Degradation of repellent DEET by *Pleurotus ostreatus* laccases

Degradação do repelente DEET pelas lacases do *Pleurotus ostreatus*

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REVISA

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RESUMO

Objetivo: Avaliar a capacidade de remediação do DEET em meio líquido, pelo fungo de decomposição branca *Pleurotus ostreatus* usando como indutor enzimático os resíduos sólidos do cacau e realizar bioensaios de toxicidade com as amostras pós-tratamento, para aplicações em tratamentos de águas. **Método:** Foi realizada a produção enzimática com resíduos do Cacau. A biorremediação com o caldo enzimático foi realizada em erlenmeyers de 250mL, contendo a solução do composto, tampão acetato de sódio pH 5 e o caldo batata, incubados à 28°C, com rotação de 120 rpm, por 48 horas. Já com o fungo ativo, o mesmo foi incubado a 28 ºC e teve em seu meio a adição do composto. As amostras foram quantificados em Cromatografia líquida de alta performance (CLAE). O teste de adsorção foi feito com o fungo autoclavado e analisado após 14 dias. **Resultado:** O composto se apresentou possivelmente tóxico e a remediação mostrou uma tendência linear de degradação com o fungo de 39%. **Conclusão:** *Pleurotus ostreatus* é um candidato promissor para o tratamento de contimanações geradas por DEET.
Descritores: Remediação; *Pleurotus ostreatus*; DEET; Cromatografia Líquida de Alta Pressão; Lacase.

ABSTRACT

Objective: We evaluated the remediation capacity of DEET in liquid medium by the white decomposition fungus *Pleurotus ostreatus* using the solid residues of cocoa as an enzymatic inducer and performed toxicity bioassays with the post-treatment samples, for water treatment applications. **Method:** Enzymatic production with cocoa residues was performed. Bioremediation with the enzyme broth was performed in a 250mL erlenmeyer flasks, containing the solution of the compound, sodium acetate buffer pH 5 and the potato broth, incubated at 28 °C, with rotation of 120 rpm, for 48 hours. With the active fungus, the same was incubated at 28 ºC and had in its culture medium the addition of the compound. The samples were quantified in high performance liquid chromatography (HPLC). The adsorption test was performed with the autoclaved fungus and analyzed after 14 days. **Results:** The compound was possibly toxic and the remediation showed a linear tendency of degradation of 39% with the fungus. **Conclusion:** *Pleurotus ostreatus* is a promising candidate for the treatment of contaminants generated by DEET.
Descriptors: Remediation; *Pleurotus ostreatus;* DEET; Chromatography, High Pressure Liquid; Laccase.

ORIGINAL

Introduction

Constant growth of the demand and offer of new chemical products increased the variety and amount of many chemical compost originating from industrial effluents, sewage and other anthropogenic activities in the various environmental compartments.[1-2]

Due to demographic and socioeconomic growth occurred in the last decades, the rates of pollution of water resources have increased, causing loss of water quality in relation to physical, chemical and bacteriological parameters.[2,18]

Contamination of surface and groundwater has been one of the major problems of modern society and a growing concern due to the risks that such compounds pose to biota and to humans, due to their deleterious effects.[10,14]

The steady progress of analytical chemistry in the last decades, and particularly the increasing sensitivity, has drastically improved the detection of traces of organic contaminants even within matrices as complex as wastewater.[3] From liquid chromatography and tandem mass spectrometry (UHPLC-MS /MS), many laboratories routinely and reliably quantify dozens of drugs and personal care products in various water samples at concentrations below 1 ng. L^{-1}.[15]

Among the commonly monitored organic contaminants in water, the diethyltoluamide repellent (DEET) became of particular interest when the first survey recognition of wastewater contaminants in US streams revealed its occurrence in 74% of the samples analyzed.[8] Since then, subsequent research has confirmed its occurrence in several water matrices in Europe, Asia, America and Africa.[17]

Such presence of DEET in environmental waters and their detection in drinking water has raised public health concerns and the efficacy of water treatment for attenuation.[4] and increasing interest in reuse of drinking water have further stimulated research into the removal of various wastewater contaminants, including DEET, by alternative, conventional and advanced treatment processes. Among these treatments may include biological, adsorption, membrane and oxidative technologies.[12]

The aim of this study is to evaluate the DEET remediation capacity of the laccase from the white Pleurotus ostreatus decomposition fungus using the vegetal biomass of cocoa from industrial residues as a potential enzymatic inducer and to carry out toxicity bioassays with the samples after their treatments.

Method

Experiments were developed during the year of 2018 in the Laboratory of Enzymology and Bioactive Materials (LENZIBIO) in the Pharmacy faculty of Federal University of Goiás (UFG). For the accomplishment of the experiments the fungus *Pleurotus ostreatus* was used. The strain is part of the collection of LENZIBIO and was provided by the Laboratory of Systematics and Microbial Physiology - Department of Food Sciences – FEA/UNICAMP. The N, N-diethyl-meta-toluamide (DEET) repellent was kindly donated by the pharmaceutical company Cifarma Científica Farmacêutica, whose batch is

USHA047984, net weight of 0.300 kg and gross weight of 0.317 kg. The stock solution of the repellent for use in the bioremediation was prepared using 10 μg of the repellent diluted in 1000 ml of distilled water, resulting in a final concentration of 10 μg.L-1 of DEET. After preparation, the stock solution was stored in a 1000 mL vial in the refrigerator at 4 ° C.

The solid culture medium used in the research was BGA (potato, glucose and agar) and the liquid was Handmade potato broth and Dextrose (CBD), with 1% cocoa as the enzymatic inducer. After preparation of the BGA solid culture medium, it was autoclaved and in laminar flow hood, about 20 ml of the medium was dispensed into Petri dishes (10 cm in diameter) until solidified. A 5 mm diameter disk of fungus extracted from a matrix plate was added to each plate and then incubated in a BOD Greenhouse (TE-391, Tecnal, São Paulo, Brazil) at 28 ° C for 7 days, sufficient time for the optimal fungal growth.[5] After this period, the fungi were stored in a refrigerator at 4 ° C to evaluate the growth.

Enzymatic production. The production of Lacases by the fungus Pleurotus ostreatus was carried out for a period of six days in which there is the maximum peak of enzymatic activity. The erlenmeyers were taken to the laminar flow hood and the fungus was transferred to the liquid culture medium, with 1% cacao, properly autoclaved. 250 ml erlenmeyers were used, each containing 60 mL of liquid medium and 5 5 mm disks of fungus. The erlenmeyers were placed in a chilled incubator (TE-421, Tecnal, São Paulo, Brazil), rotating at 120 rpm, for six days at 28 ° C and during this time period, laccase production was evaluated.

The methodology used to evaluate the production of Lacases by the fungus Pleurotus ostreatus uses seringaldazine as substrate of the enzyme.[21] The reaction mixture is composed of: 10μL of the enzyme broth (erlenmeyer supernatant), 890μL of 50 mmol.L-1 sodium acetate buffer (pH 5.0) and 100μL seringaldazine 1.0 mmol.L $^{-1}$ prepared in absolute ethanol. The enzymatic reaction is based on the oxidation of seringaldazine (ε = 65.000 L.mol^{-1}.cm^{-1}) by Lacases and was initiated by adding seringaldazine to the mixture. The oxidation rate was monitored for 5 minutes at 525 nm in a spectrophotometer (SP-2000 UV Meter, Spectrum, Shanghai, China). White was performed using 10μL of the enzyme broth and 990μL of sodium acetate buffer solution 50 mmol.L^{-1} (pH 5,0) without adding seringaldazine.

To determine the calculation of enzymatic activity, Equation:[11]

$$U = \frac{10^6 \ x\, Abs}{\varepsilon \, x \, R \, x \, t}$$

In which:
Abs = Absorbance at specific wavelength (nm);
ε = Molar extinction coefficient for each substrate (L.mol^{-1}.cm^{-1});
R = Gross amount of extract (mL);
t = Reaction time (min).
U= Amount of enzyme needed to oxidize 1 μmol.L^{-1} of substrate per minute.
The result is expressed in U.mL^{-1}.

Evaluation of enzymatic stability at different temperatures and optimum pH. For the determination of the optimal temperature of the crude extract of Lacases, the following methodology[5] was modified by the laboratory:

the enzymatic activity was evaluated at different temperatures ranging from room temperature (approximately 20 ° C) to 80 ° C, with an interval of 10 ° C analyzed every 10 min for 1 hour, and the seringaldazine was used as a substrate. In the determination of the best pH for activity of the crude lactose extract, 50 mM acetate buffer was used for pH 3.0 to 5.0 and phosphate buffer for pH 6.0 to 8.0.

Bioremediation of DEET with Enzyme Broth. After 7 days in the chilled incubator with shaking, the erlenmeyers were removed from the incubator and the enzyme broth was filtered and stored in a refrigerator for performing the bioremediation. Before initiating the enzymatic treatment, the enzymatic activity of laccase was calculated to determine the volume of enzyme broth that would be used in bioremediation.

The bioremediation of the repellent followed an already proposed methodology12 with some modifications. A total of 27 250 ml erlenmeyers were used, which included the test samples, negative control (inactivated enzyme), white and zero time, all in triplicate (Table 1).

Table 1 - Substances used in bioremediation of repellent

Components	Test	Control (inactivated enzyme)	White	Time Zero
Sodium Acetate Buffer Solution (50mmol.L^{-1} ;pH 5,0)	10mL	10mL	20mL	10mL
DEET Stock Solution (10µg.L-1)	10mL	10mL		10mL
Enzyme extract (200 and 400U)	*	Inactivated Enzyme **	*	*

* The amount of the enzyme extract was chosen based on the enzymatic activity of the sample to obtain 200 and 400 U

** The enzyme was inactivated by microwave heating to the start of boiling of the enzyme broth (30 seconds).

Bioremediation with fungus. Bioremediation with the active fungus was also performed. The fungi were grown in Petri dish, using standard BDA for seven days. Subsequently, 5 discs were transferred to the liquid culture medium and incubated at 28°C ± 2°C for 7 days and DEET was added on the concentration of 10 µL/L under laminar flow in all erlenmeyers and withdrew the aliquots on days 0, 3, 5, 7,10, 12 e 14. These samples were stored in a freezer and then taken for analysis on HPLC.

Adsorption test. The adsorption test is used to evaluate if fungi have the ability to reduce the concentration of DEET by the adsorption mechanism in the biomass.[17] The fungi were grown in Petri dish, using standard BDA for seven days. Subsequently, 5 discs were transferred to the liquid culture medium and incubated at 28 ° C ± 2° C for 7 days and thereafter heated for 6 minutes in a microwave to kill the fungus and eliminate activities of enzymes. Upon cooling to room temperature, DEET was added to the concentration of 10µg/L under laminar flow to maintain sterile conditions. The flasks were again incubated for 14 days and at the end of this period the samples were withdrawn for analysis of the final DEET concentration.

Repellent quantification and detection. The quantification of the repellent was carried out in partnership with the Nucleus of Toxic-Pharmacological Studies and Research (NEPET). High Performance Liquid Chromatography (LC-20A, Shimadzu, Prominence Series, Kyoto, Japan) with UV / Vis detector in Photodiode Array Detector (PDA) SPD-M20A. The methodology followed was that described with the following chromatographic conditions. [7]

Column: C18 (250mm x 4,6mm e 5µm);
Wave-length: 350nm for Oxytetracycline in PDA;
Mobile phase: Formic acid 0,2 % : Acetonitrile (35:65);
Flux: 1mL/min;
Wave-length: 240nm;
Injection volume: 50µL.

The calibration curve was constructed with the following concentrations of DEET: 0,05 $\mu g.mL^{-1}$, 0,10 $\mu g.mL^{-1}$, 0,25 $\mu g.mL^{-1}$, 0,5 $\mu g.mL^{-1}$, 1,0 $\mu g.mL^{-1}$, 2,5 $\mu g.mL^{-1}$

Toxicity test with *Allium cepa*. To develop the toxicity test with *Allium cepa* (onion), several onion bulbs that varied from 65 to 90 g were adquired commercially. The old roots were removed and the bulbs disinfected superficially with 70% ethyl alcohol.[19] They were then placed to germinate in distilled water in the dark at room temperature. Only the base of the bulb remained immersed in the water, which was renewed every 24h.

After two days the bulbs were then transferred to beakers of 100 mL, where they received the solution with the compound of interest. Dilutions were prepared with distilled water and performed in duplicate. In parallel, negative control tests with distilled water were carried out. After 24 hours of treatment (acute treatment), 3 roots per bulb were randomly selected and collected. The test system returned to the same previous conditions, where it remained until it completes 72 hours of treatment (sub-chronic treatment) and another 3 roots were collected. During the test, the test solution was renewed every 24 hours.

After the collection, the bulbs were placed again in distilled water and after 48 hours (recovery period), the root growth potential was evaluated. The roots were fixed in Carnoy I (ethyl alcohol-acetic acid, 3: 1) at 4° C for 24 hours.

Preparation of slides for microscopic analysis. Three roots were removed from the total roots fixed by bulb, and were sent for washing and recovery of normal hydration. Subsequently, the roots were subjected to acid hydrolysis (HCl1N at 60 °C in the oven) for approximately 10 minutes. After acidic hydrolysis, the roots were washed again for the first stage of the staining, where they were immersed in Schiff Reactive and kept in the dark for 2 hours. After staining, the roots were washed and separated the meristematic regions, which will be individually supported on the slide, covered with a drop of 1% acetic Orcein and crushed between blade and cover slip, for analysis under the microscope.

Sheet Analysis. The analysis of the slides was performed under a common optical microscope. The 3 roots per bulb were observed, both after the first 24 hours and after the 72 hours of treatment with the test solution.

At least 500 cells were analyzed by root, in each case, totaling 1500 cells. The microscopic parameters analyzed were the division and phase indexes for the evaluation of cytotoxic potentials and morphological changes (mitotic

aberrations), also as an indicator of genotoxic potential. They were defined as follows:
Division Index (DI - %) – number of cells in division/number of cells observed X 100;
Phase Index (PI - %) – number of cells in a certain mitotic phase/number of cells in mitosis X 100.

Results and discussion

Enzymatic production. The cocoa residue was used as plant material with the potential to induce the production of *Pleurotus ostreatus*. The parameter for choosing the cocoa residue was to increase the activity of this enzyme in the culture medium. There was the production of Lacases by *Pleurotus ostreatus* with the liquid culture medium and its production was higher on the 7th day of incubation.

Determination of the optimum temperature. Lacases are tolerant to high temperature and pH ranges and are ideal for application in industrial processes.[20] The optimum temperature value was 20 ° C for the crude extract tested, presenting maximum activity of 3184,62 U.mL-[1].

Determinação do pH ótimo. O valor de pH ótimo foi de 5,0 para o extrato bruto testado, tendo atividade enzimática 3966,15 U.mL-[1].

Biorremediação com o caldo enzimático. O caldo com 200U de atividade enzimática mostrou a tendência de não realizar a biorremediação do repelente apesar das variações experimentais, sendo que resultados iguais foram obtidos com 400U de atividade enzimática (dados não demonstrados).

Bioremediation by Laccase of the fungus. The fungus showed a linear trend of degradation (bioremediation) of the DEET showing a removal of up to 39% of the repellent considering the highest point with the lowest point.

Adsorption test. The test showed a 14 day mean area of 77500 indicating no adsorption by the fungus.

Tocixity test with *Allium cepa.* A stimulus of the onion bulbs for root growth was observed only after the exchange of the solution with DEET by water. The microscopy obtained in the DEET test demonstrated cellular alterations when compared to the microscopy obtained in the test with distilled water where one could observe even the mitotic phases.

Because it is a lignocellulosic material, plant residues have the potential to contribute to the growth of wood decomposing fungi, as well as to the greater production of their enzymes as observed with the use of cocoa.

The use of plant residues as inducers of Lacasses is a promising approach, since it is an alternative strategy to the synthetic inductors, hitherto used due to the need to obtain high amounts of the enzyme for the supply of its various applications, besides becoming economically viable, helps to solve the environmental problems arising from the accumulation of synthetic inductors in nature.

Optimum temperature. The optimum temperature value can be observed in Figure 1. Based on the ANOVA statistical test, temperatures of 20 ° C, 30 ° C and 40 ° C are statistically similar. At temperatures of 70 ° and 80 ° C, no enzymatic activity was observed, indicating that from 70 ° C the protein is denatured.

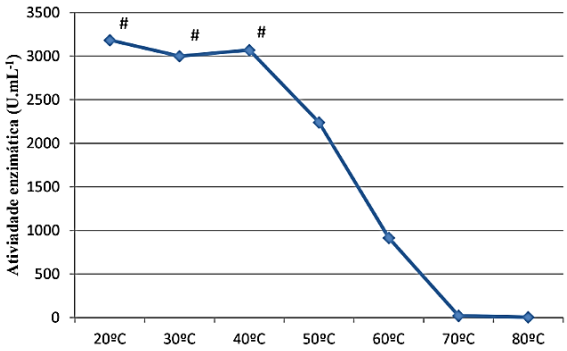

Figure 1 - Enzymatic activity in crude enzymatic extract at different temperatures.
#- Statistically similar groups

optimum pH. The optimum pH of fungal laccases is generally acidic, although this depends on the substrate used.[23] The optimum pH value was 5.0 for the crude extract tested (Figure 2). Pleurotus ostreatus lacases also showed high activity at pH 6 and pH 7 with low activity at pH 8. According to the statistical ANOVA and Tukey test with p = 0.0036, pHs 6 and 7 were statistically similar, whereas pH 5 showed higher enzymatic activity 3966,15 U.mL-[1].

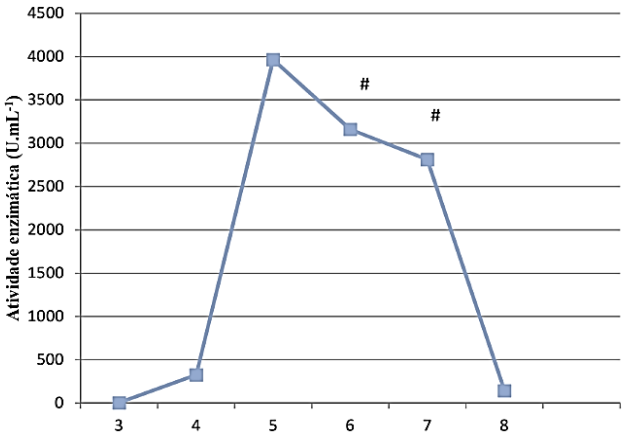

Figure 2 - Enzymatic activity in crude enzymatic extract at different pH's.
#- Statistically similar groups

Bioremediation. It is possible to observe on Figure 3A that bioremediation of repellent does not tendo t happen. There were some experimental variations, such as on the 4 hours point. Bioremediation with the active fungus (Figure 3B), it is possible to observe by the means of the areas of the Peaks of the chromatograms a linear tendency of degradation (biorremediation) of the DEET with the time presenting a removal of up to 39% of the repellent considering the greater point with the smaller point.

As can be corroborated with previous studies in which the experimental results showed that DEET was not removed by Lacase alone, one of the possible factors that may be related to the low efficiency of DEET removal by Lacases and the variation of results may be due to presence of the electronic group ($-CO-N[CH_2-CH_3]_2$) in the chemical structure of DEET.[22]

The bioprocesses developed in this work can be improved through the

use of mediators and applied in the treatment of water supply and wastewater, such as effluents from pharmaceutical industries and sewage treatment stations, thus promoting an improvement in the action of Lacases on repellent.[22]

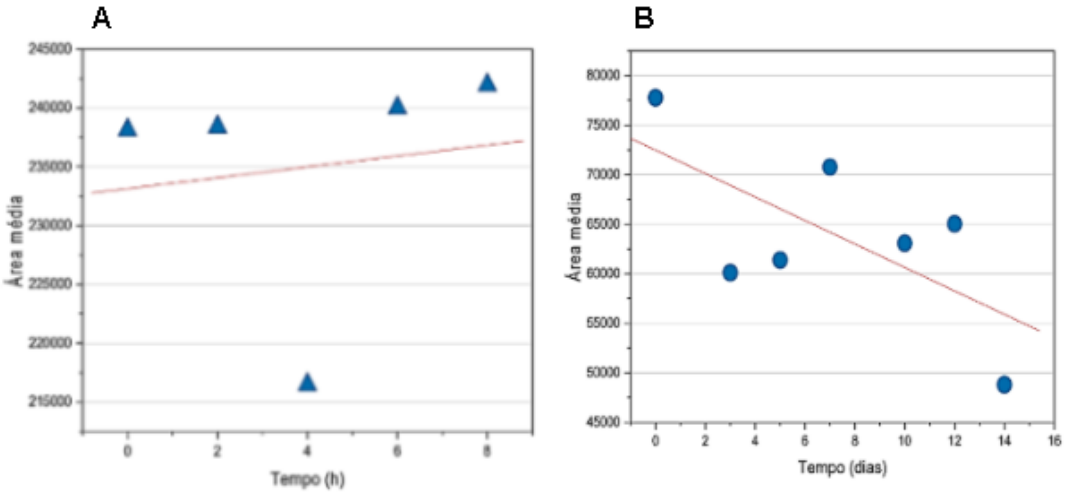

Figure 3- DEET degradation with 200U enzymatic activity broth (A) and with the active fungus (B) measured by the peak areas of the chromatograms.

Toxicity test with *Allium cepa*. The solution with water only (Figure 4A) allowed to observe the division phases and the framework of the bulb cells. If the solution is compared with DEET (Figure 4B), it is possible to observe cellular alterations. It can then be induced that the DEET reacts with the cells, being a compound with possible toxic capacity.

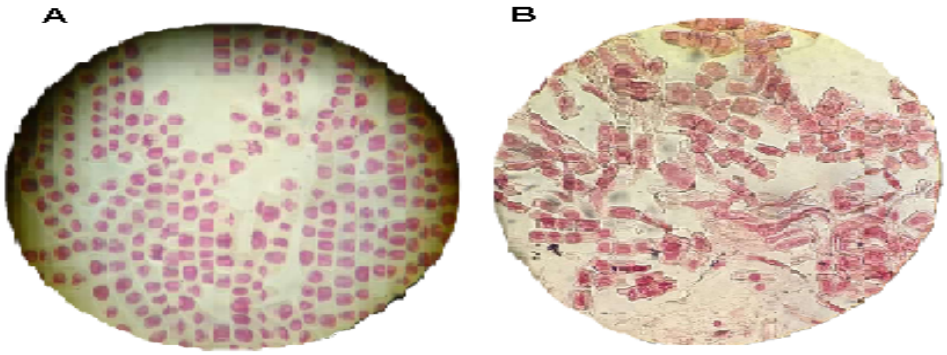

Figure 4- Microscopy obtained from the Allium strain with water (A) and DEET (B)

Conclusion

The results of the experimental part in solid culture medium with the Pleurotus ostreatus fungus indicated a tendency to apply it in bioremediation of environments contaminated with this type of repellent. However, due to the variation of the results, it is necessary to carry out more studies with a more effective approach to the methodology, once, also, that the results obtained in the present study were not feasible for the treatment of water with the enzymatic broth, but the treatment with the active fungus can be promising, especially in industrial effluents contaminated by products intended for personal care, such as repellents.

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