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Biodegradation of Chlorpyrifos by Whole Cells of Marine-Derived Fungi *Aspergillus sydowii* and *Trichoderma* sp

Natália Alvarenga[1], Willian G Birolli[1], Marcia Nitschke[1], Maria Olimpia de O Rezende[1], Mirna H R Seleghim[2], André L M Porto[1,*]

[1]*Instituto de Química de São Carlos, Universidade de São Paulo, Brazil*

[2]*Laboratório de Ecologia e Microbiologia Aquática, Universidade Federal de São Carlos, Brazil*

Abstract

This paper describes the screening of the growth of seven marine-derived fungi strains in the presence of chlorpyrifos in solid medium. The strains that showed best growth were *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932. Biodegradation reactions were performed in 10, 20 and 30 d in liquid medium containing commercial chlorpyrifos and mycelia from the selected strains. In 30 d, *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 were able to degrade on average 63% and 72% of chlorpyrifos, respectively, and reduce the concentration of 3,5,6-trichloro-2-pyridinol, the metabolite formed by the enzymatic hydrolysis of chlorpyrifos. In 30 d, *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 could use chlorpyrifos as sole source of carbon with low biodegradation percentages, 24% and 5%, respectively. Spontaneous hydrolysis was evaluated in malt medium, with the complete disappearance of chlorpyrifos. In distilled water, 61% of chlorpyrifos was hydrolyzed in 30 d.

Keywords: Organophosphate pesticides; 3,5,6-Trichloro-2-pyridinol; Marine fungi; Enzymatic hydrolysis

Introduction

Different highly toxic organic compounds have been released in the environment over the past decades [1]. Among these compounds, pesticides play an important role in modern agriculture, since they are effective in pest control and increase productivity and food quality. Millions of tons of pesticides are applied annually, but it is believed that only a small amount effectively reaches target species [2].

The fate of pesticides in the environment is influenced by processes that determine their persistence and mobility [3]. Biodegradation is the main mechanism for degradation in soil, as a result of the microbial metabolism in the pesticides' molecules. Chemical processes of degradation, such as oxidation/reduction, hydrolysis and photolysis may also occur in the environment, but they are dependent on the physical and chemical characteristics of the pesticide [4].

Microorganisms are the main biological agents capable of removing waste and degrading materials, promoting their recycling in the environment [5]. Since conventional treatments for pesticide residues include the removal of contaminated materials for incineration or disposal in landfills, biological remediation *in situ* has emerged as a safe, less harmful and more cost-effective alternative [6].

Organophosphate pesticides (OPs) are among the most important classes of pesticides used worldwide. They have emerged as an alternative to organochlorine compounds, due to their low cost, easy synthesis, increased biodegradability and limited accumulation in living organisms, in comparison to organochlorines [7]. However, OPs are also highly toxic, since they are potent irreversible inhibitors of acetylcholinesterase (AChE), an enzyme that exerts a profound effect on the nervous system of exposed organisms, including humans [8].

Chlorpyrifos (CP), *O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate is one of the major commercialized organophosphate pesticides [9]. According to the U.S. Environmental Protection Agency (EPA), between the years 1987 and 1998, approximately 21 to 24 million pounds were used annually in the U.S., of which approximately 11 million pounds were applied to non-agricultural purposes [10]. CP is effective against pests in a variety of food crops, greenhouses, turf and ornamentals plants. It can also be applied to the control of indoor and structural pests and as an active ingredient in pet collars [11].

In the environment, CP is degraded to 3,5,6-trichloro-2-pyridinol (TCP) and an alkyl phosphorotioate moiety [12]. Although TCP cannot act as an inhibitor of the AChE enzyme, it still is a pollutant compound. According to the database of pesticides (PAN Pesticides Database), no toxicological class has been defined by the World Health Organization or other regulatory organizations for TCP [13,14]. TCP has higher water solubility than CP, therefore, it can be more easily carried into the environment and contaminate soils and water bodies [9]. It is believed that TCP not only is persistent towards biodegradation, but also limits the CP metabolization, preventing the proliferation of degrading microorganisms since it exhibits antimicrobial properties [9,15].

A number of enzymes capable of detoxifying OPs have been discovered and the majority belongs to the class of phosphotriesterases (PTE). The biodegradation of OPs involving bacteria has been widely reported in the literature [9,16-18]. However, fungi biodegradation has been underexplored in comparison with studies on bacteria.

Fungi degrade a large variety of compounds through a process known as mycodegradation. It involves the xenobiotic degradation to smaller molecules, which may be toxic or non-toxic [19]. Phosphotriesterases from marine-derived fungi are a promising source of degrading enzymes that exhibit unique properties, once they are derived from organisms whose natural habitat is brackish or saline [20,21]. The final destinations of many xenobiotic compounds are the sea and ocean, where they can be further degraded [22].

***Corresponding author:** André L M Porto, Instituto de Química de São Carlos, Universidade de São Paulo, Brazil, Tel: +55 16 3373 8103; Fax: +55 16 3373 9952; E-mail: almporto@iqsc.usp.br

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Previous studies on the biodegradation of pesticides by marine-derived fungi were successfully conducted by our group. The organochlorine pesticide, DDD, was completely biodegraded by marine-derive fungi *Trichoderma* sp. CBMAI 932 in the presence of hydrogen peroxide [23]. Methyl parathion was completely degraded by *Penicillium decaturense* CBMAI 1234 and *Aspergillus sydowii* CBMAI 935 in 20 and 30 d, respectively [22]. Strains of *A. sydowii* CBMAI 935 and *Penicillium raistrickii* CBMAI 931 were capable of degrading profenofos in 30 d at rates of 72% and 97%, respectively, and also promoted an almost full degradation of the main profenofos metabolite, 4-bromo-2-chlorophenol, in the same period [24]. High conversion rates (70%) were achieved in the biotransformation of organochlorine, dieldrin, by *P. miczynskii* CBMAI 930 after only 4 d of incubation [25].

The present study reports results of the biodegradation of a commercial formulation of CP (Lorsban 480 BR) and its main hydrolysis product, TCP, promoted by marine-derived fungi strains of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932.

Materials and Methods

Chemicals

The commercial formulation of chlorpyrifos, named "Lorsban 480 BR" (48% m/v of chlopyrifos and 66.9% m/v of inert ingredients), was obtained from Dow AgroSciences (São Paulo, Brazil). Chlorpyrifos (99.9%) and 3,5,6-trichloro-2-pyridinol (99.3%) analytical standard were purchased from Sigma-Aldrich (São Paulo, Brazil). Reagents, methanol HPLC grade and ethyl acetate for extraction procedures were obtained from Sigma-Aldrich and Synth (São Paulo, Brazil). Malt extract and agar were purchased from Acumedia (São Paulo, Brazil) and Himedia (Paraná, Brazil). Salts for the preparation of artificial seawater were obtained from Synth (São Paulo, Brazil).

Marine-derived fungi

The Brazilian marine-derived fungal strains of *Aspergillus sydowii* CBMAI 934, *Aspergillus sydowii* CBMAI 935 and *Penicillium raistrickii* CBMAI 931 were isolated from the sponge *Chelonaplysilla erecta; Aspergillus sydowii* CBMAI 1241, *Penicillium decaturense* CBMAI 1234 and *Penicillium raistrickii* CBMAI 1235 were isolated from the sponge *Dragmacidon reticulatum* and *Trichoderma* sp. CBMAI 932 was isolated from the sponge *Geodia corticostylifera*. The sponges were collected by Prof. Roberto G. S. Berlinck (Chemistry Institute of São Carlos, University of São Paulo, IQSC-USP, Brazil) in São Sebastião, South Atlantic Ocean in the northern coast of São Paulo state, Brazil, The marine-derived fungi were isolated and purified in the microbiology laboratory of the Department of Ecology and Aquatic Microbiology supervised by Mirna H. R. Seleghim (UFSCar, Brazil) [26]. They were identified by both 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* (WDCM 823 - Coleção Brasileira de Microrganismos de Ambiente e Indústria - CBMAI - http://webdrm.cpqba.unicamp.br/cbmai/).

Composition of culture media and artificial sea water

Artificial sea water (ASW) (1 L): $CaCl_2.2H_2O$ (1.36 g), $MgCl_2.6H_2O$ (9.68 g), KCl (0.61 g), NaCl (30.0 g), Na_2HPO_4 (0.014 mg), Na_2SO_4 (3.47 g), $NaHCO_3$ (0.17 g), KBr (0.1 g), $SrCl_2.6H_2O$ (0.040 g), H_3BO_3 (0.030 g). -

Solid culture for fungal screening: The best strains for the chlopyrifos degradation were selected in a solid culture composed of agar (20 g L^{-1}) and malt extract (20 g L^{-1}) in ASW (1 L). The medium was adjusted to pH 5 with 0.7 KOH solution.

Liquid culture: Biodegradation reactions between the selected strains and CP were performed in a liquid medium composed of malt extract (20 g L^{-1}) in ASW (1L) adjusted to pH 7 with 0.7 KOH solution.

Liquid mineral medium supplemented with KNO_3: Biodegradation reactions were performed in a mineral medium composed of KNO_3 (12.5 mg L^{-1}), commercial CP (50 mg L^{-1}), in ASW (1 L) adjusted to pH 7 with 0.7 KOH solution.

The culture media were sterilized in an autoclave (121°C, 1.5 kPa) for 20 minutes. All manipulations involving the marine-derived fungi were carried out in a laminar flow cabinet under sterile conditions (Veco).

Growth of marine-derived fungi in a solid medium: Qualitative screenings for the fungal selection were carried out in Petri dishes containing a solid culture medium without pesticide (control culture) and a medium supplemented with commercial CP in 96, 192 and 288 mg L^{-1} (5, 10 and 15 µL) per plate (25 mL) and solubilized in 100, 200 and 300 µL of DMSO, respectively. Fungal mycelia from recent cultures were transferred to the surface of the agar plates with an inoculation loop. Strains were incubated at 32°C for 10 d and the tolerance of the fungus to the pesticide was estimated by comparing the size of the colony grown on the plates with that of the control cultures.

Growth of marine-derived fungi in a liquid medium: Spore solutions of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 were prepared by growing the fungi for 7 d at 32°C in tubes containing solid culture medium and 50 mg L^{-1} of commercial CP. 10^5 Spores mL^{-1} of each strain were inoculated in a 250 mL Erlenmeyer flask containing 100 mL of a liquid medium and cultivated in an orbital shaker (Technal model TE-421) for 4 d at 32°C and 130 rpm. After the strains growth, the liquid medium containing fungi mycelia was supplemented with 50 mg L^{-1} of commercial CP and the reaction was incubated in the orbital shaker for 10, 20 and 30 d (130 rpm, 32°C). After the reaction period, the culture was filtered in a Buchner apparatus and the mycelial mass obtained was rinsed and suspended in 20 mL of water and ethyl acetate (1:1). The mixture was magnetically stirred for 30 minutes and filtered again in a Buchner funnel. The mycelia dry weight was determined after drying at 32°C for 24 h. The samples were analyzed by different methods:

i) On the 10th and 30th d of reaction, the extracted mycelia and the liquid medium were placed in an Erlenmeyer flask, acidified to pH 6 and extracted with ethyl acetate (3 x 25 mL). In the organic phase, anhydrous Na_2SO_4 was added, filtered and evaporated under vacuum and the residue was resuspended in 10 mL of methanol HPLC grade.

ii) On the 20th d of reaction, the extractions of mycelial mass and liquid medium were analyzed together and separately. The extraction procedures were the same as those described in item *i*.

The experiments were carried out in duplicate. CP and TCP were quantified by HPLC-UV (in comparison with standard solutions) and the samples were analyzed by GC-MS. The results are summarized in Tables 2-5.

Biodegradation of CP in a liquid medium: Studies were conducted during 30 d for the evaluation of different parameters of the CP biodegradation reactions.

i. **Growth of marine-derived fungi *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 in the absence of chlorpyrifos (fungus control):** The reactions were performed in 250 mL Erlenmeyer flasks containing 100 mL of a liquid medium and 10^5 spores mL^{-1} of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, and incubated in an orbital shaker for 30 d (130 rpm, 32ºC). Extraction and analyses were performed as described in item *i*. The experiments were carried out in duplicate.

ii. **Biodegradation of 3,5,6-trichloro-2-pyridinol (main metabolite) by *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 in a liquid medium:** The reactions were performed in 250 mL Erlenmeyer flasks containing 100 mL of a liquid medium and 10^5 spores mL^{-1} of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, and incubated in an orbital shaker for 4 d (130 rpm, 32ºC). After the strains growth, the liquid medium was supplemented with 50.0 mg L^{-1} of TCP and incubated in an orbital shaker for 30 d (130 rpm, 32ºC). Extraction and analyses were perfomed as described in item *i*. The experiments were carried out in duplicate.

iii. **Biodegradation of chlorpyrifos by *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 in a liquid minimal medium supplemented with KNO_3:** The reactions were performed in 250 mL Erlenmeyer flasks containing 100 mL of a liquid mineral medium supplemented with KNO_3, 10^5 spores mL^{-1} of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 and 100 mg L^{-1} of commercial CP and incubated in an orbital shaker for 30 d (130 rpm, 32ºC). Extraction and analyses were perfomed as described in item *i*. The experiments were carried out in duplicate.

iv. **Degradation of chlorpyrifos in the absence of marine-derived fungi (abiotic control):** Two conditions of an abiotic control experiment were tested. The reactions were performed in 250 mL Erlenmeyer flasks containing 100 mL of a liquid medium of malt extract (2% w/v) and also in 100 mL of sterilized distilled water containing 50 mg L^{-1} of commercial CP. They were incubated in an orbital shaker for 30 d (130 rpm, 32ºC). Extraction and analyses were perfomed as described in item *i*. The experiments were carried out in duplicate.

Analytical procedures and quantification of CP and TCP: High-performance liquid chromatography (HPLC) analyses of the biodegradation reactions were conducted in a Shimadzu Prominence series equipped with a photodiode array detector (SPD-M20A). The HPLC (equipped with a 0.46 x 25 cm ODS (M) C18 column) conditions were: isocratic mobile phase of acetonitrile and water (70:30), detection wavelength of 280 nm, 10 µL injection volume, flow rate of 1.0 mL min^{-1}, oven at 40°C and run time of 20 min. The retention times for TCP and CP were 4.4 and 16.5 min, respectively. CP and TCP concentrations were quantified by an analytical curve of standard solutions for the evaluation of the percentage of pesticide degraded and TCP formed (SI, Figure S1). Gas chromatography-mass spectrometry (GC-MS) analyses of the fungal metabolites and biodegradation products were performed in a Shimadzu GC2010plus coupled to a mass selective detector (Shimadzu MS2010plus) in electron ionization (EI, 70 eV) mode. The GC-MS (equipped with a 30 m x 0.25 mm x 0.25 µm J&W Scientific DB5 column) conditions were: oven temperature started at 50ºC and kept for 1 min, increased to 250ºC at 5ºC min^{-1} and held for 10 min; injector and detector temperature maintained at 200ºC; injector split ratio of 1:1 and helium used as the carrier gas at 60 kPa pressure and run time of 32 min. The retention times for TCP and CP were 12.4 and 17.7 min, respectively.

Results and Discussion

Screening of marine-derived fungi in a solid medium

The screened marine-derived fungi were *A. sydowii* CBMAI 934, *A. sydowii* CBMAI 935, *A. sydowii* CBMAI 1241, *P. decaturense* CBMAI 1234, *P. raistrickii* CBMAI 931, *P. raistrickii* CBMAI 1235 and *Trichoderma* sp. CBMAI 932. The screening was carried out in a solid medium of malt extract (2% w/v) in the presence of CP for the selection of the marine-derived fungi able to grow in increasing concentrations of the pesticide. The fungi were grown in a solid culture medium at pH 5, which is an optimal value for the cultivation of most fungi [24].

The fungi biodegradation potential was evaluated by comparing the colonies grown on plates with different pesticide concentrations to those in control cultures (absence of pesticide) after 10 d of incubation. The sizes of the colonies were measured between the furthest points and the qualitative results are shown in Table 1.

All fungal strains showed excellent growth in the control plates

Marine-derived fungi	Strains growth (cm)			
	Control culture*	96 mg L^{-1}	192 mg L^{-1}	288 mg L^{-1}
A. sydowii CBMAI 934	2.0 x 2.0	2.0 x 2.0	1.5 x 1.5	1.0 x 1.0
P. raistrickii CBMAI 931	3.5 x 3.0	4.0 x 2.5	3.5 x 3.0	3.0 x 2.5
A. sydowii CBMAI 935	All plate	All plate	All plate	2.0 x 1.5
P. raistrickii CBMAI 1235	5.0 x 3.0	4.0 x 2.5	3.0 x 2.0	1.5 x 1.5
P. decaturense CBMAI 1234	4.0 x 3.0	4.0 x 2.5	3.0 x 2.5	4.0 x 2.5
A. sydowii CBMAI 1241	5.0 x 3.0	3.5 x 2.5	3.0 x 3.0	1.0 x 1.0
Trichoderma sp. CBMAI 932	All plate	All plate	4.0 x 3.0	4.0 x 3.0

*Absence of chlorpyrifos

Table 1. Growth of marine-derived fungi in a solid medium of malt extract (2% w/v) in the absence (control culture) and presence of chlorpyrifos (32 °C, 10 d, pH 5).

Reaction time (d)	Extraction	Fungal dry mass (g)	[a] TCP (mg L^{-1})	[a] CP (mg L^{-1})	% of CP degraded
A. sydowii CBMAI 935 (50 mg L^{-1} of chlorpyrifos)					
10	(liquid medium + mycelia)	0.41 ± 0.01	3.0 ± 3.0	31.3 ± 5.90	37
20*	(liquid medium + mycelia)	0.40	6.3	24.6	51
20*	(liquid medium)	-	4.9	1.0	
20*	(mycelia)	0.38	-	26.0	46[b]
30	(liquid medium + mycelia)	0.36 ± 0.01	9.1 ± 0.40	18.6 ± 2.30	63
30	(fungus control)	0.34 ± 0.01			
Trichoderma sp. CBMAI 932 (50 mg L^{-1} of chlorpyrifos)					
10	(liquid medium + mycelia)	0.40 ± 0.01	-	27.1 ± 4.50	46
20*	(liquid medium + mycelia)	0.40	-	17.7	65
20*	(liquid medium)	-	-	2.5	
20*	(mycelia)	0.39	-	24.6	46[b]
30	(liquid medium + mycelia)	0.38 ± 0.01	2.9 ± 2.4	13.9 ± 0.07	72
30	(fungus control)	0.43 ± 0.01			

[a] = concentration determined by HPLC-UV.

[b] = % of CP biodegraded was measured by the sum of the concentration remaining in both liquid medium and mycelia.

* Not performed in duplicate.

Table 2. Quantitative biodegradation of chlorpyrifos by *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, on the 10th, 20th and 30th d of reaction in a liquid medium.

Fungi	Fungal dry mass (g)	[a] TCP (mg L^{-1})	% of TCP degraded
A. sydowii CBMAI 935	0.34 ± 0.01	37.3 ± 1.8	25
Trichoderma sp. CBMAI 932	0.19 ± 0.01	30.7 ± 2.1	39

[a] = concentration determined by HPLC-UV.

Table 3. Quantitative biodegradation of 3,5,6-trichloro-2-pyridinol (50 mg L^{-1}) by *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, on the 30th d of reaction in a liquid medium.

Fungi	Fungal dry mass (g)	[a] TCP (mg L^{-1})	[a] CP (mg L^{-1})	% of CP degraded
A. sydowii CBMAI 935	*	4.9 ± 0.01	76.5 ± 16.6	24
Trichoderma sp. CBMAI 932	*	3.7 ± 2.2	94.5 ± 5.5	5

[a] = concentration determined by HPLC-UV.

* = fungal dry mass was not sufficient to be quantified.

Table 4. Quantitative biodegradation of chlorpyrifos (100 mg L^{-1}) by *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, on the 30th d of reaction in a liquid mineral medium supplemented with KNO_3.

Reaction time (d)	[a] CP (mg L^{-1})	% of CP not detected
liquid medium of malt extract (2% w/v)		
10	6.0 ± 1.5	88
20	3.6 ± 1.1	93
30	-	100
Sterilized destilled water		
10	46.2 ± 2.9	8
20	36.1 ± 1.0	28
30	19.5 ± 0.9	61

[a] = concentration determined by HPLC-UV.

Table 5. Quantitative degradation of chlorpyrifos (50 mg L^{-1}) by spontaneous hydrolysis on the 10th, 20th and 30th d of reaction in a liquid medium.

(absence of pesticide) (Figure 1). The fungal growth in the presence of CP may indicate the pesticide metabolization, which may occur as a defense mechanism of the microorganisms with the elimination of the xenobiotic compound, or the use of this substance as a nutrient source for the fungal development. Previous studies with methyl parathion and profenofos showed screening in solid culture medium as an excellent tool for the selection of fungi with biocatalytic potential for biodegradation reactions in a liquid medium [22,24].

The strains of *P. raistrickii* CBMAI 931, *A. sydowii* CBMAI 935, *P. raistrickii* CBMAI 1235, *P. decaturense* CBMAI 1234 and *Trichoderma* sp. CBMAI 932 showed excellent growth in all concentrations evaluated. In comparison to the control culture (absence of pesticide), the fungal growth showed a weak inhibition in plates supplemented with CP, even in plates containing the highest pesticide concentration (288 mg L^{-1}). The results indicated that these strains are resistant to high CP concentrations, i.e., the fungi can adapt to the xenobiotic presence and/ or biodegrade the pesticide.

A. sydowii CBMAI 934 showed a slight growth and inhibition at the highest pesticide concentration (288 mg L^{-1}). Fungus *A. sydowii* CBMAI 1241 showed a good growth in comparison to the control culture. However, at the highest pesticide concentration, it was almost completely inhibited, which may indicate this fungus is less resistant to high concentrations of CP.

The screening in a solid culture medium showed the microorganisms selected for quantification of CP were *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932.

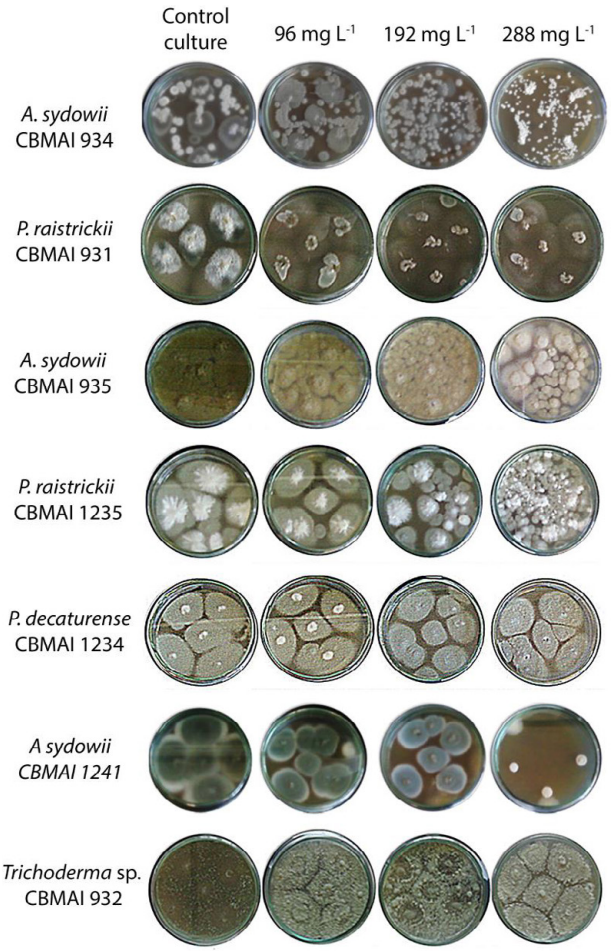


Figure 1. Marine-derived fungi strains growth in a solid culture medium containing different concentrations of chlorpyrifos (10 d, 32 ºC).

Biodegradation of chlorpyrifos by marine-derived fungi *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932

The strains selected for the biodegradation studies in a liquid medium were grown in the presence of CP so as to induce the production of phosphotriesterases or other enzyme classes capable of degrading CP.

Biodegradation reactions in the liquid medium were carried out at pH 7, since phosphotriesterases are more likely to exhibit enhanced catalytic activity at basic pH [27,28]. However, the OP chemical hydrolysis is enhanced at basic pH [8]. Aiming at the evaluation of the effect of microorganisms' enzymes involved in the biodegradation process without significant interference from chemical hydrolysis, the biodegradation experiments were conducted at pH 7, which is the limiting pH for PTE enzyme activity.

The residual CP contained in the extractions of liquid medium and mycelia was evaluated on the 10th and 30th d of reaction between *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 and CP. On the 20th d, it was also evaluated in mycelia and liquid medium separately to verify the presence of a higher concentration of the residual pesticide in the medium (liquid medium extraction) or intracellularly/adhered to the cell wall (mycelia extraction). Table 2 shows the data of CP biodegradation by both fungal strains.

On the 10th d, CP showed biodegradation averages of 37% and 46% for *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, respectively, with no or low formation of the main hydrolysis metabolite, TCP (Table 2). *Trichoderma* sp. CBMAI 932 was very efficient in short reaction times, since almost half of the pesticide was degraded in 10 d.

On the 20th d, a high concentration of CP was found in the mycelial mass (*A. sydowii* CBMAI 935, 26.0 mg L^{-1} and *Trichoderma* sp. CBMAI 932, 24.6 mg L^{-1}), whilst a low amount of the residual pesticide was present in the liquid medium (*A. sydowii* CBMAI 935, 1.0 mg L^{-1} and *Trichoderma* sp. CBMAI 932, 2.5 mg L^{-1}). TCP was quantified (4.9 - 6.3 mg L^{-1}) only in the presence of *A. sydowii* CBMAI 935 (Scheme 1, Figures S2, S3).

Since the major concentration of the residual CP was found in the mycelial mass, it is possible to assume either the fungi have absorbed the pesticide to metabolize this xenobiotic compound, or the pesticide was adhered to the cell's wall surface. The literature supports these data since the PTE enzymes are described to reside intracellularly [29], requiring the transportation of xenobiotics into the cell for their metabolization.

By averaging the two reactions performed for chlorpyrifos, in 20 d, it could be observed a CP degradation of 49% for *A. sydowii* CBMAI 935 and 56% for *Trichoderma* sp. CBMAI. Therefore, 20 d can be inferred as the half-life of CP in the presence of an enzymatic system of the selected marine-derived fungi (Table 2).

On the 30th d of reaction, *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 promoted a satisfactory CP degradation, i.e., 63% and 72% respectively. In comparison to studies of organophosphate pesticides biodegradation developed by our group [22,24], in which marine-derived fungi were successfully employed for the degradation of methyl parathion and profenofos, CP showed to be the most recalcitrant pesticide to fungi metabolization.

The resistance to the biodegradation of CP may be due to its low water solubility and/ or toxicity of TCP against fungi strains. To increase the CP solubility in water, which is 0.39 mg L^{-1} in the pure form of the active ingredient, Lorsban is commercialized as an emulsifiable concentrate with a higher water solubility (1.4 mg L^{-1}) [13,30]. The limitation of CP solubility in water may have restricted the supply of pesticide carbon sources, which has hampered the biodegradation process. Moreover, TCP, has anti-microbial activity and may have caused the death of the fungi cells along its formation and affected the CP degradation during the reaction [15].

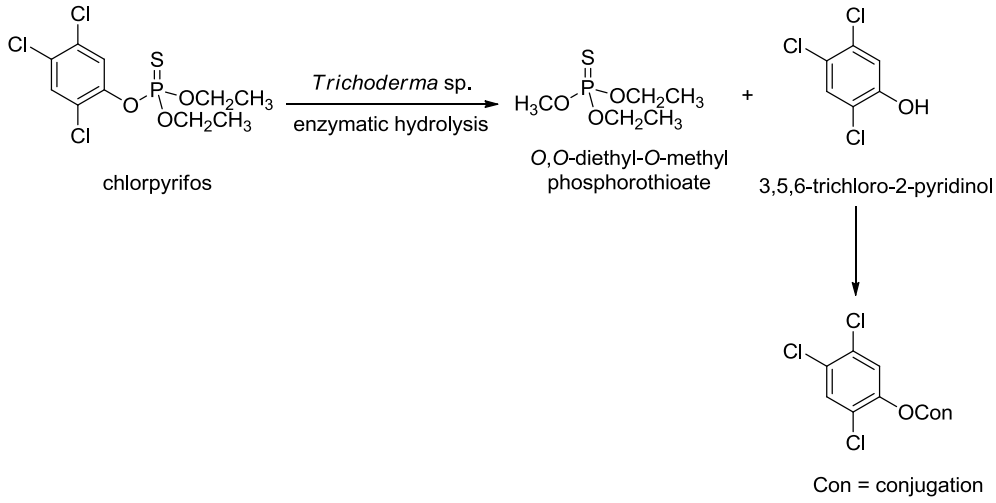
The comparison between the mycelial mass obtained in 30 d for fungus *A. sydowii* CBMAI 935 grown in the absence of CP (fungus control, 0.34 g) (Table 2) and the biodegradation reaction showed the mycelial mass slightly increased when the fungus was in the presence of CP. However, the comparison between the mycelial mass obtained in 30 d for fungus *Trichoderma* sp. CBMAI 932 grown in the absence of CP (fungus control, 0.43 g) (Table 2) and the biodegradation reaction showed a slight reduction in the mycelial mass. The dry mass reduction indicates either an inhibition in the fungus growth or the cell death caused by the pesticide and/or TCP.

A small formation of TCP was observed only on the 30th d of reaction in the presence of *Trichoderma* sp. CBMAI 932 (2.9 mg L^{-1}). For *A. sydowii* CBMAI 935, there was a small accumulation of TCP in the reaction course, from 3.0 mg L^{-1} on the 10th d to 9.1 mg L^{-1} on the 30th d.

Once the main presumable CP hydrolysis product is TCP, the formation of this substrate in a proportional amount of the CP hydrolyzed was expected. However, both fungi showed a small amount of TCP, which might be an evidence of the metabolite degradation. In order to investigate if *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932 were also capable of degrading the TCP, quantification reactions were performed in a liquid medium in the presence of only TCP and the strains studied. The results showed both strains could reduce the TCP concentration (Table 3).

Trichoderma sp. CBMAI 932 showed good TCP degradation (39%). However, the comparison between the fungal dry mass obtained in the absence of pesticide (fungus control) (Table 2) and the mass obtained in the presence of TCP (Table 3) showed a decrease in the mycelial mass produced. Since this fungus promoted better TCP biodegradation, the microorganism may have metabolized this compound as a defense against its xenobiotic action, followed by cellular death and reduction in the fungal dry mass.

TCP showed a lower degradation in the presence of *A. sydowii*



Scheme 1. Proposed pathway of chlorpyrifos biodegradation by *Trichoderma* sp. CBMAI 932.

CBMAI 935 (25%) than in the presence of *Trichoderma* sp. CBMAI 932. No decrease in the fungal dry mass was observed when TCP were in the presence of *A. sydowii* CBMAI 935 (Tables 2 and 3). Since TCP was not completely degraded by *A. sydowii* CBMAI 932 and *Trichoderma* sp. CBMAI 932, it can be inferred that other reactions may have acted on the TCP metabolization. Similar results were obtained in the biodegradation reactions of methyl parathion by marine-derived fungi [22]. The metabolic biotransformation of xenobiotics can proceed with the formation of conjugated products, in which endogenous functional groups are introduced in the degradation products, usually resulting in more polar compounds [22,31].

The fungi enzymatic system may promote conjugation reactions after the hydrolysis of CP and formation of a more polar derivative of TCP, which cannot be identified by the analysis performed in our study. Moreover, the CP resistance to the complete biodegradation may be attributed to the toxic effects of TCP on fungi strains.

To complement the biodegradation studies, experiments were conducted in a liquid mineral medium supplemented with KNO_3 for the evaluation of a possible use of CP as a carbon source by the fungi (Table 4). As CP is the only source of nutrient for the fungi development in a mineral medium, a higher concentration of this pesticide (100 mg L^{-1}) was required.

The percentage of CP biodegradation by *A. sydowii* CBMAI 935 was satisfactory, since a higher concentration of the pesticide was employed in comparison to the previous experiments. In the presence of *Trichoderma* sp. CBMAI 932, the pesticide showed a quite small degradation. The production of fungal dry mass was not sufficient to be quantified (Table 4).

The reaction of CP in the absence of fungi (abiotic control) showed its spontaneous hydrolysis in a liquid medium of malt extract. Experiments of CP spontaneous hydrolysis were also conducted in sterilized distilled water in 10, 20 and 30 d. Data from averages of duplicate reactions are shown in Table 5.

Approximately 90% of CP had disappeared after 10 d of incubation in a liquid medium of malt extract (Scheme 1, Figure S4). The aggregation with sugars or other molecules present in the medium is the most likely hypothesis for the disappearance of CP in such a short period of time. The hypothesis of spontaneous hydrolysis seems unlikely since TCP was not detected in the abiotic control in the malt extract. Xu et al. [16] reported a CP half-life in soil from 10 to 120 d, depending on the soil characteristics [15]. In water, the volatilization of CP is the most likely route of loss for this pesticide, with half-lives of 3.5 and 20 d estimated for pond water [32].

No pesticide aggregation occured in the presence of *A. sydowii* CBMAI 935 and *Trichoderma* sp. CBMAI 932, since these microorganisms are capable of absorbing CP to the cellular interior and make it less available in the medium for aggregation with other molecules.

In sterilized distilled water, the degradation of CP reached 61% on the 30th d of reaction, in agreement with technical data regarding CP volatilization. On the 10th and 20th d, the CP degradation rate was much more prominent in the presence of the fungi (Table 2) than in the abiotic control in distilled water (Table 5), which indicates the efficiency of the biodegradation process. Although this difference in the degradation rate is not so pronounced on the 30th d, the fungi enzymatic system is still a promising source, because it also promotes TCP degradation/conjugation reactions.

The GC-MS analyzes in scan mode revealed peaks corresponding to *Trichoderma* sp. CBMAI 932 metabolites (Scheme 1, Figure S5). By overlaying the chromatograms of the CP biodegradation and the *Trichoderma* sp. CBMAI 932 fungus control (absence of pesticide), it is possible to observe a slight decrease in the fungal metabolites peaks in the chromatogram of CP degradation. The decrease in the fungal metabolite peaks may indicate the toxicity of the pesticide/metabolite to this microorganism, as confirmed by the decrease in the mycelial mass of *Trichoderma* sp. CBMAI 932. The chromatograms of *A. sydowii* CBMAI 935 in both presence and absence of CP showed the same peak patterns.

The GC-MS analyzes of the biodegradation reaction of CP by *Trichoderma* sp. CBMAI 932 in a liquid mineral medium identified a low intensity peak of *O*,*O*-diethyl-*O*-methyl phosphorothioate (similarity of 93% with Wiley 09 Mass Spectral Data) (Scheme 1, Figures S6 and S7), the methylated form of the CP phosphorothioate moiety, *O*,*O*-diethyl-*O*-hydrogen phosphorothioate. The presence of *O*,*O*-diethyl-*O*-methyl phosphorothioate in the medium indicates the fungus has not promoted the bioactivation of CP to its oxon form. In biodegradation reactions of methyl parathion by marine-derived fungi, the strains promoted the bioactivation of methyl parathion to methyl paraoxon followed by further hydrolysis [22].

Based on the metabolites identified in CG-MS and HPLC-UV chromatograms in the reaction between CP and *Trichoderma* sp. CBMAI 932, it was suggested a possible pathway for the CP biodegradation involving its hydrolysis, yielding *O*,*O*-diethyl-*O*-methyl phosphorothioate and TCP, which is further subjected to conjugation reactions (glycosylation, alkylation, etc.) (Scheme 1).

Conclusions

A. sydowii CBMAI 935 and *Trichoderma* sp. CBMAI 932 were selected as best CP degrading strains in a screening with seven marine-derived fungi in a solid medium. Biodegradation assays in a liquid medium showed both strains were capable of degrading CP in a high percentage and also reducing the concentration of TCP. In a mineral liquid medium, *Trichoderma* sp. CBMAI 932 used CP as a sole source of carbon and, based on the CG-MS analysis of the reaction, a pathway has been suggested for the CP biodegradation by this strain. Studies regarding the conjugation reactions of the phenolic metabolite are in progress in our research group.

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