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Original Research Paper

Biodegradation of pentachlorophenol by marine-derived fungus *Trichoderma harzianum* CBMAI 1677 isolated from ascidian *Didemnun ligulum*

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ABSTRACT

In this study, the marine-derived fungi *Aspergillus sydowii* DL6A, *Apergillus versicolor* DL5A, *Cladosporium oxysporum* DL5G, *Fusarium proliferatum* DL11A and *Trichoderma harzianum* CBMAI 1677 isolated from marine ascidian *Didemnun ligulum* were evaluated according to their growth in the presence of pentachlorophenol (PCP). The colonies were assessed in 10, 20, 30, 40 and 50 mg L^{-1} of PCP in a solid culture medium (3% malt). The fungus *T. harzianum* CBMAI 1677 showed the best growth at 50 mg L^{-1}, which suggests its potential for biodegradation, therefore, this strain was selected for quantitative experiments in 3% malt liquid medium (initial concentration of 20 mg L^{-1} of PCP) using a validated method. After 7 d of incubation, PCP was not detected and an increasing concentration of pentachloroanisole (PCA) and 2,3,4,6-tetrachloroanisole (2,3,4,6-TeCA) was observed. In a second step, *T. harzianum* CBMAI 1677 was employed in the biodegradation of PCA and 2,3,4,6-TeCA in a liquid medium. It was observed that both PCA and 2,3,4,6-TeCA were also biodegraded. *T. harzianum* CBMAI 1677 is a potential strain for bioremediation studies since it was able of biodegrade not only PCP at 20 mg L^{-1}, but also its main metabolites PCA and 2,3,4,6-TeCA.

1. Introduction

The contamination of the environment by a variety of toxic compounds such as pesticides is worrisome (Baird, 2002). Among all pesticides used in agriculture, those belonging to the organochlorine class cause serious concern due to their high toxicity and resistance to biotic and abiotic degradation (Singh and Walker, 2006). The most part of the organochlorine pesticides have been studied because of their persistence and extensive use in the past, since these compounds have been banned from agriculture (Kayser et al., 2001). However, the organochlorine compound Pentachlorophenol (PCP) has been still used as insecticide, fungicide, herbicide and wood preservative (Salmerón-Alcocer et al., 2007). Furthermore, it is a byproduct of different processes such as paper bleaching, water treatment (which containing phenols and chlorine or sodium hypochlorite), incineration of urban waste and chlorination of wastewater (Chandra et al., 2006).

The use of microorganisms as degrading agents of dyes, cosmetics, detergents, medicines and agricultural chemicals (Harms et al., 2011) is an effective method to reduce environmental harm, since biodegradation by microorganisms is the main process of pesticide dissipation in soil. Once pesticides are toxic, it is necessary to know if the degradation products are also toxic and will be subsequently degraded to less aggressive compounds (Kanekar et al., 2004). Thus studies have described the PCP biodegradation by microorganisms (Tortella et al., 2005; Yadid et al., 2013).

Some microorganisms characteristics such as: development under extreme conditions (Kumar et al., 2011), fast growth, low cultivation cost and capacity of transforming a wide variety of non-natural chemicals are very important to their use in bioremediation (Simões and Tauk-Tornisielo, 2005; Yu et al., 2011). Dash et al. (2013) reported that marine microorganisms may show

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these characteristics, producing unique enzymes in different environmental conditions.

Marine-derived fungi can produce compounds different from their respective representatives of terrestrial environment (Saleem et al., 2007). Thereforea wide variety of enzymes with specific activities were identified in marine microorganisms isolated from inorganic materials, marine invertebrates and vertebrates, and plants (Conceição et al., 2005; Rocha et al., 2013; Ferreira et al., 2014) for use in biotransformation and secondary metabolites studies (Rateb and Ebel, 2011).

Studies about enzyme production by filamentous marine-derived fungi are important for future applications in bioremediation of contaminated environments. This manuscript deals with the biodegradation of PCP, an organochlorine pesticide, by fungi strains isolated from ascidian *Didemnun ligulum*.

2. Materials and methods

2.1. Reagents

PCP (98%, analytical standard) and pentachloronitrobenzene (99%, PCNB) were commercially obtained from Sigma-Aldrich, (São Paulo, Brazil). 2,3,4,6-Tetrachlorophenol (98%, analytical standard) was obtained from SUPELCO. The salts of the artificial sea water and ethyl acetate were acquired from Synth and Vetec. Malt extract and soy peptone were obtained from ACUMEDIA (São Paulo, Brazil).

2.2. Isolation of marine-derived fungi

Marine-derived fungi were isolated from marine ascidian *D. ligulum* according to the protocol described by Kossuga et al. (2012) and stored according to Castellani (1967). Details are presented in Supplementary material-1.

2.3. Growth of strains in a solid medium

Five strains of marine-derived fungi were used: *Trichoderma harzianum* CBMAI 1677, *Aspergillus sydowii* DL6A, *Apergillus versicolor* DL5A, *Cladosporium oxysporum* DL5G and *Fusarium proliferatum* DL11A. *T. harzianum* was deposited in the *Brazilian Collection of Environmental and Industrial Microorganisms* (CBMAI, WDCM823—http://webdrm.cpqba.unicamp.br/cbmai/), deposite number 1677.

They were cultivated on Petri plates containing 3% malt solid culture medium using artificial seawater with the following composition: malt extract (30.0 g L^{-1}), soy peptone (3.0 g L^{-1}) and agar (20.0 g L^{-1}). The pH was adjusted to 8 (pH of the sea coast) with KOH solution (0.1 mol L^{-1}) (Kossuga et al., 2012). Artificial seawater composition was: $CaCl_2 \cdot 2H_2O$ (1.36 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 (0.10 g L^{-1}), $SrCl_2.6H_2O$ (0.04 g L^{-1}) and H_3BO_3 (0.03 g L^{-1}) (Menezes et al., 2010).

2.4. Selection of strains resistant to pesticide PCP

Solid culture media (3% malt) with (10, 20, 30, 40 and 50 mg L^{-1}) of PCP and without PCP (control experiments) were prepared and sterilized in autoclave at 121 °C for 20 min. The pesticide was added to the media at 40–50 °C to prevent thermal degradation. Agar plates were inoculated at a central insertion point and incubated at 32 °C. The colony diameters were measured after 7, 14 and 21 d of cultivation as performed by Birolli et al. (2014). The strain that showed the best radial growth was selected for the biodegradation and metabolites identification of PCP in a liquid medium. The experiments were performed in triplicates.

2.5. Biodegradation of PCP/PCA/2,3,4,6-TeCA in a liquid medium by *Trichoderma harzianum* CBMAI 1677

The strain was cultivated in inclined test tubes containing 5 mL of a solid culture medium for 7 d at 32 °C for the preparation of the spores solution. After the culture growth, 2 mL of a 1.0% Tween 80 solution were added to each tube. The spores were released from the mycelium using the inoculation loop and transferred to a 100 mL Erlenmeyer flask containing previously sterilized glass beads. Subsequently, the solution was stirred for a few minutes and filtered using glass fiber with a sterile Buchner filtration apparatus. After successive washes with sterile distilled water for the complete removal of Tween 80, a hemocytometer (Neubauer chamber) was used for the spores count.

Erlenmeyer flasks of 250 mL containing 100 mL of a 3% malt liquid medium at pH 8 (the pH of the seawater) were sterilized in an autoclave at 121 °C for 20 min. After cooling, each flask was inoculated with 1 mL of 10^5 spores mL^{-1} of strain *T. harzianum* CBMAI 1677 and incubated in orbital shaking for 3 d (32 °C, 130 rpm). Subsequently, 2 mg (20 mg L^{-1}) of PCP, PCA or 2,3.4.6-TeCA dissolved in 200 μL ethyl acetate were added. The degradation experiments were performed in an orbital shaker (32 °C, 130 rpm) in triplicates for 7, 14 and 21 d.

2.6. Control experiments

Fungus control was performed for the assessment of the natural metabolites under the same conditions of the biodegradation experiments, however, without the pesticide. To evaluate the pesticide stability during the cultivation period, abiotic control experiments were performed in 250 mL Erlenmeyer flasks containing 3% malt culture medium and PCP (20 mg L^{-1}). After 21 d, the samples were extracted and analyzed by gas chromatography (GC-FID). Each experiment was carried out in triplicate.

2.7. Extraction of PCP from the culture medium

For the chromatographic analyses, the samples were fractionated and extracted in three different ways: *analysis of the culture broth*, *analysis of the mycelial extract* and *analysis of total extract* (culture broth plus mycelial extract).

For the samples extraction in the *analysis of total extract*, the mycelium was vacuum-filtered using filter paper and a Büchner funnel coupled to a 250 mL Kitassato. After filtration, the culture broth was separated and the filtrated mycelium was transferred to a 250 mL Erlenmeyer flask containing 60 mL of distilled water and ethyl acetate (1:1) and subjected to vigorous magnetic stirring for 30 min for the fungal cells lysis. It is noteworthy that a mechanical (stirring) and a chemical (solvent) method were employed simultaneously for the cell lysis.

The cells were filtered in a Büchner funnel and the mycelial extract was added to the culture broth of the first filtration. Subsequently a liquid–liquid extraction was performed in three steps of 30 mL ethyl acetate each. Anhydrous Na_2SO_4 was added to the organic phase, which was filtered and evaporated in vacuum for a final volume of 1.0 mL ethyl acetate.

The *analysis of the culture broth* was performed with the supernatant of the first filtration (without the addition of the mycelial extract), whereas the *analysis of the mycelial extract* was carried out in the lysed mycelial mass.