Fungal Isolates

The selected isolates (S47 and S69) were identified by molecular taxonomy. Genomic DNA from 7-day growth cultures was extracted by physical lysis with glass beads (425–600 μm diameter) following a combined protocol from Moeller et al. (1992) and Gerardo et al. (2004). After DNA extraction, the ITS region was amplified with the primer pair ITS4 and ITS5 for isolate S69 and ITS1 (White and Lee 1990) and UniR (MähnB et al. 2005) for isolate S47. Amplification reactions consisted of 0.2 mM of each dNTP, 5× KCl buffer, 1.5 mM $MgCl_2$, 0.5 μM of each primer and 1 U of Taq polymerase (Promega) in a final volume of 25 μL. Amplicon purification was performed using the Wizard® SV Gel and PCR Clean-up System kit (Promega) and quantified in the NanoDrop® (Thermo Scientific). DNA sequencing was performed using the BigDye® Terminator Cycle Sequencing kit v.3.1 (Life Technologies) according to the manufacturer's protocol. Forward and reverse sequences were compiled in contigs in BioEdit v7.1.3. The contigs were queried in the NCBI-GenBank (www.ncbi.nlm.nih.gov) of the Fungal Biodiversity Centre (CBS, www.cbs.knaw.nl) databases for homologous sequences of closely related species.

2.6 Benzo(a)pyrene Biodegradation

The biodegradation assays were based on the method proposed by Passarini et al. (2011a) and performed according to the cultivation, inoculation, and incubation methodology described in item 2.4. After 72 h of incubation, 125 mL Erlenmeyer flasks containing 30 mL of MEB2 were supplemented with 1 mg of BaP (Assay I) and 2 mg of BaP (Assay II), diluted in 0.5 mL dimethylsulfoxide and incubated for 14 days. The experiments were performed in duplicate and the control group consisted of 2 % MEB supplemented with BaP. Biodegradation quantification was conducted after 7 and 14 days of BaP supplementation in liquid medium.

2.7 Extraction and Sample Preparation

Extraction of BaP and its metabolites was performed using the Ultra-Turrax system (IKA Labortechnik, Germany) and ethyl acetate. To each Erlenmeyer flask, 50 mL of ethyl acetate was added and the cell biomass was subjected to disintegration at 14,500 rpm for 2 min. The material was transferred to a 125-mL separatory funnel and subjected to vigorous agitation for 1 min. The organic phase was collected and the aqueous phase was again extracted by the addition of 40 mL of ethyl acetate. In the collected organic phase anhydrous sodium sulfate was added and after filtration the samples were subjected to vacuum rotaevaporator at 45 °C until reduction of the volume of the sample to approximately 2 mL. The concentrate was transferred to a 10 mL volumetric flask and diluted with ethyl acetate. An aliquot of 1 mL of this solution was diluted with another 10 mL volumetric flask containing 20 μg mL^{-1} of an internal standard solution. The volume of 1 μL of the sample was injected into GC-MS.

The internal standard and *surrogate* solutions were also made up of PAHs with a purity ≥ 98 % (Sigma-Aldrich, 98 %) (St. Louis, MO, USA). The internal standard (pyrene) was inserted in the samples diluted in a volumetric flask before injected into GC-MS. The *surrogate* (chrysene) was inserted in Erlenmeyer flasks before starting the extraction process and contained the same BaP concentration supplemented in the trials. The use of these patterns is needed to recognize possible losses during the extraction process. Thus, the extraction recovery rate was given by the formula: $[(AC_{ii}/AC_i] \times 100$, where AC_i corresponds to the chromatographic area of the internal standard and the AC_{ii} pattern for the chromatographic area of the *surrogate*. The percentage of BaP degradation was given by the formula: $[(c_i - c_r)/c_i] \times 100$, where c_i is the BaP concentration in the control test and c_r the remaining concentration of BaP from bioassays (Machín-Ramírez et al. 2010). The mean and standard deviation were calculated to higher accuracy of the BaP concentration obtained after completion of the extraction procedures, chromatographic area of the analyte is corrected by making a calibration curve

using different BaP concentrations: 1, 5, 10, 15, 20, 25, and 30 μg L^{-1}.

2.8 CG-MS Analysis

Analyses were performed on a gas chromatograph (Shimadzu 2010) (Tokyo, Japan) coupled to a mass spectrometer (Shimadzu QP 2010 Plus) and equipped with a Restek fused silica capillary column (RTX-5MS 30 m × 0.25 mm × 0.25 μm) (Bellefonte, PA, USA). The GC temperature program started at 180 °C and remained for 5 min, followed by a heating rate of 10 °C min^{-1} until reaching 295 °C where it remained for 11 more minutes. The injector temperature was 295 °C and was in the *splitless* injection mode, with helium as a carrier gas at a rate of 1.56 mL min^{-1}. Mass fragments were recorded by selective ion monitoring (SIM). The ions used were as follows: benzo(a)pireno (*m/z* 252, 250), internal standard (*m/z* 202, 200), and *surrogate* (*m/z* 228, 226).

2.9 Benzo(a)pyrene Metabolites

The resulting samples of BaP biodegradation assays were subjected to qualitative analysis to determine the presence of two metabolites recently reported in literature: 1-hydroxy-2-naphthoic acid and coumarin (Table 2) (Hadibarata and Kristanti 2012b). Therefore, the metabolite fragmentation pattern was recorded by the GC-MS and the retention time from injection of standards (Sigma-Aldrich, ≥98 %). The test and standard samples were injected for analysis in the "*scan*" method with an initial column temperature of 60 °C, remaining for 1 min. The heating rate was 10 °C min^{-1} to 280 °C and remained in isotherm for 10 min. The injector temperature was 280 °C and the injection mode was *splitless*, with helium being the carrier gas.

Table 2 Benzo(a)pyrene mass fragment and metabolite retention time

Metabolites	Ion fragments (*m/z*)	Retention time (min)	Reference
1-Hydroxy-2-naphthoic acid	144, 115, 58	12.29	Hadibarata and Kristanti (2012b)
Coumarin	118, 146, 89, 90, 63, 45	11.24	

2.10 Statistical Analysis

The recovery results of the extraction method and rate of biodegradation were expressed as a percentage. To compare the enzymatic activities, variance analysis was applied (one-way ANOVA) followed by Tukey's test at a significance level of 5 % ($p < 0.05$) using the R program (California, Los Angeles, USA).

3 Results

3.1 Isolation and Screening of Filamentous Fungi

By means of the Negro River sediment samples, it was possible to obtain 146 filamentous fungi. The region of Panair Port showed the greatest number of isolates (49), highlighting PA2 (21) and PA1 (19), sites that showed considerable contamination by total PAHs (892.7 to 995.5 ng g^{-1}). The environments with the largest contamination by total PAHs (5273.8 to 2528.5 ng g^{-1}) presented a total of 51 isolates distributed among SR1 (15), SR3 (13) CE3 (12), and CE1 (11). The biodegradability test using DCPIP showed positive results for 93 strains (63.7 %), being eight positive results in 2 h, 57 after 24 h, and 28 after 48 h. Among these fungi, 21 isolates (22.6 %) had positive responses for the gallic acid reaction (Table 3). Most isolates showed brown form quinones (BR) and only four isolates (S06, S29, S42, and S47) were classified with dark brown form quinones (DB). From sampling sites SR1 and CE1, both sites with high contamination by total PAHs (5273.8 to 2375.3 ng g^{-1}) and 16 priority PAHs by USEPA (1187–407 ng g^{-1}), seven isolates were capable of oxidizing the gallic acid (S03, S10, S15, S53 and S29, S49, S50, S55). Site PA1, with considerable concentration of total PAHs (892.7 ng g^{-1}) (Souza et al. 2015), showed three isolates (S21, S42, and S47) with positive results (Table 3).

In the assays without the addition of BaP (Fig. 1a), MnP activity was observed in 10 isolates (S03, S15, S47, S53, S55, S65, S67, S72, S132, and S140) and laccase in only 2 (S10 and S47). Higher MnP activity was produced by the isolate S132 (14.96 ± 5.29 U L^{-1}) with a significant difference ($p < 0.05$) from the production by the other isolates.

Table 3 Gallic acid reaction results and the influence of the absence and presence of benzo(a)pyrene in the production of ligninolytic enzymes

Isolated selected	Sampling sites	Gallic acid reaction	MEB2 without BaP			MEB2 with BaP		
			Laccase	LiP	MnP	Laccase	LiP	MnP
S03	SR1	YB	−	−	+	−	−	−
S06	IR1	DB	−	−	−	−	−	−
S10	SR1	YB	+	−	−	+	−	−
S11	IR1	BR	−	−	−	−	−	+
S15	SR1	YB	−	−	+	+	−	−
S21	PA1	BR	−	−	−	−	−	−
S29	CE1	DB	−	−	−	−	−	−
S42	PA1	DB	−	−	−	+	−	−
S47	PA1	DB	+	−	+	+	−	+
S50	CE1	BR	−	−	−	−	−	+
S53	SR1	BR	−	−	+	−	−	+
S55	CE1	BR	−	−	+	−	−	−
S65	PA2	BR	−	−	+	−	−	−
S67	CE2	BR	−	−	+	−	−	+
S69	CE2	BR	−	−	−	−	−	+
S72	CE2	BR	−	−	+	−	−	+
S123	SR3	BR	−	−	−	−	−	+
S125	SR3	YB	−	−	−	−	−	−
S132	IR3	BR	−	−	+	−	−	+
S140	MM3	BR	−	−	+	−	−	+
S143	CE3	BR	−	−	−	−	−	−

DB dark brown, *BR* brown, *YB* yellowish brown, + presence of enzyme activity, − absence of enzyme activity, *MEB 2 %* Agar Malt Extract 2 %

Laccase activity was low and non-existent for most isolates, being the highest activity (3.82 ± 0.99 U L^{-1}) produced by the isolate S47. In the assays supplemented with BaP (Fig. 1b), four isolates (S10, S15, S42, and S47) presented positive response for laccase activity, suggesting that the xenobiotic served as inductor to increase the activity of this enzyme. The isolate S47 ($22{:}15 \pm 5.39$) produced the highest amount of laccase ($p < 0.05$). Another 10 isolates (S11, S47, S50, S53, S67, S69, S72, S123, S132, and S140) were able to produce MnP. The presence of BaP resulted in different responses in MnP activity among the isolates: decrease (S132 and S72); inhibition (S03, S15, S55 and S65), increase (S47, S49, S53, S67, and S140), and induction (S11, S50, S69, and S123). The isolate S69 stood out with the highest activity ($30.20 \pm 8{:}54$ U L-1) ($p < 0.05$) (Fig. 1). There was no LiP activity (Table 3). Based on these results, the isolates S47 and S69 were selected and submitted to the BaP biodegradation assays.

3.2 Taxonomic Identification of Selected Fungi

Following a morphological examination of isolates S47 and S69 no reproductive structures could be found in the cultures even after one-month growth in MA2% plates. ITS sequencing revealed *Megasporoporia* sp. (accession #: KP013029) as the closest related fungus for isolate S47 with 97 % of similarity. For isolate S69 the closest related fungus was an unidentified Sordariales species (accession #: AB986410) with 91 % of similarity. Because of the low similarity with homologous sequences and the absence of reproductive structures, strain S69 was considered as an unidentified fungus from the Sordariales order and may represent a putative new species.

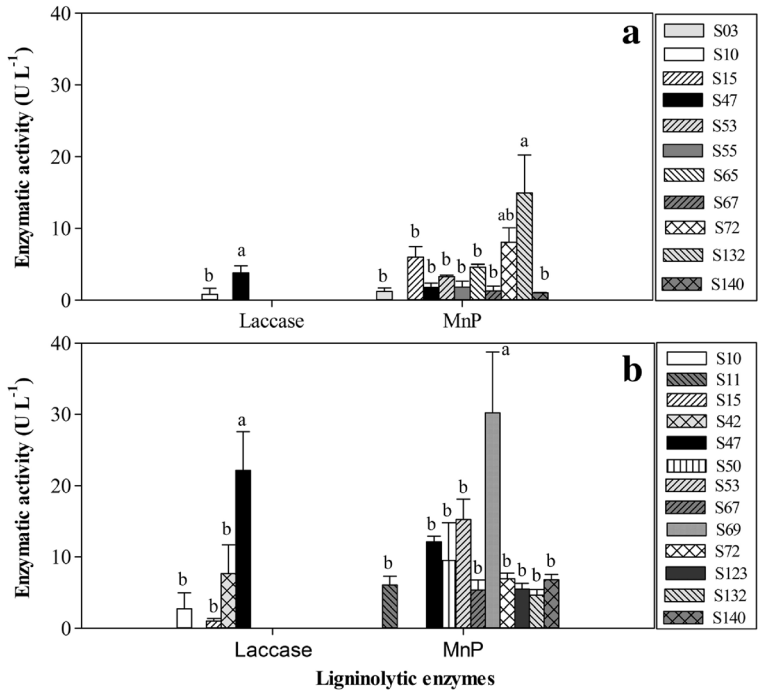

Fig. 1 Production of ligninolytic enzymes by filamentous fungi in the absence (**a**) and presence (**b**) of benzo(a)pyrene. The *vertical bars* show the standard error. *Different letters* are significantly different at $p < 0.05$ by Tukey's test

3.3 Benzo(a)pyrene Biodegradation

The BaP biodegradation potential by the selected fungi is shown in Fig. 2. In assay I, *Megasporoporia* sp. S47 showed a degradation rate of 49.9 ± 0.6 % over a period of 7 days and 54.4 ± 3.6 % after 14 days. Unidentified Sordariales S69 degraded only 28.2 ± 0.5 %. In assay II, the results were similar, confirming the greatest potential of *Megasporoporia* sp. S47 for biodegradation, which reached a maximum degradation of 36.5 ± 5.3 % at the end of the experiment. Unidentified Sordariales S69 degraded only 17.2 ± 9.3 %. The chromatogram of BaP degradation by *Megasporoporia* sp. S47 is shown in Fig. 3. The recovery of the extraction method ranged from 75.5 to ≥99 %. The metabolites (1-hydroxy-2-naphthoic acid and coumarin) were not identified in the BaP biodegradation samples.

4 Discussion

The selection of microorganisms that have potential for PAH degradation is a key step in bioremediation programs. Therefore, combining information obtained in different screening tests is essential. In this study, the biodegradability test using the DCPIP redox indicator allowed us to quickly recognize the fungal isolates that had the ability to perform diesel hydrocarbon oxidation. The positive results were faster than some cited in

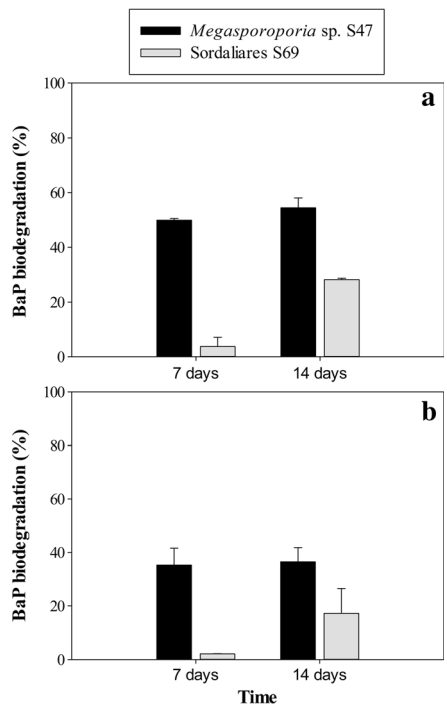

Fig. 2 Benzo(a)pyrene biodegradation by filamentous fungi isolated from samples of the Amazon region. **a** Assay supplemented with 1 mg of BaP and **b** assay supplemented with 2 mg of BaP

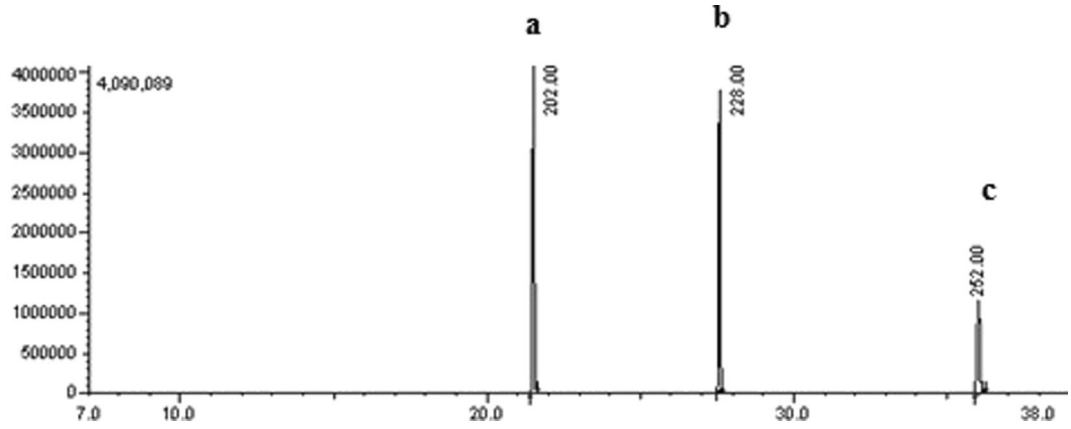

Fig. 3 Chromatogram related to benzo(a)pyrene biodegradation by *Megasporoporia* sp. S47. (*a*) pyrene (*m/z* 202, 200); (*b*) chrysene (*m/z* 228, 226); and (*c*) benzo(a)pyrene (*m/z* 252, 250) after biodegradation

literature (Mariano et al. 2007, 2008). This may possibly be related to the presence of extracellular enzymes that remain in the culture used in the experiments. The results indicate that the method is effective for initial tests and quick for the selection of fungi with potential for hydrocarbon degradation.

The gallic acid reaction is a simple and convenient qualitative method that has been recommended in literature as the best indicator, compared with the use of Remazol Brilliant Blue R (RBBR) dye and tolerance tests to classify fungi by its ability to degrade xenobiotics (Lee et al. 2014). Gallic acid is a natural reagent that when oxidized by the action of the phenoloxidase enzymes form quinones, yielding a brown halo around the mycelium (Erden et al. 2009). In this paper, the use of this technique resulted in the selection of 22.6 % of the fungal isolates, which was similar to other researchers related to the selection of ligninolytic (basidiomycetes) and non-ligninolytic fungi with abilities to proceed in studies on PAHs biodegradation (Conceição et al. 2005; Lee et al. 2014).

Together with the application of the aforementioned qualitative tests, it is necessary to evaluate the production of microbial ligninolytic enzymes, since these enzymes are directly involved in the degradation of several recalcitrant phenolic compounds (Sette et al. 2008; Harms et al. 2011). The ability of fungi to produce ligninolytic enzymes in malt extract with the presence or absence of inducing substrates (Bonugli-Santos et al. 2009) and in the presence of diesel oil (Maciel et al. 2010) has been reported. The best results have been observed to the basidiomycetes fungi in regard to the production of these enzymes and their relationship to the discoloration of the RBBR dye (Bonugli-Santos et al. 2012; Machado et al. 2005) and to degrade PAHs, such as phenanthrene, pyrene, anthracene, benzo(a)anthracene, and benzo(a)pyrene (Wu et al. 2010; Ting et al. 2011; Hadibarata and Kristanti 2012a).

In the present study, there was low production of ligninolytic enzymes. However, it was enough to show that some fungal isolates are able to express these enzymes. The use of malt extract to evaluate the activity of ligninolytic enzymes has being carried out because this medium is rich in tryptophan, aromatic amino acids, and tyrosine (Arora and Gill 2001). However, for screening of species that are effective in PAH degradation, conducting enzyme assays without the presence of the substrate of interest has been cited as inadvisable (Lee et al. 2014). It is necessary to take into consideration that aromatic compounds and synthetic dyes can act as agents responsible for the regulation and manifestation of these enzymes (Yang et al. 2011; Casas et al. 2013). This fact corroborates the results obtained in this study, where it was observed that the highest laccase activity and MnP occurred in assays supplemented with BaP (Table 3).

Data from literature showed that the increased activity of ligninolytic enzymes (especially MnP and laccase) by the white-rot fungi of the genera *Armillaria*, *Anthracophyllum*, *Polyporus*, and some leaf litter decomposers, is directly related to the degradation of high molecular weight PAHs (Steffen et al. 2003; Acevedo et al. 2011; Hadibarata et al. 2009; Hadibarata and Kristanti 2012a, b). These enzymes are proving to be key enzymes in PHAs degradation process. Among the fungi selected in this study *Megasporoporia* sp. S47, a

white-rot fungus from Polyporaceae family, proved to be the most promising. The fungus was able to produce higher amounts of laccase in the presence of the xenobiotic and showed higher potential to BaP biodegradation, supporting the literature findings that indicate a direct relationship between laccases and BaP degradation. Currently, it is known that the regulation of the laccase transcription can be different for each species of fungus (Rivera-Hoyos et al. 2013); thus, new studies about laccase production by *Megasporoporia* sp. S47, as well as it purification and characterization are needed to determine the potential of the fungus/enzyme for being used in industrial bioprocesses and future applications in bioremediation.

The *Megasporoporia* genus Ryvarden & JE Wright was described by Ryvarden et al. (1982) based on *Poria setulosa* Henn. Representatives of this genus are characterized by having ressupinated basidiocarps containing large or small pores, basidiospores often exceeding 10 mm in length, occurring mainly in angiosperm branches and causing white rot (Ryvarden et al. 1982; Li and Cui 2013). These are fungi from the Polyporales order, Polyporaceae family. In the state of Amazonas, a representative of this genus, *Megasporoporia cavernulosa* (Berk.) Ryvarden, was already reported by Gomes-Silva and Gibertoni (2009). In the present study, *Megasporoporia* sp. S47 was obtained from the sediment collected at the Negro River in the Amazon, located in a popular port area mainly built with wood. Since representatives of this genus are able to degrade wood, it is reasonable to believe that pieces of timber in this region are the colonization source of these kind of fungi. Furthermore, there is some plant material in the sediments that can be transported and deposited on the bottom. Although a wide variety of white-rot fungi is capable of degrading low molecular weight PAHs such as anthracene and phenanthrene, literature reports the existence of few genus that degrade BaP (Juhasz and Naidu 2000). It is important to highlight that to our knowledge there is no data in the literature related to the BaP degradation by representatives of the genus *Megasporoporia*, been this study the first one to report this source of data. However, other representatives of the Polyparaceae family, such as *Polyporus* sp., are known as efficient decomposers of the chrysene and BaP (Hadibarata et al. 2009; Hadibarata and Kristanti 2012a).

In the present study, results of the biodegradation assays suggest that *Megasporoporia* sp. S47 is a promising species in degrading HMW PAHs, since in 14 days 54 % of BaP was degraded. In the literature, BaP degradation has been reported by ligninolytic and non ligninolytic fungi. Passarini et al. (2011a) showed that *Aspergillus sclerotiorum* and *Mucor racemosus* (non ligninolytic fungi) were able to degrade 51.7 to 76.6 % BaP, respectively, after 16 days of incubation. Wu et al. (2010) reported 60 % of benzo(a)anthracene degradation by *Fusarium solani* in a period of 40 days. Among the ligninolytic fungi (white-rot basidiomycetes), *Polyporus* sp. and *Armillaria* sp. presented high potential for BaP degradation, reaching between 73 and 76 % after 30 days of incubation. The use of energetic substrates in these studies showed an increasing in the degradation by cometabolism (Hadibarata and Kristanti 2012a, b).

It is important to mention that the potential of PAHs biodegradation can be maximized by the otimization of some parameters such as temperature, pH, carbon and nitrogen sources, and by the use of inductors. Another factor that influences the xenobiotic degradation process is the toxicity of the compound. Since BaP is toxic, mutagenic and carcinogenic, and seeing that it is known that *Megasporoporia* sp. S47 has degradation abilities, further studies are necessary to evaluate the degradation process over a longer time period and diversifying the compound concentration.

PAH metabolites originating from fungi of white rot (ligninolytic) are generally quinone (Volkering and Breure 2003). In a recent study, Hadibarata and Kristanti (2012a) evaluated BaP degradation by *Polyporus* sp. S133 and suggested that laccase and 1.2-dioxygenase have an important role in BaP transformation. *Polyporus* sp. S133 transformed BaP into BaP-1.6-quinone, which is degraded in two other ways, forming 1-hydroxy-2-naphthoic acid, and coumarin. The presence of these metabolites indicates the possibility of cleavage forming benzoic acid, which can also be degraded by the formation of tricarboxylic acid, followed by mineralization.

In this study, the analysis of BaP metabolites reported by Hadibarata and Kristanti (2012a) was not identified. However, considering that *Megasporoporia* sp. S47 belongs to the ligninolytic group of fungi (white hot) and that laccase production was induced by the presence of BaP it is probable that this fungus carry out a similar method for HPAs degradation like *Polyporus* sp. S133. Therefore, further studies on the production of metabolites must be performed in order to confirm this similarity between fungi of the same taxonomic family.

Megasporoporia sp. S47 and Unidentified Sordariales S69 selected in this study were obtained from environments with considerable contamination of total PAHs (PA1 and CE2) (Table 1). Fungi isolated from environments with high PAHs contamination showed no potential for BaP degradation. Probably the fungi from these sites are tolerant to the presence of PAHs, but do not have the ability to degrade them. It is also necessary to take into consideration the special conditions of the Negro River sediments regarding their granulometry, as mentioned by Souza et al. (2015), and the small amounts of substrates for fungal colonization. Gomes et al. (2011) and Da Silva et al. (2003) reported that the diversity of substrates and nutrient availability are abiotic factors that determine the presence, permanence and survival of mycobiota. It is important to mention that of the 21 isolates selected for their oxidation capacity of gallic acid, nine isolates (S03, S10, S15, S29, S50, S55, S123, S125, S143) belong to the regions of Porto São Raimundo and Ceasa, regions characterized by Souza et al. (2015) to be highly contaminated. Among these isolates, three (S03, S15 and S55) showed MnP production and one isolate (S10) produced laccase in MEB 2 %. In the presence of BaP, this production was completely inhibited. Although the production of these enzymes was much smaller than that found in the isolates selected in this study, it is possible to advise that further studies should also be carried out with these isolates in order to check for increased production of these enzymes in optimal conditions and using other PAHs with smaller toxicity.

5 Conclusion

The approaches used in this study were effectives and enabled the selection of the white-rot fungus *Megasporoporia* sp. S47, which in the presence of BaP, increased the production of laccase and MnP and presented a great ability to degrade this recalcitrant environmental pollutant. There are no data in the consulted literature related to the potential of BaP degradation by representatives of the genus *Megasporoporia*; therefore, the fungus *Megasporoporia* sp. S47 can be considered as a promising genetic resource for future studies related to the optimization of culture conditions targeting the increase of the enzymatic production and PAHs degradation rates. Additionally, this study also demonstrates the importance of the microbial selection stage, even to those collected in areas contaminated with PAHs, ensuring the obtainment of genetically promising species for biodegradation of environmental pollutants with a view to their use in bioremediation programs.

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