Marine Pollution Bulletin xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

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

Biodegradation of anthracene and several PAHs by the marine-derived fungus *Cladosporium* sp. CBMAI 1237

Willian G. Birolli[a], Darlisson de A. Santos[a], Natália Alvarenga[a], Anuska C.F.S. Garcia[b], Luciane P.C. Romão[b], André L.M. Porto[a,*]

[a] Laboratório de Química Orgânica e Biocatálise, Instituto de Química de São Carlos, Universidade de São Paulo, Av. João Dagnone, 1100, Ed. Química Ambiental, J. Santa Angelina, 13563-120, São Carlos, SP, Brazil
[b] Laboratório de Matéria Orgânica Natural, Departamento de Química, Universidade Federal de Sergipe, 49100-000, São Cristovão, SE, Brazil

ARTICLE INFO

Keywords:
Marine fungi
Polycyclic aromatic hydrocarbons
Biotransformation
Organic pollutant
Fluorene
Acenaphthene

ABSTRACT

The biodegradation of polycyclic aromatic hydrocarbons (PAHs) by marine-derived fungi was reported in this work. Marine-derived fungi (*Trichoderma harzianum* CBMAI 1677, *Cladosporium* sp. CBMAI 1237, *Aspergillus sydowii* CBMAI 935, *Penicillium citrinum* CBMAI 1186 and *Mucor racemosus* CBMAI 847) biodegraded anthracene (14 days, 130 rpm, 50 mg mL^{-1} initial concentration in malt 2% medium). *Cladosporium* sp. CBMAI 1237 was the most efficient strain and biodegraded more anthracene in the presence (42% biodegradation) than in the absence (26%) of artificial seawater, suggesting that the biodegradation of PAHs may be faster in seawater than in non-saline environment. After 21 days, *Cladosporium* sp. CBMAI 1237 biodegraded anthracene (71% biodegradation), anthrone (100%), anthraquinone (32%), acenaphthene (78%), fluorene (70%), phenanthrene (47%), fluoranthene (52%), pyrene (62%) and nitropyrene (64%). Previous undocumented metabolites were identified and, anthraquinone was a common product of different PAHs biodegradation. The marine-derived fungus *Cladosporium* sp. CBMAI 1237 showed potential for bioremediation of PAHs.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more fused benzene rings generated by thermal decomposition of organic molecules (Haritash and Kaushik, 2009). These compounds have low vapor pressure, high melting and boiling points, low aqueous solubility and, bioavailability that decreases with the increasing of molecular weight, which reduces the microbial degradation and promote their accumulation in solids (Johnsen et al., 2005).

PAHs are mainly produced by pyrosynthesis and pyrolysis during incomplete combustion. At over 500 °C, the molecular bonds are broken and form free radicals, which combine with acetylene and condense in aromatic ring structures (Ravindra et al., 2008). Moreover, PAHs can also be produced at lower temperatures (100–150 °C) during oil maturation, and cause freshwater and oceanic contamination during transportation, storage or use of crude oil and its products (Abdel-Shafy and Mansour, 2016). As other pollutants, PAHs are widespread in the atmosphere due to their volatility, bounding to particulate matter and consequently polluting the entire environment (Kim et al., 2013). Ingestion and inhalation usually occurs by contaminated food and airborne contaminants, generating several health problems such as cancer, endocrine disruption and, birth and reproductive problems (Ball and Truskewycz, 2013).

Studies in marine environment have showed that biodegradation is one of the most important processes in the weathering of oil (Bacosa et al., 2015; Liu et al., 2012). However, this process is slow and takes years, although it has been shown that contaminated areas present increased population of PAH-degrading bacteria, as reported in the Gulf of Mexico (Bacosa et al., 2016; Liu et al., 2012; Sammarco et al., 2013). Therefore, the introduction of microorganisms adapted to the marine environment to increase the biodegradation rate of PAHs may be an important approach to decrease these compounds concentration in a contaminated site.

Studies have been performed aiming the understanding of the role of marine bacteria in the biodegradation of oil products such as PAHs. Bacterial strains, mainly the phyla Proteobacteria (α-, β- and γ-classes), Actinobacteria, Cyanobacteria, Bacteriodetes and Firmicutes capable of biodegrade PAHs have been isolated from different marine environments (Louvado et al., 2015; Yuan et al., 2015). Spills generate a response on the oil-degrading bacterial community, from which strains are usually isolated, such as the *Marinobacter* strains that showed potential for biodegradation of PAHs (Lamendella et al., 2014; Vila et al.,

* Corresponding author.
E-mail address: almporto@iqsc.usp.br (A.L.M. Porto).

http://dx.doi.org/10.1016/j.marpolbul.2017.10.023
Received 11 May 2017; Received in revised form 27 September 2017; Accepted 9 October 2017

2010), *Mycobacterium* sp. strain AP1 that degraded three- and four-ring PAHs (Vila and Grifoll, 2009) and *Pseudomonas* strains isolated from Patagonian coasts (Isaac et al., 2015). Generally, PAHs biodegradation studies are carried out progressively from pure cultures to environmental communities, explaining implications in complex systems of the environment (Vila et al., 2015).

Fungi may possess increased capacity to degrade pollutants due to their ability to extend mycelial networks and low specificity of catabolic enzymes. Studies about biodegradation of PAHs by several terrestrial fungi were already described in the literature in different conditions (Kadri et al., 2017). Such as the biodegradation of different PAHs by *Pleurotus ostreatus* (oyster mushroom) in lab experiments (Pozdnyakova et al., 2016), in soil (Marquez-Rocha et al., 2000) and its spent mushroom substrate as bulking agent in a dynamic biopile pilot plant (Di Gregorio et al., 2016).

However, marine-derived fungi have not been extensively employed for bioremediation (Harms et al., 2011). Although different strains of marine bacteria have been studied for biodegradation of PAHs, only few studies about marine-derived fungi have been reported. Marine fungi strains were isolated and screened for PAHs biodegradation experiments, and *Aspergillus sclerotiorum* CBMAI 849 degraded 85% of pyrene and 61% of benzo[*a*] pyrene after 4 and 8 days of reaction, respectively (Da Silva et al., 2008; Passarini et al., 2011). Whereas *Cochliobolus lunatus* strain CHR4D isolated from a contaminated area at Gujarat (India) biodegraded 93% of chrysene in the 4th day of reaction (Bhatt et al., 2014).

In another interesting work, fungi of the *Ascomycota* phylum were isolated from oil-soaked sand patties collected from beaches following the Deepwater Horizon oil spill (United States of America) and 42–84% PAHs were biodegraded (Simister et al., 2015). Therefore, marine-derived fungi are a promising source of biocatalysts for PAHs biodegradation/bioremediation and the exploration of these microorganisms for decontamination is desirable.

Marine-derived microorganisms are naturally adapted to extreme temperature, acidity, pressure and/or salt concentration in the ocean; therefore, they can be an important source of new enzymes with interesting characteristics, i.e., high salt tolerance, hyperthermostability, barophilicity and cold adaptivity (Dash et al., 2013; Rocha et al., 2012). Marine fungi enzymes may show unique properties and great potential for biodegradation of toxic compounds such as PAHs. Different classes of persistent organic pollutants were biodegraded by marine-derived fungi, including pesticides such as methyl parathion (Alvarenga et al., 2014b), pentachlorophenol (Vacondio et al., 2015), and dyes as Reactive Blue 4 (anthraquinone-derived compound) (Verma et al., 2012).

This work is aimed to investigate the biodegradation of anthracene, a starting member of the PAHs chemical group and one of the simplest, by marine-derived fungi in the presence of artificial seawater (ASW) and in low concentration of minerals. In addition, the most efficient strain *Cladosporium* sp. CBMAI 1237 was employed in the biodegradation of several PAHs.

2. Materials and methods

2.1. PAHs, reagents and solvents

Anthracene (99%), acenaphthene (99%), phenanthrene (97%) and pyrene (98%) were obtained from Acros Organics. Whereas anthraquinone (97%), fluorene (98%), fluoranthene (98%) and nitropyrene (99%) were acquired from Sigma-Aldrich and anthrone (97%) from Vetec. Salts, reagents and solvents were obtained from Sigma-Aldrich and Synth (São Paulo, Brazil). Malt extract and agar were purchased from Kasvi (Paraná, Brazil).

2.2. Marine-derived fungi

The marine-derived fungi employed in this work were isolated from marine sponges obtained at a non-contaminated site at São Sebastião (São Paulo state coast, Brazil), identified by conventional and molecular methods at the Chemical, Biological and Agricultural Multidisciplinary Research Center (CPQBA/UNICAMP, Brazil) and deposited in the *Brazilian Collection of Environmental and Industrial Microorganisms* (CBMAI - http://webdrm.cpqba.unicamp.br/cbmai/, WDCM823).

The genomic DNA was extracted according to the literature (Aamir et al., 2015). The amplification of the region ITS1-5.8S-ITS2 was performed by PCR methodology with the primers ITS-1 and ITS-4. The amplification product was purified in column (GFX PCR DNA and Gel Band Purification Kit, GE Healthcare) and directly submitted to sequencing (automated sequencer ABI3500XL Series, Applied Biosystems).

A consensus DNA sequence was generated for the phylogenetic analysis and compared with the Genbank (http://www.ncbi.nlm.nih.gov/) and CBS (http://www.westerdijkinstitute.nl/) databank. The DNA sequence were aligned employing the software CLUSTAL X (Thompson et al., 1997) and the phylogenetic analysis were carried out by the software MEGA 6.0 (Tamura et al., 2007). The evolutionary distance was calculated according to the Kimura model (Kimura, 1980) and the phylogenetic three were carried out with the Neighbor-Joining methodology (Saitou and Nei, 1987), with bootstrap values calculated by 1.000 resamples, employing the software MEGA 6.0.

The strains *Cladosporium* sp. CBMAI 1237, *Aspergillus sydowii* CBMAI 935, *Penicillium citrinum* CBMAI 1186 and *Mucor racemosus* CBMAI 847 were isolated, identified and deposited by Kossuga et al. and *Trichoderma harzianum* CBMAI 1677 by Vacondio et al. as reported in the literature (Kossuga et al., 2012; Vacondio et al., 2015). It is noteworthy that the marine-derived fungi were isolated in different media without the presence of PAHs or any xenobiotic. Subsequently, these strains were adapted to grow in malt 2% medium in the presence of PAHs.

2.3. Growth of marine-derived fungi on solid medium

Marine-derived fungi were cultivated in malt 2% medium composed of malt extract (20 g L^{-1}) and agar (20 g L^{-1}) in distilled water or ASW with pH adjusted to 7.0. The ASW composition was: $CaCl_2{\cdot}2H_2O$ (1.3 g L^{-1}), $MgCl_2{\cdot}6H_2O$ (9.68 g L^{-1}), KCl (0.61 g L^{-1}), NaCl (30.0 g L^{-1}), Na_2HPO_4 (0.014 mg L^{-1}), Na_2SO_4 (3.47 g L^{-1}), $NaHCO_3$ (0.17 g L^{-1}), KBr (0.1 gL^{-1}), $SrCl_2{\cdot}6H_2O$ (0.040 g L^{-1}) and H_3BO_3 (0.030 g L^{-1}) (Kjer et al., 2010; Kossuga et al., 2012). The solid culture media were inoculated with an inoculation loop and incubated at 32 °C (B.O.D. 411D, Nova Ética, Brazil).

2.4. Growth of marine-derived fungi in liquid medium

250 mL Erlenmeyer flasks containing 100 mL of 2% (w/v) malt liquid medium were employed for fungi cultivation in liquid medium. The inoculations were carried out with seven circular slices (0.5 cm diameter) from a fungus culture incubated for 7 days at 32 °C (B.O.D. 411D, Nova Ética, Brazil). The biodegradation reactions were carried out with different strains in 2% malt liquid medium with ASW (high concentration of minerals) and in low concentration of salts, in which distilled water was employed instead of ASW. The cultures were incubated in orbital shaker for 5 days (32 °C, 130 rpm) and after that, 50 mg L^{-1} of anthracene (previously dissolved in 200 µL dichloromethane, which does not interfere in the fungal cells because this solvent is insoluble in water and evaporates very quickly) were added and the reactions were incubated in orbital shaker for 14 days (32 °C, 130 rpm). All biodegradation experiments were performed in duplicate.

Cladosporium sp. CBMAI 1237 was also employed in biodegradation experiments with different PAHs (50 mg L^{-1}) in malt 2% liquid medium incubated in orbital shaker for 21 days (32 °C, 130 rpm). In addition, fungal control experiments (without PAHs) to identify the natural metabolites of the employed strains and, abiotic control