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ORIGINAL PAPER

Isolation of Brazilian marine fungi capable of growing on DDD pesticide

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Abstract The fungi *Aspergillus sydowii* Ce15, *Aspergillus sydowii* Ce19, *Aspergillus sydowii* Gc12, *Bionectria* sp. Ce5, *Penicillium miczynskii* Gc5, *Penicillium raistrickii* Ce16 and *Trichoderma* sp. Gc1, isolated from marine sponges *Geodia corticostylifera* and *Chelonaplysylla erecta*, were evaluated for their ability to grow in the presence of DDD pesticide. Increasing concentrations of DDD pesticide, i.e., 5.0 mg (1.56×10^{-12} mmol), 10.0 mg (3.12×10^{-2} mmol) and 15.0 mg (4.68×10^{-2} mmol) in solid and liquid culture media were tested. The fungi *Trichoderma* sp. Gc1 and *Penicillium miczynskii* Gc5 were able to grow in the presence of up to 15.0 mg of DDD, suggesting their potential for biodegradation. A 100% degradation of DDD was attained in liquid culture medium when *Trichoderma* sp. Gc1 was previously cultivated for 5 days and supplemented with 5.0 mg of DDD in the presence of hydrogen peroxide. However, the quantitative analysis showed that DDD was accumulated on mycelium and biodegradation level reached a maximum value of 58% after 14 days.

Keywords DDD · Marine fungi · Biodegradation · *Trichoderma* sp. · *Penicillium miczynskii*

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Introduction

1,1-Dichloro-2,2-bis-(4-chlorophenyl) ethane (DDD) is a chlorinated aromatic hydrocarbon insecticide highly persistent in the environment and its use was banned in most countries during the 1970s (Foght et al. 2001). However, its presence is still evidenced in soil, water and food, not only through its direct use but also as a product of DDT breakdown under reductive conditions (Aislabie et al. 1997). In Brazil, DDT is still used in the Amazon area to control the malaria mosquito (D'Amato et al. 2002). Due to their recalcitrant nature, the enhancement of the degradation process of organochloride pesticides by microorganisms has gained increasing attention in recent decades and a range of bacteria and fungi have been demonstrated to contribute to the degradation process (Bumpus and Aust 1987; Kamanavalli and Ninnekar 2004; Huang et al. 2007; Purnomo et al. 2008). Additionally, bioremediation using bacteria and fungi

has been shown to be a cost-effective method of treating various pesticides including DDD (Garrison et al. 2000). Microorganisms from many different genera are capable of degrading DDT, promoting the loss of HCl via reductive dechlorination and producing DDD as a metabolite, and DDD can undergo reductive dehalogenation (Subba-Rao and Alexander 1985) or hydroxylation of aliphatic moieties (Bumpus and Aust 1987). Hay and Focht (2000) have demonstrated that DDD can be also transformed via dioxygenation and ring fission. Biodegradation using fungi has received relatively little attention compared to the process using bacteria, however, the proposed mechanism of degradation was similar to that in bacteria. The ligninolytic fungus *Phanerochaete chrysosporium* was able to perform DDE mineralization and the lignin-degrading enzymatic system was suggested to be involved in degradation (Bumpus et al. 1993; Reddy et al. 1998). In contrast, Purmono et al. (2008) reported the ability of brown-rot fungi which did not produce ligninolytic enzymes to degrade DDT. The authors suggested that the fungi degraded DDT via Fenton reaction. The dehalogenation of lindane by the *Penicillium camemberti* was reported by Taseli (2006). Huang et al. (2007) described the uptake and degradation of DDT by ectomycorrhizal fungi.

The discharge of waste through deep water outfalls can contribute to contaminating marine environments with toxic contaminants (Eganhouse et al. 2000). The potential of marine microorganisms to degrade organochloride pesticides has not been explored yet although these organisms are adapted to survive in such complex and diverse conditions of pressure, salinity and temperature developing unique metabolite and physiological capabilities which can be explored for many applications. The objective of the present study was to select marine fungi capable of degrading DDD under "in vitro" conditions.

Materials and methods

General

DDD in isomer mixture, 2,2′-bis(*o*-chlorophenyl)-1,1-dichloroethane and 4,4′-bis(*p*-chlorophenyl)-1,1-dichoroethane, was purchased from Sigma–Aldrich. All manipulations involving the marine fungi were carried out under sterile conditions in a laminar flow cabinet (Veco). Marine sponges *Geodia corticostylifera* and *Chelonaplysylla erecta* were collected by R.G.S. Berlinck off the coast of São Sebastião, in the north of São Paulo state, Brazil (Rocha et al. 2009). All the culture media were sterilized in autoclave (Phoenix) heated at 121°C for 15 min.

Isolation of marine fungi

The isolation of fungi strains was performed as previously described (Rocha et al. 2009). The marine fungi *Aspergillus sydowii* Gc12, *Penicillium miczynskii* Gc5 and *Trichoderma* sp. Gc1 were obtained from the sponge *G. corticostylifera*.

Aspergillus sydowii Ce15, *Aspergillus sydowii* Ce19, *Bionectria* sp. Ce5 (*Bionectria* cf. *orchroleuca*) and *Penicillium raistrickii* Ce16 were isolated from the sponge *C. erecta.* These fungi were isolated and purified at the Department of Ecology and Evolutionary Biology, Federal University of São Carlos (UFSCar-Brazil) and identified at the Chemical, Biological and Agricultural Research Center (CPQBA/UNICAMP, Brazil), using both conventional and molecular methods (Rocha et al. 2009).

Composition of culture media and artificial sea water

Solid culture: Stock cultures of marine fungi were stored in solid culture medium (prepared with artificial sea water): agar (15.0 g l^{-1}), malt extract (30.0 g l^{-1}), soy peptone (3.0 g l^{-1}), and medium pH was adjusted to 8 with 3 M KOH solution.

Liquid culture: (prepared with artificial sea water): malt extract (30.0 g l^{-1}), soy peptone (3.0 g l^{-1}), and medium pH was adjusted to 8 with 3 M KOH solution.

Artificial sea water (1l): $CaCl_2{\cdot}2H_2O$ (1.36 g), $MgCl_2{\cdot}6H_2O$ (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), $SrCl_2{\cdot}6H_2O$ (0.040 g), H_3BO_3 (0.030 g).

Growth of marine fungi in solid medium

Qualitative screenings were carried out in Petri plates (10 cm × 1 cm) containing solid culture medium supplemented with 5.0, 10.0 and 15.0 mg of DDD per

plate. The pesticide was dissolved in 100 µl of DMSO for each 5.0 mg of DDD. One small slice of agar (0.5 cm × 0.5 cm) containing the mycelium of fungus was inoculated in the center of an agar plate. The fungi were grown at 32°C and the tolerance of pesticide was estimated by the growth diameter after 5 days of incubation. All the assays were carried out in duplicate and assays without the addition of pesticide were used as control. The results are summarized in Table 1.

Growth of marine fungi in liquid medium

A small slice of solid medium (0.5 × 0.5 cm) bearing mycelia of the marine fungi was cut from the stock culture and inoculated into 250 ml Erlenmeyer flasks containing 100 ml of liquid medium. In addition, the liquid culture was supplemented with 5.0, 10.0 and 15.0 mg of DDD per flask. The fungi were incubated at 32°C for 5 days on a rotary shaker (Technal model TE-421) at 150 rpm. After growth of strains in liquid medium, the mycelia were filtered using a Buchner apparatus and cell dry weight was determined after drying at 70°C for 24 h. The experiments were carried out in duplicate. The results are summarized in Table 2.

Table 1 Growth of marine fungi in solid medium with and without addition of pesticide (32°C, 5 days)

Marine fungi	Halo diameter (cm)	
	Plate without addition of pesticide	Plate with addition of pesticide
Addition of 5.0 mg of DDD (100 µl of DMSO)		
Aspergillus sydowii Ce15	>9.0	4.5
Aspergillus sydowii Ce19	4.5	3.5
Bionectria sp. Ce5	5.0	3.0
Penicillium raistrickii Ce16	5.5	4.5
Aspergillus sydowii Gc12	>9.0	>9.0
Penicillium miczynskii Gc5	3.5	3.0
Trichoderma sp. Gc1	>9.0	>9.0
Addition of 10.0 mg of DDD (200 µl of DMSO)		
Aspergillus sydowii Gc12	>9.0	3.0
Penicillium miczynskii Gc5	3.5	3.0
Trichoderma sp. Gc1	>9.0	>9.0
Addition of 15.0 mg of DDD (300 µl of DMSO)		
Trichoderma sp. Gc1	>9.0	7.0
Penicillium miczynskii Gc5	3.0	1.5

Biodegradation of DDD in liquid medium by *Trichoderma* sp. Gc1

Different studies were performed in liquid medium to determine the best conditions for DDD degradation by *Trichoderma* sp. Gc1:

(a) The microorganism was grown in liquid medium for 5 days and after this time, 5.0 and 10.0 mg of DDD dissolved in 100 and 200 µl of dimethyl sulphoxide, respectively, were added and incubation was continued for 14 days at 32°C, 150 rpm.
(b) To the liquid medium, 5.0 and 10.0 mg of DDD were added, dissolved in 100 and 200 µl of dimethyl sulphoxide, respectively, inoculated with microorganism and incubated for 14 days at 32°C, 150 rpm.
(c) The experiment was conducted as described in (a) with the addition of 100 µl of H_2O_2 (30 V).

Table 2 Growth of marine fungi in liquid medium with and without addition of pesticide (32°C, 150 rpm, 5 days)

Marine fungi	Cell dry weight (g) with addition of pesticide	Cell dry weight (g) without addition of pesticide
Addition of 5.0 mg of DDD (100 µl of DMSO)		
Aspergillus sydowii Ce15	0.95	1.66
Aspergillus sydowii Ce19	0.44	1.66
Bionectria sp. Ce5	0.58	0.69
Penicillium raistrickii Ce16	0.31	0.43
Aspergillus sydowii Gc12	0.79	0.82
Penicillium miczynskii Gc5	0.64	0.65
Trichoderma sp. Gc1	0.54	0.74
Addition of 10.0 mg of DDD (100 µl of DMSO)		
Aspergillus sydowii Ce15	0.72	1.9
Aspergillus sydowii Gc12	1.0	1.0
Penicillium miczynskii Gc5	1.1	2.2
Trichoderma sp. Gc1	0.64	0.75
Addition of 15.0 mg of DDD (100 µl of DMSO)		
Aspergillus sydowii Ce15	0.64	0.76
Aspergillus sydowii Gc12	0.93	1.0
Penicillium miczynskii Gc5	1.0	1.1
Trichoderma sp. Gc1	0.69	0.69

(d) The experiment was conducted as described in (b) with the addition of 100 µl of H_2O_2 (30 V).

After incubation the liquid medium was filtered for cell removal and extracted with ethyl acetate (3 × 20 ml). To the organic phase Na_2SO_4 was added before filtering and the solvent was evaporated under vacuum. The residue was analyzed by GC-FID and GC-MS in comparison with standard solutions. The results of the biodegradation are summarized in Table 3.

Biodegradation of DDD on *Trichoderma* sp. Gc1 mycelium

The fungus *Trichoderma* sp. Gc1 was grown in 250 ml Erlenmeyer flasks containing 150 ml of liquid medium for 5 days with the addition of 100 µl of H_2O_2 (30 V), and after this time, 5.0 mg of DDD dissolved in 100 µl of dimethyl sulphoxide were added and biodegradation was evaluated during 2–14 days at 32°C, 150 rpm. The results are summarized in Table 4.

After each time interval, the cells were separated using a Buchner filter and the resulting mycelium mass was stirred for 30 min with 50 ml of distilled water, then, 50 ml of ethyl acetate were incorporated and the mixture was stirred for 30 min. The cells were harvested by filtration and the filtered liquid was extracted with ethyl acetate (3 × 20 ml). To the organic phase Na_2SO_4 was added before filtering and the solvent was evaporated under vacuum. The residues were analyzed by GC-FID and GC–MS. The analytical methods were used for identify the DDD residual. For quantitative measurement of biodegradation, standard solutions with the following concentrations of DDD (l) were prepared: 0.010; 0.015; 0.020; 0.030; 0.040; 0.050 and 0.060 g in ethyl acetate and submitted to GC analysis.

Table 3 Quantitative degradation conditions of DDD by *Trichoderma* sp. Gc1

DDD (mg)	H_2O_2 (µl)	Initial DDD (g/l)	Final DDD (g/l)	% of DDD residual	% of DDD degraded
With simultaneous addition of pesticide DDD					
5	–	0.033	0.026	79	21
10	–	0.066	0.062	94	6
5	100	0.033	0.005	15	75
10	100	0.066	0.033	50	50
With addition of pesticide DDD after 5 days growth					
5	–	0.033	0.019	58	42
10	–	0.066	0.040	61	39
5	100	0.033	0.000	0	100
10	100	0.066	0.006	9	91

Experimental conditions are describes in material and methods

Table 4 Quantitative analysis of DDD degradation by *Trichoderma* sp. Gc1 mycelium

DDD (mg)/H_2O_2 (µl)	Times (days)	Initial DDD (g/l)	Final DDD (g/l)	% of DDD residual	% of DDD degraded
5/100	2	0.033	0.033	99	1
5/100	4	0.033	0.033	100	0
5/100	6	0.033	0.031	94	6
5/100	8	0.033	0.019	57	43
5/100	10	0.033	0.025	76	24
5/100	12	0.033	0.019	57	43
5/100	14	0.033	0.014	42	58

Experimental conditions are described in "Materials and methods"

Analytical methods

All the reactions were analyzed by gas chromatography to evaluate the percentage of pesticide degraded.

Gas chromatography (*GC-FID*): the analyses were performed in a Shimadzu GC2010plus gas chromatograph equipped with a FID detector, using a DB5 fused silica column (J&W Scientific 30 m × 0.25 mm × 0.25). The oven temperature was programmed at 200°C for 2 min, followed by a linear increase of 3°C min^{-1} to 250°C, kept at 250°C for 5 min. The injector and detector temperatures were 200°C; injector split ratio was 1:20 and nitrogen was used as the carrier gas at a pressure of 60 kPa. Retention time for *o,o*-DDD (10.15 min) and *p,p*-DDD (11.51 min).

Gas chromatography—mass spectrometry (*GC–MS*): a Shimadzu GC2010plus gas chromatography system coupled to a mass selective detector (Shimadzu MS2010plus) in electron ionization (EI) mode was used. The GC oven was fitted with a DB5 fused silica column (J&W Scientific 30 m × 0.25 mm × 0.25). The oven temperature was programmed from 50 to 250°C at an increasing rate of 5°C min^{-1}. The injector and detector temperatures were maintained at 200°C; injector split ratio was 1:20 and helium was used as the carrier gas at a pressure of 60 kPa.

Results and discussion

The isolates screened were *Aspergillus sydowii* Ce15, *Aspergillus sydowii* Ce19, *Aspergillus sydowii* Gc12, *Bionectria* sp. Ce5, *Penicillium miczynskii* Gc5, *Penicillium raistrickii* Ce16 and *Trichoderma* sp. Gc1 obtained from the marine invertebrates *Geodia corticostylifera* and *Chelonaplysylla erecta*. Initially we studied the selection of marine fungi capable of growth in the presence of DDD pesticide using increasing concentrations of pesticide. The results obtained are shown in Tables 1 and 2.

The isolates *A. sydowii* Ce15, *A. sydowii* Ce19, *Bionectria* sp. Ce5 and *P. raistrickii* Ce16 grew poorly in plates inoculated with 5.0 mg of pesticide when compared to control plates (Table 1). *A. sydowii* Ce15 presented a growth inhibition of 50% when the medium was enriched with DDD.

The best result in solid media was obtained with *A. sydowii* Gc12 and *Trichoderma* sp. Gc1. These microorganisms presented excellent growth in plates with and without addition of DDD. Both the microorganisms were resistant in 5.0 mg (1.56×10^{-2} mmol) of pesticide, suggesting they have the potential to support growth at higher concentrations of DDD. The growth of these fungi occupied up to 90% of the culture area, with and without the addition of pesticide. *P. miczynskii* Gc5 presented the smallest halo diameter in solid medium (3.5 cm), however the presence of 5.0 mg of DDD reduced the halo of growth by 14% (3.0 cm) showing a good tolerance to DDD presence. As the fungi *A. sydowii* Gc12, *Trichoderma* sp. Gc1 and *P. miczynskii* Gc5 presented good growth and low inhibition with 5.0 mg of DDD, these microorganisms were selected for new qualitative assays in solid medium containing 10.0 mg of DDD. As observed in Table 1, the fungus *Trichoderma* sp. Gc1 presented an excellent development in the presence of 10.0 mg of pesticide, showing the same halo diameter as the control plates. As observed in the experiment with 5.0 mg of DDD, the growth of *P. miczynskii* Gc5 was poorly inhibited with 10.0 mg of pesticide. However, *A. sydowii* Gc12 presented a strong inhibition of growth when the concentration of DDD was increased from 5.0 mg to 10.0 mg.

The selected fungi *Trichoderma* sp. Gc1 and *P. miczynskii* Gc5 were grown in the presence of 15.0 mg of DDD, and a decrease in the growth of the fungi was observed at these concentrations (Table 1). Although an inhibition was observed, *Trichoderma* sp. Gc1 was selected as a potentially DDD degrading microorganism for further studies. The growth of the fungi *A. sydowii* Ce15, *A. sydowii* Ce19, *A. sydowii* Gc12, *Bionectria* sp. Ce5, *P. miczynskii* Gc5, *P. raistrickii* Ce16 and *Trichoderma* sp. Gc1 in liquid medium supplemented with 5.0 mg (1.56×10^{-12} mmol), 10.0 mg (3.12×10^{-2} mmol) and 15.0 mg (4.68×10^{-2} mmol) of DDD are summarized in Table 2.

The fungi *A. sydowii* Gc12, *P. miczynskii* Gc5 and *Trichoderma* sp. Gc1, that showed good growth in the presence of pesticide in solid medium, presented similar behavior in liquid medium. The fungi *A. sydowii* Ce15, *A. sydowii* Ce19, *Bionectria* sp. Ce5 and *P. raistrickii* Ce16 presented a significant increase in inhibition in liquid medium with the addition of 5.0 mg of pesticide.

Similar behaviors were observed when marine fungi where were supplemented with 10.0 and 15.0 mg of pesticide in liquid medium (Table 2). In

conclusion, the fungi *A. sydowii* Ce15, *A. sydowii* Gc12, *P. miczynskii* Gc5 and *Trichoderma* sp. Gc1 showed potential to catalyze the biodegradation of DDD pesticide.

For further experiments we selected *Trichoderma* sp. Gc1 as the standard microorganism, given that it showed good resistance to DDD in both solid and liquid medium.

To verify the potential of this isolate to perform DDD biodegradation "in vitro" we varied the assay conditions (Table 3). Hydrogen peroxide was utilized, once it promotes the activity of peroxidases (Agha et al. 2009; Osborne et al. 2006).

In the experiments where 5.0 mg of DDD pesticide was added concomitantly with the fungus, a degradation of 21% of the pesticide was obtained. In the experiment with 10.0 mg of DDD, only 6% of the DDD was degraded (Table 3). The addition of H_2O_2 to the reaction promoted an excellent degradation of the pesticide, i.e., 75% (with 5.0 mg of DDD) and 50% degradation with 10.0 mg of DDD.

In the experiments where DDD was added after 5 days of *Trichoderma* sp. Gc1 growth, we observed the best degradation levels. In accordance with the data in Table 3, in the assay with 5.0 mg, the pesticide was degraded about 42%. While in the assay with 10.0 mg pesticide degradation reached 39%. The addition of hydrogen peroxide promoted the total degradation of the 5.0 mg of DDD and 91% degradation of the 10.0 mg of DDD. In addition, GC-MS analyses were undertaken to detect the metabolites and percentage of DDD degraded (Fig. 1). These analyses did not identify the products of biodegradation on chromatograms (Fig. 2).

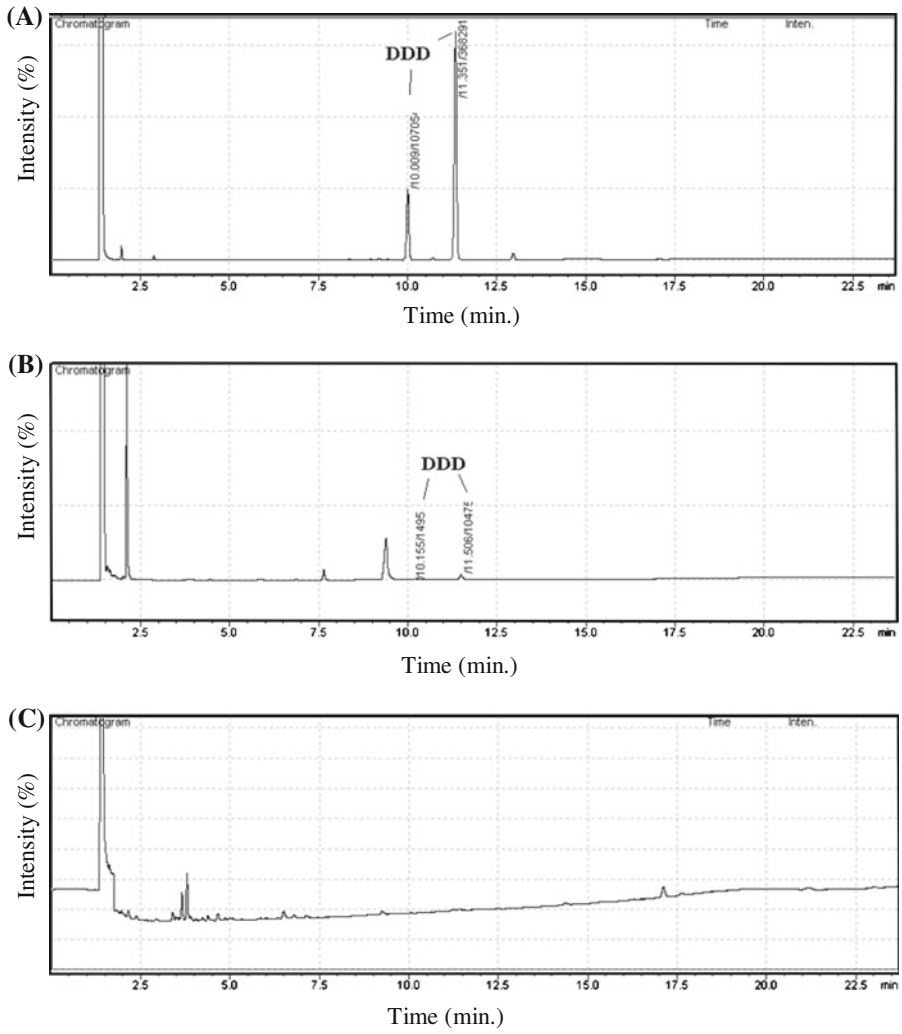

Fig. 1 GC-FID Chromatograms: **a** standard pesticide (mixture of *ortho*- and *para*-DDD); **b** biodegradation of DDD on *Trichoderma* sp. Gc1 in culture medium with 5.0 mg of DDD; **c** biodegradation of DDD on *Trichoderma* sp. Gc1 in culture medium with 5.0 mg of pesticide after 5 days growth and with addition of 100 µl of hydrogen peroxide

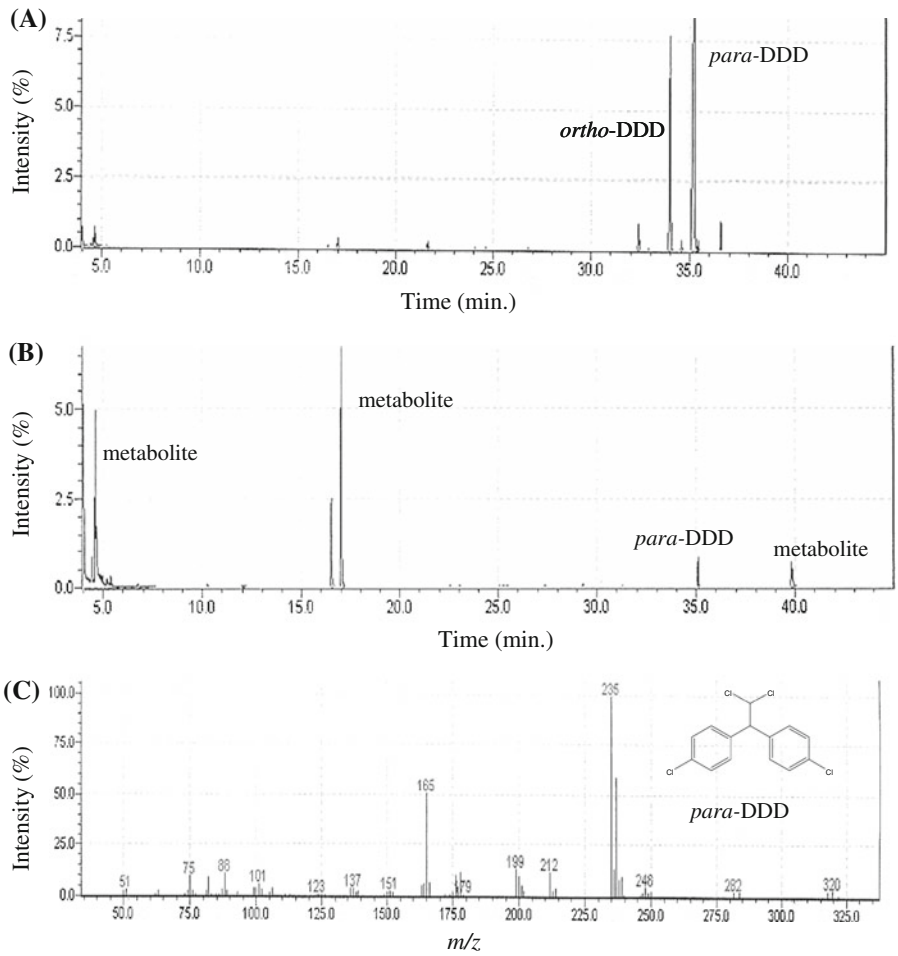

Fig. 2 GC-MS analyses: **a** chromatogram of standard pesticide (mixture of *ortho*- and *para*-DDD); **b** chromatogram of biodegradation of DDD on *Trichoderma* sp. Gc1 in culture medium with 5.0 mg of pesticide after 5 days growth and with addition of 100 µl of hydrogen peroxide; **c** mass spectrum standard of *para*-DDD. Metabolites produced by *Trichoderma* sp. Gc1

No intermediary products of DDD degradation were detected under experimental conditions using GC-MS analysis. This fact is important for bioremediation purposes since complete pesticide degradation or mineralization is desirable and avoids the presence of more toxic or recalcitrant biotransformation-derived compounds. Similar results were obtained by Benimeli et al. (2003) which reported that the aquatic Streptomycete strain M7 was able to remove aldrin from medium, however, no intermediary compound originating from pesticide degradation was found in the culture broth. Cellular lysis did not increase pesticide concentration in the medium, so the authors suggested that aldrin was removed by metabolic reactions or by bioaccumulation into cell insoluble components. In addition, the compounds can be biotransformed in polar organic compounds soluble in water.

To verify if *Trichoderma* sp. Gc1 accumulated DDD on the mycelium without degrading it, other experiments were conducted and the results are summarized in Table 4. As previously observed, the DDD analysis of culture medium suggest it was 100% biodegraded (Table 3), however, the analysis of biomass extracts showed that DDD was accumulated on mycelium. The pesticide accumulated presented an increase of the biodegradation after 8 days, reaching a maximum value of 58% after 14 days (Table 4).

These experiments show that, although the fungus has retained part of the pesticide in its cells, it was capable of degrading it after 8 days of incubation. Huang et al. (2007) also observed that 40–50% of the DDT supplemented in culture medium was not degraded but accumulated in mycelia of ectomycorrhizal fungi. These authors suggested that the slime layer around hyphae works as a buffer where the

toxic chemicals were sequestrated. The fungus *Trametes hirsutus* was also reported to accumulate intracellular lindane without degrading it, suggesting no intracellular enzyme was involved in pesticide degradation (Singh and Kuhad 1999).

The fact that DDD was better degraded after mycelium growth can be related to the accumulation of the pesticide on fungal cells, nevertheless, the assays revealed that part of DDD accumulated was degraded suggesting that secondary metabolism reactions are probably involved in biodegradation.

We did not detect intermediary compounds that would make it possible to propose a degradation pathway for DDD by *Trichoderma* sp. Gc1. However, the increase in degradation in the presence of H_2O_2 suggests the involvement of peroxidases (Agha et al. 2009). Osborne et al. (2006) reported that a chloroperoxidase from *Caldariomyces fumago* catalyzes H_2O_2-dependant dehalogenation reactions.

This is the first report on the biodegradation of DDD by the marine fungi *Trichoderma* sp. Gc1, further work needs to be carried out in order to investigate the pathway of DDD degradation by this microorganism.

Conclusion

In conclusion, the isolation of *A. sydowii* Ce15, *A. sydowii* Gc12, *Penicillium miczynskii* Gc5 and *Trichoderma* sp. Gc1 in the presence of DDD indicates a potential application for these fungi in the biodegradation of organochlorine pesticides. These marine fungi were able to grow in DDD concentrations ranging from 5.0 to 15.0 mg, even in solid or liquid medium. Degradation was improved when 5.0 mg of DDD was added to previously cultivated medium with *Trichoderma* sp. Gc1 and in the presence of hydrogen peroxide.

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