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SCREENING MITOSPORIC FUNGI FOR ORGANOCHLORIDES DEGRADATION

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ABSTRACT

Fifty-five isolates of filamentous fungi were studied regarding their ability to decolorize Remazol brilliant blue R dye. The fungi were isolated from soil in the Baixada Santista region, which is contaminated with industrial residues containing a mixture of organochlorine compounds, mainly hexachlorobenzene. The fungi were grown in liquid malt extract medium with 0.02% of dye and shaken at 200 rpm for 14 days at 28 ± 2ºC. Two types of behavior regarding the dye were observed: adsorption and degradation. *Eupenicillium baarnense* SSP1951 and SSP1952 and *Eupenicillium crustaceum* SSP1953 presented high RBBR decolorization and were then analyzed regarding their ability to degrade ^{14}C-hexachlobenzene (4138.31 mg HCB per kg soil) during a 56 days culture at 28 ± 2ºC. *Eupenicillium crustaceum* SSP1953 was able to reduce n-hexane soluble ^{14}C-compounds (24.6%) and to form non-extractable ^{14}C-residues (20.5%). The same behavior was also observed in the two *E. baarnense* strains (SSP1951 and SSP1952) but the percentages were lower than those obtained for *Eupenicillium crustaceum*. The main action of *Eupenicillium* spp on HCB is to transform it into non-extractable ^{14}C-residues as confirmed by the gas chromatography results.

Key words: RBBR decolorization, soil bioremediation, organochlorine degradation, xenobiotics

INTRODUCTION

Hexachlorobenzene (HCB) is a xenobiotic compound stable in the environment and insoluble in water. HCB has been widely used in agriculture and industry, but has been shown to be highly persistent in natural environments and possibly to be mutagenic and carcinogenic, thus posing a risk to human health (26). In Brazil, areas in the Baixada Santista region, São Paulo, were contaminated with industrial residues containing high concentrations of HCB among other organochlorine compounds.

Bioremediation studies employing Brazilian lignocellulolytic basidiomycetous fungi have shown the capacity of these fungi to reduce the concentration of HCB by transforming the molecule as demonstrated by the formation of chloride ions (17). Recently, optimization of the inoculum conditions has yielded up to 12% mineralization of HCB by *Psilocybe castanella* (18).

Enzymes of the lignocellulolytic complex of fungi have been related to the degradation of various xenobiotic pollutants when used in combination with mediators and reactive radicals (15,24). Studies have shown that some mitosporic fungi produce ligninolytic enzymes such as lignin peroxidase isoenzymes (H7, H8, H10), laccase, glyoxal oxidase and aryl alcohol dehydrogenase, as well as reactive oxygen, hydroxyl radicals, and Fe^{2+}-containing non-protein low molecular weight substances (10,13,19,22). The ability of this group of fungi to degrade organochlorine compounds has been demonstrated by the methylation of pentachlorophenol into pentachloroanisol by *Trichoderma virgatum*, *Aspergillus sydowi* and *Penicillium* sp. (7,8). *T. harzianum* has been shown to degrade various organochlorine pesticides, with an oxidase being involved in the metabolism of endosulfan (12).

Methods based on the decolorization of dyes have been used to select microorganisms able to degrade xenobiotics.

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Polymeric dyes and Remazol brilliant blue R (RBBR) have been found to be efficient in the selection of basidiomycetes, actinomycetes, ascomycetes and deuteromycetes with the ability to degrade polluting organic compounds (4,16,21).

The objective of the present study was to identify native mitosporic fungi in Baixada Santista's contaminated soil, able to biotransform HCB, to be used in subsequent evaluations. The mitosporic fungi will be tested with other basidiomycetes concomitantly (mixed culture), or after a pre-treatment with the basidiomycetes (sucessive culture) in future projects of soil bioremediation.

MATERIALS AND METHODS

Fungi

Fifty-five fungi were isolated from a soil contaminated with industrial residues containing organochlorine compounds, collected inside the area of a Rhodia chemical plant, located in the Baixada Santista, São Vicente, SP (23:58S and 46:23W). The three strains selected for the biodegradation tests were identified as *Eupenicillium baarnense* (Beyma) Stolk & Scott (SSP1951 and SSP1952) by the "Centraalbureau voor Schimmelcultures", The Netherlands, and *Eupenicillium crustaceum* (Ludwig, SSP1953). These three specimens were deposited in the culture collection of the Institute of Botany, São Paulo. The cultures were maintained in 2% malt extract agar.

RBBR decolorization

Five disks (5mm diameter) of a fungal culture in malt extract agar (7 days of incubation at 28ºC) were inoculated, in triplicate, into Erlenmeyer flasks containing 100 mL 2% malt extract broth. After 48 h incubation under shaking at 200 rpm, RBBR solution was added to a final concentration of 0.02% (v/v) and the flasks were incubated for an additional period of 14 days. Every day in the frist 6 days, and after that each 4 days, aliquots were removed, diluted 1/10 with distilled water, and absorbance read at 580 and 500 nm. The ability to decolorize RBBR was assessed based on the determination of the absorbance ratios (580/500), absorption spectrum of RBBR in the visible light region (500-1000 nm) (9,27) and visual analysis. Non-inoculated flasks were used as negative control. Flasks inoculated with the basidiomycetes *Trametes villosa* CCB176 served as positive control (16).

Biodegradation experiments

Soil: the soil, collected on the Rhodia plant, at the same site where the fungi were isolated, was characterized (17) as follows: 98% sand, pH 3.6, cation exchange capacity of 5.5 mEq 100 g^{-1} soil, 2.3% organic matter, 0.06% nitrogen, 1.0 $\mu g.g^{-1}$ phosphorus, and 0.01 mEq 100 mL^{-1} potassium. The organochlorine concentrations (per kg soil) were measured by gas chromatograph: 4138.3 mg HCB, 429.6 mg pentachlorobenzene (PeCB), 26.9 mg 1,2,4,5-tetrachlorobenzene (1245TCB), and 6.8 mg 1,2,3,4-tetrachlorobenzene (1234TCB).

Incubation Conditions: HCB with all carbon ring radiomarked uniformly (HCB-UL-^{14}C), with especific activity of 11.46GBq $mmol^{-1}$ and 97% of purity ("International Isotopes Münich", Alemanha), was utilized. The soil sample was air dried, sieved through a 2-mm mesh. Then, $CaSO_4$ (95:2.5 per dry weight) was added to increase the fungi growing conditions (5), the mixture was homogenized and sterilized with methyl bromide gas. This mixture was treated with 60 Bq g^{-1} of the ^{14}C-HCB and was placed in 250 mL flasks containing sterile sugar cane bagasse (2.5% dry weight). The sugar cane bagasse was included because it is usefull as nutrient supply when basidiomycetes are tested. The humidity was adjusted to 60% of the maximum water retention capacity.

Five grams of wet fungi micelium, obtained in liquid malt extract medium (2%) under shake for 48hs and homogenized in a blender for 20 seconds, were used as inoculum. A non-inoculated system served as abiotic control. The samples were incubated at 28 ± 2ºC for 56 days in a dark room. The experiment was carried out in triplicate.

Mass Balance Calculation: the initial amount of radiocarbon applied to the samples was determined by combustion of 500 mg soil in a biological oxidizer (OX-600 Harvey Instrument) and by liquid scintillation spectrometry using a Packard Tri-Carb 1600TR apparatus according to Andrea *et al*. (2). The mass balance of ^{14}C-HCB was calculated as the percentage of the radioactivity recovered determined by the mineralization of ^{14}C-HCB, where the $^{14}CO_2$ produced was captured in soda lime traps and extracted (1). The next steps were the n-hexane extraction to determine the production of volatile ^{14}C captured in polyurethane foam (20), and the soluble ^{14}C-compounds according to Andrea *et al*. (3), using 180 kW microwaves in 16 cycles of 60s each. The non-extractable ^{14}C-residues were measured by combustion of 0.5 g aliquots of extracted soil.

High-resolution gas chromatography: the extracts obtained from the radioactive experiments were utilized to measure the organochloride concentrations before and after fungi treatment. The extracts were dilluted in n-hexane (UV-residue analysis, Merck), filtered through a 13 mm Millex HV membrane (0.45 µm Millipore), and then 1 µL aliquots were injected into a Varian 3400 chromatograph under the following conditions: solid phase 50% phenyl and 50% methyl polysiloxane megabore column (0.5 µm x 0.53 mm x 30 m) and detection by electron capture. The operational temperatures were 220ºC in the injector and 300ºC in the detector. The temperature program of the column was 188ºC for 21.5 h, followed by increases of 10ºC/min up to 238ºC for an additional 18.5 h. The mobile phase was nitrogen (1.5 $mL.min.^{-1}$ flux).

The organochlorine compounds identification was done by the retention time of a standard mixture of: HCB, PeCB, 1245TCB, 1234TCB. The quantification was calculated by regression analysis and the standard curve of each compound.

Statistical analysis: The percentages of RBBR decolorization, reduction in extractable ^{14}C-compounds, formation of non-extractable ^{14}C-residues and reduction in HCB concentration were submitted to variance analysis (α=0.05) using the ANOVA application of the Minitab program. When a significant effect was observed, means were compared by the Tukey test to the level of significance set at P≤0.05, except if stated otherwise.

RESULTS AND DISCUSSION

RBBR decolorization

During the 55 fungi grow the RBBR dye decoloration was observed through the mycelium coloration (adsortion) or by the molecule degradation when the medium change the colour to light blue, green and yellow. Color changes during fungal growth in the presence of dyes have been previously reported by Cookson (6). The RBBR decoloration ranged from 19.8 to 90.5%. *T. villosa* CCB176, the positive control, promoted 89.6% of the RBBR decoloration, and the non-inoculated control (abiotic degradation) only 9.2%. Statistical analysis revealed a significant difference among the majority of fungi and non-inoculated control (P≤0.05), except for four fungi.

According to Glenn and Gold (9) both, the adsortion and the degradation, are able to change the RBBR coloration. When the ratio of 580 and 500nm of wave-length is constant with time, the dye adsortion by the fungus is observed, and the characteristic spectrum peak diminish; when the ratio decrease, the degradation happenes, and the characteristic spectrum peak disappear. Twenty-three isolates adsorbed the dye, SSP 20 being an example this group, showing constant absorbance ratios along time (Fig. 1A), and a decrease in the characteristic RBBR peak (Fig. 1B). The decolorization due to adsorption ranged from 19.8 to 56%, and differed significantly from *T. villosa* (P≤0.05). Twenty-two fungi promoted RBBR degradation in various degrees (decolorization - 58.7 to 90.5%), with no statistical differences compared to the positive control. Among theses, eight promoted the degradation of the dye at the same level as *T. Villosa*, (decoloration 78.2 to 90.5%). *E. crustaceum* SSP1953 is an example of this group, being able to degrade the dye in culture medium after 2 days of incubation, with 84.2% of decoloration (Fig. 2A), and to cause the disappearance of the characteristic RBBR peak (Fig. 2B). This same result was observed for *T. villosa* after 6 days of growth. The other fourteen fungi, such as *E. baarnenses* SSP1951 with 76.6% of decoloration, promoted parcial RBBR degradation, and a residual color was noted in the culture medium after 14 days of incubation. This behavior was

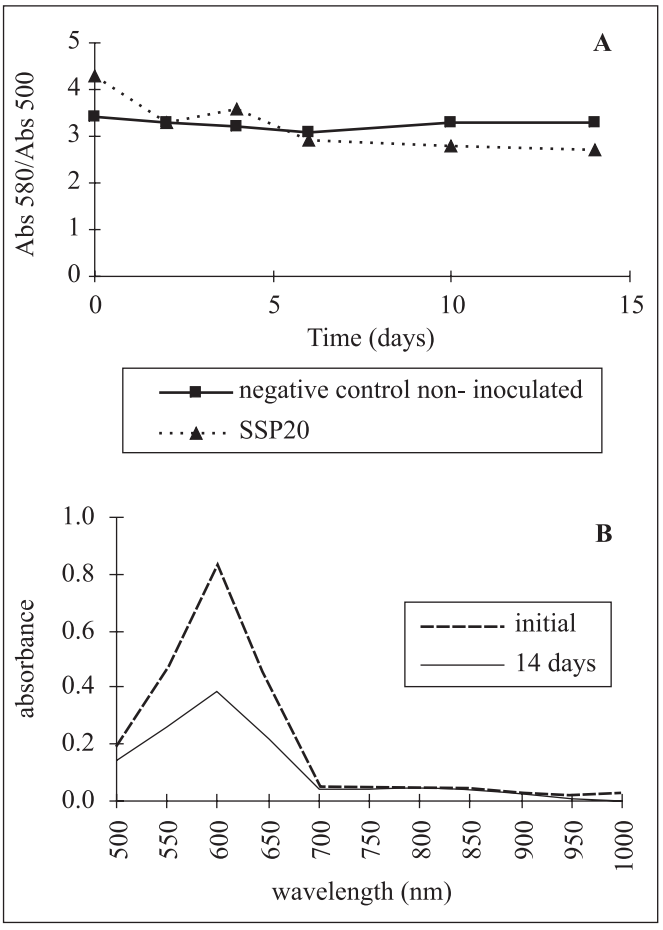

Figure 1. Absorption of Remazol brilliant blue R (RBBR) by fungus SSP20, in culture in malt extract after 14 days of incubation at 28 ± 2ºC under shaking. A) 580/500 absorbance ratio. B) Visible absorption spectrum of RBBR.

characterized by a decline in the absorbance ratios up to the sixth day of growth, with values remaining constant thereafter until the end of the incubation period (Fig. 3A), and by the absence of the characteristic RBBR peak (Fig. 3B). Various mitosporic fungi are able to decolorize RBBR and laccase activity has been demonstrated in some of them (2,4). Soares *et al*. (25) suggested that fungi producing laccase plus a corresponding natural redox mediator may be more easily detected using RBBR.

Ten fungi were excluded from the statistical analysis because they promoted intensified color of the culture medium after the fungal growth.

E. baarnense (SSP1951 and SSP1952) and *E. crustaceum* SSP1953 were selected for futher HCB biodegradation tests: they decolorized RBBR at the same percentages as *T. villosa*, are able to grow in soil containing 3770 mg HCB per kg soil and

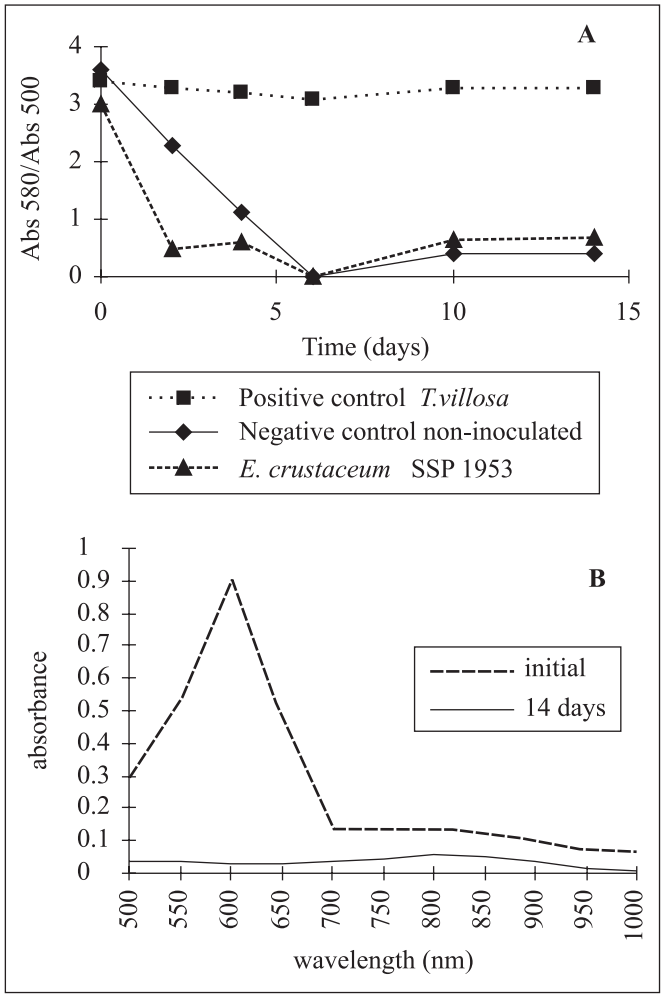

Figure 2. Degradation of Remazol brilliant blue R (RBBR) dye by *Eupenicillium crustaceum* SSP 1953, after 14 days of culture in malt extract at 28 ± 2ºC under shaking. A) 580/500 absorbance ratios. B) Absorption spectrum of RBBR.

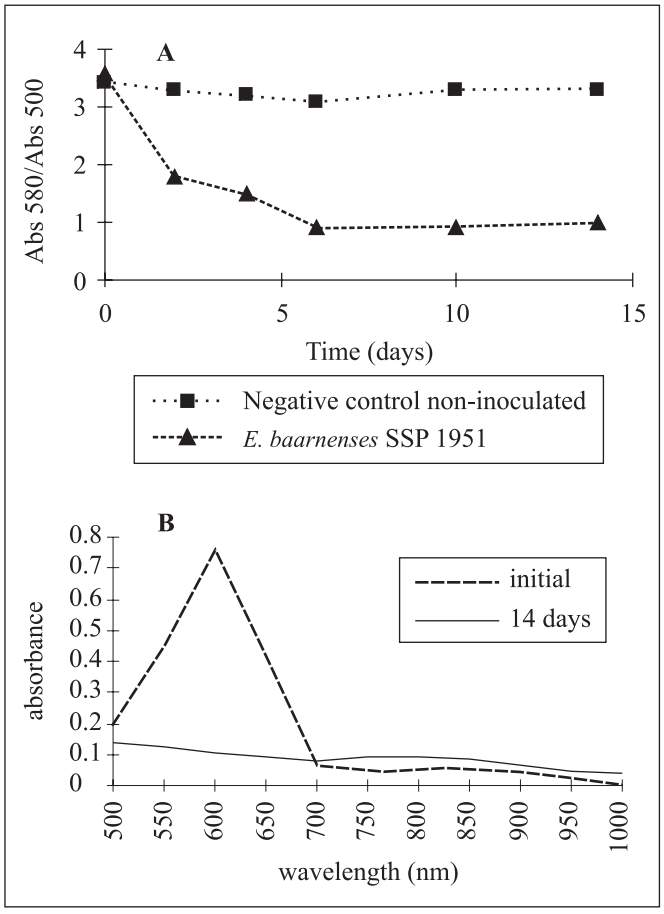

Figure 3. Parcial degradation of Remazol brilliant blue R (RBBR) by *Eupenicillium baarnense* SSP1951, after 14 days of culture in malt extract at 28 ± 2ºC under shaking. A) 580/500 absorbance ratios. B) Absorption spectrum of RBBR.

1719 mg PCP per kg soil (unpublished data), and are easily handled in the laboratory.

Biodegradation of ^{14}C-HCB

The mass balance of ^{14}C-HCB (Table 1) ranged from 97.1 to 104.5%, being these values in the acceptable limits for a biodegradability test (1). No significant mineralization of ^{14}C-HCB or formation of volatile ^{14}C-compounds were observed. Radioactivity was detected in n-hexane soluble compounds (65 to 89.6%) and in non-extractable residues (8.7 to 29.5%). According to Fig. 4, subtracting the abiotic control, *E. crustaceum* showed the highest reduction of soluble ^{14}C-compounds (24.6%), as well as the formation of 20.5% of non-extractable ^{14}C-residues. The formation of these residues can

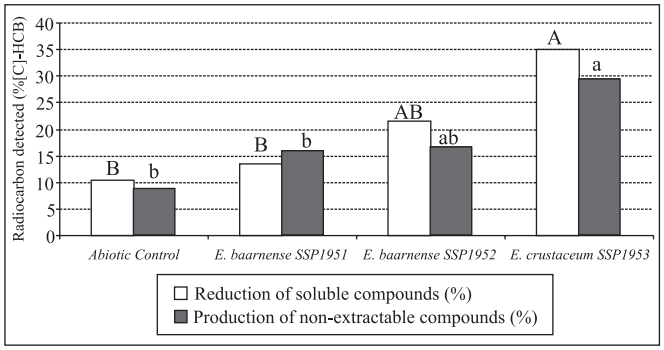

Figure 4. Reduction of detected extractable ^{14}C-compounds and production of non-extractable ^{14}C-residues (%) during the growth of fungi in soil contaminated with hexachlorobenzene for 56 days at 28 ± 2ºC. The same letters indicate statistical equality (Tukey Test, P≤0.05).

occur due to microbial action or to abiotic processes (11). The action of fungi on the HCB molecule may have caused alterations, resulting in the formation of reactive sites that bind to the soil's organic matter, originating non-extractable residues. The involvement of ligninolytic enzymes, such as laccase, in the formation of bound residues is well documented in the literature (11,14).

Reduction of organochlorine compounds

Treatments of soil with *Eupenicillium* spp resulted in a reduction on the HCB concentration (Table 2), however *E. crustaceus* SSP 1953 gave the best result: a significant reduction (35.6%) on the HCB initial concentration was observed ($P \leq 0.05$), which corresponded to a net reduction of 16%, subtracting the abiotic control ($P \leq 0.01$). This decrease in HCB concentration was close to the production of non-extractable ^{14}C-residues (20.5%, showed in Fig. 4).

In contrast, no differences in PeCB concentration were observed between fungal treatments and the abiotic control, which was responsable for a reduction of about 52% of the initial concentration, in all cases ($P < 0.000$). As to 1245TCB and 1234TCB, no significant differences between fungal treatment, the abiotic control and the initial concentrations of these compounds in soil were observed (Table 2), indicating that no the fungus tested, nor the abiotic factors, were able to promote the degradation of these two compounds.

CONCLUSION

E. crustaceum, selected based on its ability to decolorize RBBR, promoted the transformation of HCB, an environmentally stable compound, into non-extractable residues. The ability of this fungus to render the compound less bioavailable without the production of volatile compounds, using poor nutrients as substrates, makes this species interesting for soil bioremediation studies.

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Table 1. Mass balance of ^{14}C recover from the biodegradability test of ^{14}C-HCB treated soil and incubated with *Eupenicillium* spp, during 56 days at 28 ± 2ºC.

	mass balance of 14 C-HCB (%)				
	$^{14}CO2$	^{14}C volatile	soluble ^{14}C-compounds	non-extractable ^{14}C-residues	^{14}C-total
E. baarnense SP1951	0.2	1.8	86.5	15.9	104.5
E. baarnense SP1952	0.6	2.8	78.5	15.5	98.4
E. crustaceum SP1953	0.5	2.2	65.0	29.5	97.1
abiotic control	0.0	3.1	89.6	8.7	101.4

abiotic control= non-inoculated sterile system.

Table 2. Mean organochlorine concentrations in soil (mg/Kg dry weight soil) before and after incubation of *Eupenicillium* spp for 56 days, based on gas chromatography.

TREATMENT	HCB(mg/kg)	PeCB (mg/kg)	1245TCB (mg/kg)	1234TCB (mg/kg)
E. baarnense SSP1951	3015.68 ab	206.14 b	18.46 a	5.13 a
E. baarnense SSP1952	2735.24 ab	203.82 b	17.84 a	5.41 a
*E. crustaceum*SSP1953	2665.52 b	198.02 b	18.82 a	5.84 a
Abiotic control	3310.39 ab	237.19 b	23.01 a	6.19 a
Initial concentration	4138.31 a	429.6 a	26.89 a	6.84 a

The same letters indicate statistical equality (Tukey test, $P \leq 0.05$); HCB= hexachlorobenzene; PeCB = pentachlorobenzene; 1245TCB=1245-tetrachlorobenzene; 1234TCB = 1234-tetrachlorobenzene.

RESUMO

Seleção de fungos mitospóricos para degradação de organoclorados

Cinqüenta e cinco isolados de fungos filamentosos foram avaliados quanto a capacidade de descolorir o corante remazol brilliant blue R. Estes fungos foram isolados de solos da Região da Baixada Santista contaminados com resíduos industriais contendo uma mistura de organoclorados, principalmente hexaclorobenzeno. O crescimento dos fungos foi realizado em meio líquido de extrato de malte contendo 0,02% do corante, sob agitação de 200 rpm, durante 14 dias a 28ºC ± 2. Foi possível verificar, entre os fungos avaliados, dois comportamentos em relação ao corante: adsorção e degradação. *Eupenicillium baarnense* SSP1951 e SSP1952 e *Eupenicillium crustaceum* SSP1953 apresentaram altas porcentagens de descoloração do RBBR. Estes fungos foram, então, avaliados quanto a sua capacidade de degradar ^{14}C-hexaclorobenzeno (4138,31 mg de hexaclorobenzeno kg^{-1} de solo) durante 56 dias a 28ºC ± 2. *Eupenicillium crustaceum* SSP1953 foi capaz de reduzir em 24,6% os ^{14}C-compostos solúveis em n-hexano e formar 20,5% de ^{14}C-resíduos não extraíveis. As duas linhagens de *E. baarnense* (SSP1951 e SSP1952) também apresentaram o mesmo comportamento, porém com porcentagens inferiores à observada para *Eupenicillium crustaceum*. A principal ação de *Eupenicillium* spp sobre hexaclorobenzeno foi transformá-lo em ^{14}C-resíduos não extraíveis, como comprovaram os resultados da cromatografia gasosa.

Palavras-chave: descoloração de RBBR, biorremediação de solo, degradação de organoclorados, xenobióticos

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