UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

BIODEGRADATION OF THE PYRETHROID PESTICIDE GAMMA-CYHALOTHRIN BY GRUTA DO CATAO FUNGI

Fábio Rocha Rigolin

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Dissertação apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos como parte dos requisitos para obtenção do título de Mestre em Ecologia e Recursos Naturais.

Orientadora: Profª Drª Mirna Helena Regali Seleghim

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SUMÁRIO

RESUMO

A contaminação de áreas agricultáveis por pesticidas, seu uso intensivo e toxicidade para muitos organismos não alvo causam sérios problemas ambientais. Portanto, o desenvolvimento de metodologias de biorremediação para ingredientes ativos distintos, tais como piretróides, é primordial. Nesse trabalho, a biodegradação de gama cialotrina (GCH) por fungos da Gruta do Catão foi investigada. Os experimentos foram realizados com três diferentes linhagens fúngicas (*Aspergillus ustus* CBMAI 1894, *Talaromyces brunneus* CBMAI 1895 e *Aspergillus* sp. CBMAI 1926) em meio líquido malte 2% com 300 mg L^{-1} de GCH em experimentos em triplicata utilizando um método validado. Todas as linhagens biodegradaram esse inseticida e a espécie mais eficiente foi *Aspergillus ustus* com uma taxa de degradação de aproximadamente 50% da concentração inicial de GCH em 21 dias de experimento. Também conduzimos um planejamento experimental testando três variáveis: temperatura (25-35 graus), pH (5,5 - 8,5) e concentração de pesticida (50-550 mg L^{-1}), sendo que a temperatura e a concentração influenciaram no experimento. Concluímos que fungos de caverna biodegradaram GCH e podem ser empregados em estudos futuros para caracterização enzimática e biorremediação de ambientes contaminados.

Palavras-chave: *Aspergillus ustus*; Biorrremediação; Cialotrina; Inseticida; Contaminação; *Talaromyces brunneus*.

BIODEGRADATION OF THE PYRETHROID PESTICIDE GAMMA-CYHALOTHRIN BY GRUTA DO CATAO FUNGI

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Abstract

The contamination of agricultural lands by pesticides, their overuse and toxicity to many non-target organisms cause serious environmental problems. Therefore, the development of bioremediation methods for distinct active ingredients, such as pyrethroids, is primordial. In this work, the biodegradation of gamma-cyhalothrin (GCH) by fungi from the cave Gruta do Catão was investigated. Experiments were conducted with different fungi strains (*Aspergillus ustus* CBMAI 1894*, Talaromyces brunneus* CBMAI 1895 and *Aspergillus* sp. CBMAI 1926) in 2% Malt liquid medium with 300 mg L^{-1} of GCH in triplicate employing a validated method. All strains biodegraded this insecticide and the most efficient was *Aspergillus ustus* with a degradation rate of approximately 50% of the initial GCH concentration in 21 days of experiment. We also conducted an experimental design testing three variables: temperature (25-35 degrees), pH (5,5– 8,5) and pesticide concentration (50-550 mg L^{-1}) being the temperature and pesticide concentration were those that most influenced the biodegradation. So, we concluded that cave fungi biodegraded GCH and can be employed in future studies for enzymatic characterization and bioremediation of contaminated environments.

Keywords: *Aspergillus ustus*; Bioremediation; Cyhalothrin; Insecticide; Contamination; *Talaromyces brunneus*.

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1. Introduction

With population growth, one of the great challenges of society is undoubtedly to generate food that can meet the human demand without environment degradation by the use of chemical substances such as pesticides. Despite the importance of this issue, the use of chemical substances is considered fundamental for the increasing productivity of established agricultural areas, especially considering the huge losses when they do not use pesticides during the food production process (MESNAGE & SÉRALINI, 2018).

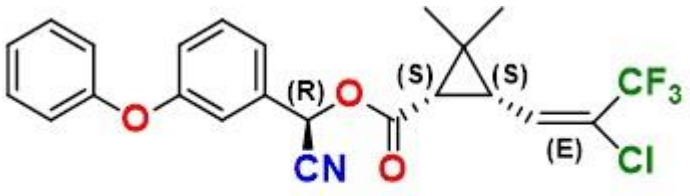
In 2008, Brazil used about 730 million tons of agrochemicals, which made this country the world's largest consumer of pesticides, position that maintain until today (ALBUQUERQUE et al., 2016; BRASIL, 2015; FAO, 2014; SOUZA et al, 2019). Pesticides can be classified according to target organisms (nematicides, insecticides, herbicides, fungicides, rodenticides, acaricides, molluscicides, algicides, etc) and the chemical group to which they belong (organochlorines, chloro-phosphors, pyrethroids, organophosphates, carbamates, triazines) (CONWAY, 2003). Despite the benefits, literature data showed that less than 0.1% of the applied pesticides actually reach their target organisms. In this way, 99.9% of these substances have the potential to translocate to undesirable regions, reaching different dimensions of the environment (SABIK et al., 2000), affecting human, fauna and flora health (RIBEIRO et al., 2008).

Throughout the history, the use in agriculture of some pesticides has been banned because of their high toxicity and their persistence in the environment after application. In this context, since 1985, organochlorines, the pioneers among the synthetic pesticides, had their agricultural use banned in Brazil. Currently the use of organochlorines is restricted to public health campaigns (SANTOS et al., 2008). For this reason, there is a demand for chemical substances that could replace them (MANNA et al., 2004) and pyrethroids became the most widely used class of insecticide (SANTOS et al., 2008).

Pyrethroids are derived from the natural pesticides pyrethrins produced by the trituration of flowers of some plants belonging to the genus *Chrysanthemum* as it is the case of *Chrysanthemum cineraiaefolium* (PIMPÃO et al., 2007) and *Chrysanthemum cocineum* (TRAMUJAS et al., 2006). Pyrethroid pesticides have low toxicity levels for mammals, reduced environmental impacts, and smaller dosages are required to fulfill their role in their target organisms. However, the use of some pyrethroids has increased risk of contamination to birds (QUEIROZ et al., 2001), fish (SINGH & SINGH, 2008), bees, lobsters and shrimps (GRISOLIA, 2005).

Cyhalothrin is a pyrethroid insecticide formed by four stereoisomers of which gamma-cyhalothrin (1*R*,3*R*,α*S*-GCH) is the most active enantiomer. In addition, GCH is one of the enantiomers present in the lambda-cyhalothrin, pesticide composed of a racemic enantiomeric mixture. (GIDDINGS et al., 2009) (Figure 1).

Fig. 1. Structural formula of the pesticide gamma cyhalothrin.



(1R,3R,αS) – Gamma-Cyhalothrin

Looking for more sustainable methodologies, extreme and unexplored environments such as deep oceans, caves and deserts are the target of studies to look for bioactive compounds and microorganisms (CHÁVEZ, 2015; JONES et al., 2015). Underground environments such as caves can be considered extreme environments (ENGEL, 2007) that stimulate unique survival strategies in organisms and can provide far-reaching improvements in secondary metabolism (ADAM et al., 2018) due to their great adaptive plasticity (SOARES et al, 2011). Thus, Moreover, according to Ghosh et al. (2017), antimicrobial and enzymatic activities of cave microorganisms are different from microorganisms elsewhere, constituting an opportunity for novel discoveries.

Efficient biodegraders of polymers from plant origin such as lignin and cellulose, the fungi also are able to degrade waxes, rubbers, phenol, benzene, toluene, xylene and xenobiotics in forest environments (BENEVIDES & MARINHO, 2015). Usually when bacterial strains are not successful in biodegradation, fungi are triggered for this role (PARTE, 2017). Barton & Jurado (2007) reported that little is known about the populations, distribution and biochemical processes of microorganisms that inhabit cave environments. This is not different, for the Brazilian scenario since studies related to the microbiota of caves are still incipient (VANDERWOLF et al., 2013).

In this context, few studies have shown the importance of biodegradation performed by fungi found in caves such as De Paula et al. (2016) that showed

the ability of these organisms for cellulolytic degradation. So, the aim of this study was to evaluate fungi strains from the Gruta do Catão cave (São Desidério, Bahia State, Brazil) for biodegradation of GCH focusing on the identification of new catalysts for biotransformation of organic compounds and biodegradation reactions for future bioremediation processes.

2. Materials and methods

2.1 Pesticides, reagents, solvents, and culture media

(±)-LCH (analytical standard, 97%) was obtained from Sigma-Aldrich and employed for obtention of analytical curves. The insecticide NEXIDE (15% w/v of GCH) was donated from FMC QUÍMICA (Brazil). Reagents and solvents were obtained from Sigma-Aldrich and Synth (São Paulo, Brazil). Malt extract and agar were obtained from Acumedia (São Paulo, Brazil). Formic acid, methanol and acetonitrile (HPLC grade) were obtained from PANREAC and TEDIA.

2.2. Cave fungi

Strains of cellulolytic fungi were isolated during the work of De Paula (2016). We tested three strains (*Aspergillus ustus* CBMAI 1894*, Talaromyces brunneus* CBMAI 1895 and *Aspergillus* sp*.* CBMAI 1926), which were deposited at the *Brazilian Collection* of *Environmental and Industrial Microorganisms* (CBMAI, WDCM823).

2.3. Culture media

The cave fungi were reactivated and cultivated in three different culture media (Malt 2%, Sabouraud agar and Potato dextrose agar) in Petri dishes. The culture medium with the best fungal growth (2% malt) was used for further experiments. The composition of this culture medium was malt extract (20 g L^{-1})

and Agar (20 g L^{-1}). After preparation, all culture media were sterilized in an autoclave (AV-50, Phoenix) at 121 °C for 20 minutes and then the microorganisms handled in an aseptic chamber previously disinfected with 70% ethanol aqueous solution and UV light.

2.4 Selection of resistant fungal strains to gamma cyhalothrin

For each fungal strain, Petri dishes were prepared in triplicate containing 2% malt solid culture medium without gamma-cyhalothrin (controls) and with the pesticide in 300 mg L^{-1} concentration. The culture media autoclaved at 121^{o}C for 20 minutes, cooled to about 40-50^{o}C, and then the stock solution of the insecticide added. The mixture was homogenized and then poured into the respective Petri dishes. Inoculations of the fungal strains on the plates were done by transferring the mycelia from the pure cultures reactivated in 2% Malt medium after 7 days of growth, with the aid of a platinum needle by an insertion point in the centre of the plate. The plates were cultivated in an incubator at 25 °C (B.O.D. 411D, Nova Ética, Brazil) and the radial growths of the fungi were observed for 21 days, determining the colony diameter on the surface of the plates, at intervals of 7 days of cultivation.

2.5 Assay in liquid culture medium containing gamma-cyhalothrin

To prepare the spore solution, each strain was cultured in malt 2% solid culture medium for 7 days at 25°C. The spores were collected from solid culture medium by using 0.1% (v/v) Tween-80 solution that was then filtered through a glass wool to remove mycelia and recover only the spores. All spores were counted employing a Neubauer chamber to adjust the concentration to 1×10^{7} spores/mL.

Erlenmeyers of 125 mL capacity containing 50 mL of liquid malt 2% culture medium were used. The pH of the medium was adjusted to 5.6 and then autoclave sterilized at 121 °C for 20 minutes. After cooling, an average of 10^7 spores per mL of the selected fungi strain was added to the media and incubated in orbital shaker (25 °C, 130 rpm) for 3 days.

After 3 days, 300 mg L^{-1} of a GCH solution (commercial emulsifiable formulation –NEXIDE- diluted 20 times with distilled water) was added to the culture medium after sterilization in autoclave. The reactions were conducted under orbital stirring, maintaining the above conditions (25 °C, 130 rpm) for 14 days, in triplicate experiments. In addition, another experiment was done to test the addition of the pesticide at the same time of the inoculation, evaluating the use of primary or secondary fungal metabolism.

The biodegradation experiments were performed with 300 mg L^{-1} of GCH at 25 °C and 130 rpm for 14 days in the absence of light, employing 50 mL of 2% malt liquid medium.

2.6 Control trials (fungal and abiotic) and growth curve

Simultaneously, control experiments were carried out to verify the production of natural metabolites of the fungus, which were cultivated under the same conditions; however, in the absence of the pesticide. To verify the stability of the pesticide (GCH) and the abiotic degradation during the incubation, abiotic controls were performed containing only the culture medium and the pesticide. After 14 days of reaction, the control media were extracted and the samples analysed by high performance liquid chromatography (HPLC). Besides, in order to study the growth of the most efficient species, a growth experiment was carried out in which the fungus was cultivated for 14 days and its dry mass

measured daily after drying at 50°C for 24h. In addition, a growth curve was obtained for the species and all tests employed in triplicate.

2.7 Extraction of the pesticide gamma-cyhalothrin and their metabolites

The extraction and quantification of GCH was performed according to the literature (BIROLLI et al., 2016). The fungal cells were filtered with a Buchner apparatus and stirred vigorously in 100 mL of water and ethyl acetate (1:1) for 30 minutes for partitioning. Thereafter, the cells were subjected to a second filtration in a Buchner apparatus and the mycelial extract was added to the enzymatic broth. Then, the pH was adjusted to 5.0 and the final extract was obtained by three-step liquid-liquid extraction with 30 mL of ethyl acetate (P.A.) each. The aqueous phase was discarded and to the organic phase it was added anhydrous sodium sulfate, than filtered and transferred to a 250 mL round bottom flask for evaporation under reduced pressure. The sample was then dissolved in methanol in a 5mL volumetric flask (BIROLLI et al., 2016).

2.8 Quantification of gamma-cyhalothrin by HPLC-UV

Standard methanol solutions of increasing concentrations of GCH were prepared for the quantification of gamma-cyhalothrin, generating a linear equation.

GCH and metabolites were quantitatively determined by a validated method with a Shimadzu 2010 high pressure liquid chromatographic system (LC-20AT pump, DGU-20A5 degasser, SIL-20AHT sampler, SPD-M20A UV-VIS detector operating in 277 nm, CTO-20A column oven and CBM-20A controller) with a 25 cm x 4.6 mm Phenomenex C18 column with 5 µm of particle size. The analyses were carried out with 0.5% formic acid in water Milli-

Q (solvent A) and 0.5% formic acid in acetonitrile (solvent B) at 1.0 mL min^{-1} and 40 °C. From 0 to 17 min 60% of B isocratic, from 17 to 18 min 50-90% linear gradient, from 18 to 30 min 90% of B isocratic. The injection volume was 10 µL and the detection was performed at 277 nm (BIROLLI et al., 2016).

Analytical curves were obtained using the external standard method for the compound analysed. Note that the samples were re-suspended in 5 mL of methanol after the liquid-liquid extraction, being concentrated 10 times (BIROLLI et al., 2016).

2.9 Method validation

The recovery and standard deviation of the method were determined with five cultures of *Aspergillus ustus* CBMAI 1894 sterilized in autoclave after 7 days of cultivation at 25°C and 130 rpm. The commercial formulation of GCH was employed in these experiments for an increased similarity with the samples of the study.

2.9 Metabolites identification

The metabolites identification was performed by gas chromatography coupled to mass spectrometry (GC-MS) in a Shimadzu GC2010plus coupled to a mass selective detector (Shimadzu MS2010plus) in electron ionization (EI, 70 eV) mode. The GC-MS (employing a 30 m × 0.25 mm × 0.25 µm J&W Scientific DB5 column) conditions were: 90°C for 4 min, increased to 280°C at 6 °C min^{-1}, held for 6 min; injector and interface temperature was maintained at 250°C; splitless 1-µL injection; helium was used as the carrier gas at a constant flow 0.75 mL min^{-1}. The run time was 40 min and the scan mode used was *m/z* 40–500 (BIROLLI et al., 2016).

3. Results and discussion

3.1 Testing the best culture media

First, we conducted a primary experiment for checking which culture medium would have better fungal biodegrading performance of the insecticide. The results of this test are shown in Table. 1.

Table 1. Percentage of fungal growth in three different culture media grown at 25ºC in the absence of light containing 300 mg L^{-1} of gamma-cyhalothrin insecticide.

Species/culture medium	*Aspergillus ustus* CBMAI 1894 (%)	*Talaromyces brunneus* CBMAI 1895 (%)	*Aspergillus* sp. CBMAI 1926 (%)
Sabouraud Agar	29%	76%	76%
Potato Dextrose Agar	38%	66%	83%
2% Malt Agar	34%	64%	87%

Note: the efficiency calculation was conducted by the average of the values (radius in centimeters) of the cultures in the presence of pesticide divided by the average values in the absence of the same.

The fungi strains were able to grow in all the evaluated media. Since the 2% malt medium presented satisfactory values for all strains, and had been isolated in previous work with it, the following experiments were conducted with the same medium.

3.2. Growth curve

Analysing the growth curve of *Aspergillus ustus CBMAI 1894* (Figure 2) it was noted that the lag phase lasts until day one, followed by the exponential phase (days 1-5), stationary phase (5-11 days) and logarithmic decline phase or death (day 11 onwards). The curve showed that in the experiments, adding the insecticide concomitantly to the fungal spores we were doing it at the lag phase while in the three-day addition of the pesticide, the culture is already in the log phase. According to Cycon and Piotrowska-Seget (2016), when the pesticide

concentration is higher, the degradation rates are usually lower with or without lag phase.

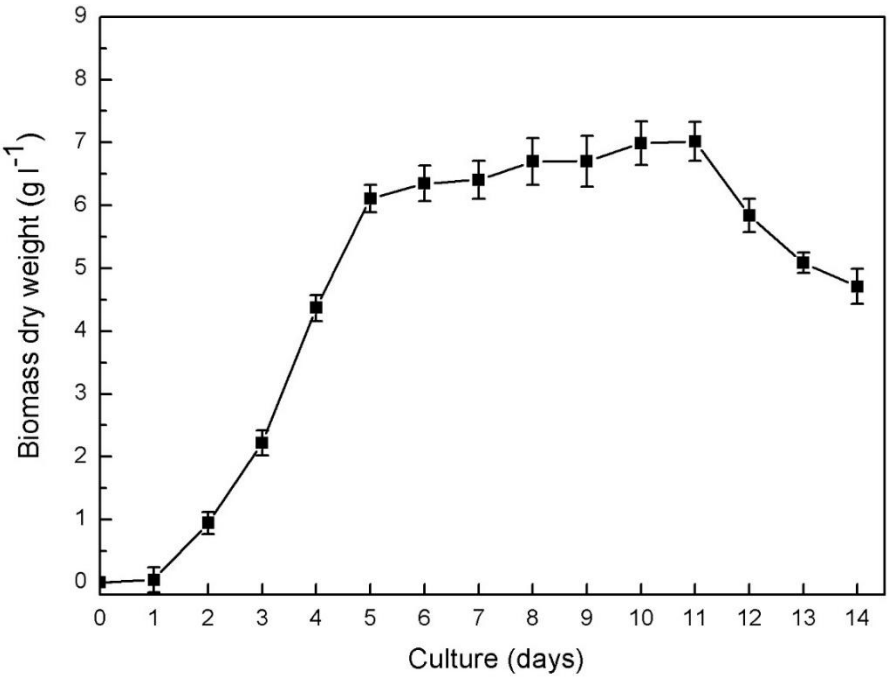


Figure 2. Growth curve of *Aspergillus ustus* during 14 days of cultivation (weight of the dry mass after drying at 50°C for 24h).

3.3. Quantification of gamma-cyhalothrin by HPLC-UV

The experiments were compared with the abiotic control and the degradation was investigated by the recovery method. This method ensure that the compound was not absorbed or adsorbed by the fungal cells or degraded abiotically. Then, it was assessed the amount of pesticide that cave fungi were able to biodegrade, showing absolute and relative values of GCH (Table 2).

Table. 2. Absolute and relative values of the gamma-cyhalothrin insecticide degradation by species isolated from cave fungi.

Sample	Degradation (absolute values, $mg.L^{-1}$)	Degradation (relative values, %)
Recovery	319.1±4.2	-6.4±4.2
Abiotic control	291.9±10.3	2.7±10.3
Aspergillus ustus CBMAI 1894 (Inoculation and GCH addition at day 0)	156.9±12.4	47.7±4.1
Aspergillus ustus CBMAI 1894 (Inoculation at day 0 and GCH addition at day 3)	211.4±9.0	29.5±3.0
Talaromyces brunneus CBMAI 1895 (Inoculation and GCH addition at day 0)	236.0±5.3	21.3±1.8
Talaromyces brunneus CBMAI 1895 (Inoculation at day 0 and GCH addition at day 3)	241.8±16.9	19.4±5.6
Aspergillus sp. CBMAI 1926 (Inoculation and GCH addition at day 0)	169.3±21.9	43.6±7.3
Aspergillus sp. CBMAI 1926 (Inoculation at day 0 and GCH addition at day 3)	187.6±24.5	37.5±21.5

The abiotic control and recovery tests are parameters to ensured that without the presence of microorganisms the amounts of the compound disappearing from the sample were very low, 2.7±10.3 and 6.4±4.2, respectively.

It is already known that microorganisms participate in various activities, including soil aggregation, formation of symbiotic relationships, decomposition of residues, control of pests and diseases, and mineralization of nutrients (MOREIRA and SIQUEIRA, 2006). However, little is known about cave microorganisms and its biotechnological contributions, such as bioremediators.

Microbial metabolism has been studied for many years and it executes important roles in detoxification or degradation of pyrethroids residues, as it can be seen, for example in bacteria (Akbar et al., 2015a; Chen et al., 2012a, 2014; Lee et al., 2016) and fungi (Chen et al., 2011a, 2012d; Saikia and Gopal, 2004). The study of novel species, as in this work, helps to understand how microorganisms that inhabit extreme environments (SOARES, 2011), as caves, may develop ways of optimize its energy obtaining.

3.4. Experimental planning

The strain that the experiments indicated that best biodegraded the GCH insecticide (*Aspergillus ustus CBMAI 1894)* was tested using an experimental design, carried out with three different variables at three levels during 21 days, as shown in Table 3. The results were also summarized by an ANOVA table (Table 4), the Pareto chart (Figure 3) and the Fitted surface figure (Figure 4), respectively.

Table 3. Box-Behnken experimental design and the response of dependent variable for GCH degradation using the *Aspergillus ustus CBMAI 1894* strain as bioremediator.

Runs	X1	X2	X3	Response	Concentration of degradation (%)
1	-1	-1	0	19.7	60.6
2	-1	1	0	480.8	12.6
3	1	-1	0	5.45	89.1
4	1	1	0	351.2	36.1
5	-1	0	-1	17.3	65.4
6	-1	0	1	471.4	14.3
7	1	0	-1	2.1	95.6
8	1	0	1	426.1	22.5
9	0	-1	-1	300.3	0.0
10	0	-1	1	123.6	58.8
11	0	1	-1	295.4	1.5
12	0	1	1	200.3	33.2

Note: X1: temperature, -1 (15^{o}C), 0 (25^{o}C), +1 (35^{o}C); X2: medium pH, -1 (5.5), 0 (7.0), +1 (8.5); X3: initial concentration, -1 (50 mg.L^{-1}), 0 (300 mg L^{-1}), +1 (550 mg L^{-1}). The table is composed of two replicates at central point.

335 **Table 4.** ANOVA analysis of experimental design.

Variation source	Quadratic sum	Degrees of freedom	Quadratic mean	F calculated	p (95%)
(1) Concentration (L)	6339,38	1	6339.380	38.69132	0.000437
Concentration (Q)	3023,34	1	3023.340	18.45244	0.003586
(2) Temperature (L)	2538,28	1	2538.281	15.49197	0.005632
Temperature (Q)	69,56	1	69.564	0.42458	0.535462
(3) pH (L)	25,56	1	25.561	0.15601	0.704617
pH (Q)	65,16	1	65.161	0.39770	0.548309
Error	1146,91	7	163.845		
Total SS	13087,43	13			

336 R-sqr =0.91237; Adj: 0.83725

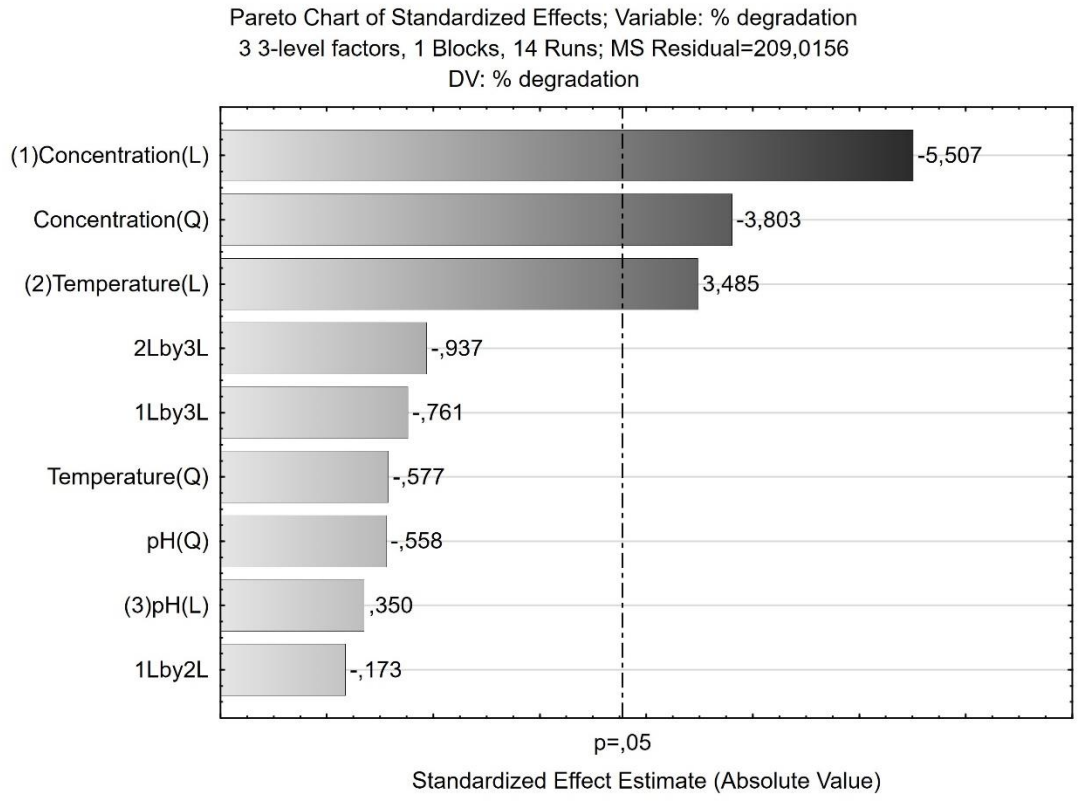


Figure 3. Pareto chart of model planning showing the responses of the variables concentration, temperature and pH.

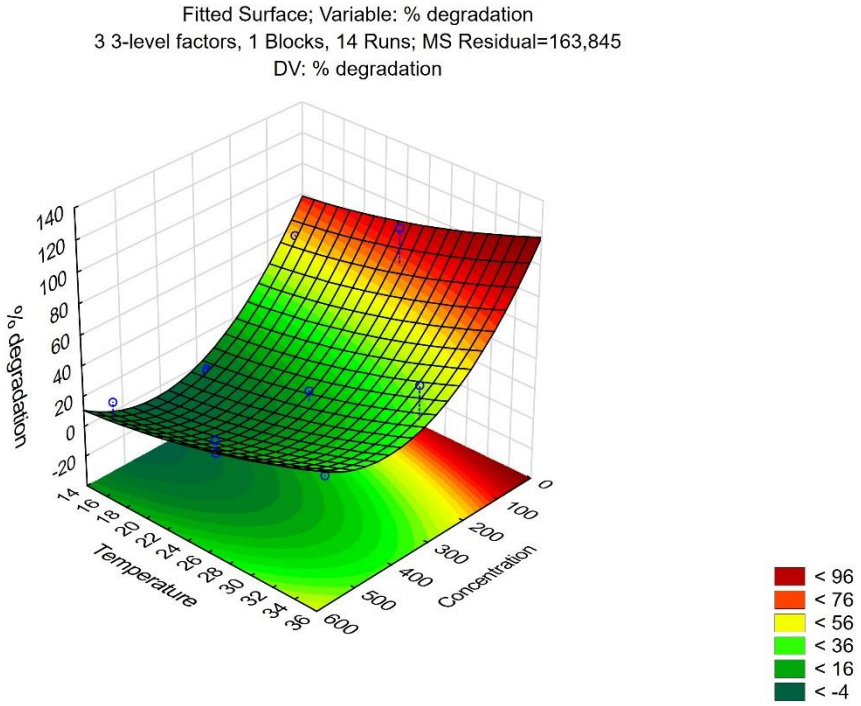


Figure 4. Response surface plot showing the effects of temperature and initial concentration on GCH for medium pH 7.0.

Note: $y = b_1 \cdot T^oC + b_3 \cdot [I] + b_{33} \cdot [I]^2$

$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x^2_{11} + b_{22}x^2_{22} + b_{33}x^2_{33} + b_{12} \cdot x_1x_2 + b_{13} \cdot x_1x_3 + b_{23} \cdot x_2x_3$

Factors such as initial pesticide concentration, pH, nutrients, organic matter content, carbon sources, temperature, microbial metabolism, and moisture substantially influence pyrethroid biodegradation in both liquid medium and soil (Saikia and Gopal, 2004; Zhang et al., 2010; Zhao et al., 2013; Cyco´n et al., 2014; Chen et al., 2015; Song et al., 2015; Akbar et al., 2015). The pH did not influenced the experimental model in any way in our work. In the literature it is claimed that alkaline or neutral pH conditions increased the degradation of pyrethroid insecticide. However, in acid conditions the degradation was reduced probably because the acid pH increases the resistance of pyrethroids against microorganisms and stability (Singh et al., 2006; Anwar et al., 2009; Cyco´n et al., 2009; Zhao et al., 2013; Chen et al., 2015).

In our work temperature showed a positive correlation with the increasing rates of GCH degradation. Although it is known that, at certain limits, temperature influence positively microbial metabolism, it is stated that conditions tending to high or low temperature are not ideal for pyrethroid biodegradation (Chen et al., 2011b; Zhang et al., 2016).

At the theoretical maximum point, the values of X1 and X3 in terms of code units were 28-30^{o}C and [100 mg.L^{-1}], respectively.

Differently from the other variables, GCH concentration showed negative correlation with biodegradation rates for both analyses (linear and quadratic). This response goes according to Cycon and Piotrowska-Seget (2016) which stated that higher concentrations lower degradation rates regardless of having a lag phase. Other authors ponder that higher concentrations would act as inhibitors for microorganisms in two ways: slower growth and adaptation to the environment; and increased lag phase as recruitment of more microorganisms to begin biodegradation (Chen et al.,2012a, 2015; Zhao et al.,2013; Cyco´n et al., 2014).

3.5. Metabolites identification

Products of the gamma-cyhalothrin metabolism by the cave fungi were identified. The metabolite 2-(3-phenoxyphenyl)acetonitrile was identified for all the evaluated strains in the biodegradation experiments with a retention time of 27.12 min and a 90% of similarity with the spectra library NIST08. This substance was absent in the abiotic control and killed-cells control experiments, showing that this compound resulted from the biodegradation process.

The 3-phenoxybenzaldehyde was also identified in the GC-MS analyses for all strains with a retention time of 23.6 min and a similarity with the spectra

library NIST 8 of 89%. This compound was confirmed with an authentic standard. The metabolite 3-phenoxybenzoic acid was identified in HPLC-UV analyses of the experimental planning experiments employing a standard. Probably this compound was absent in the GC-MS analyses because of its low concentration and high detection limit. Considering the identified metabolites, a partial biodegradation pathway was proposed (Figure 5).

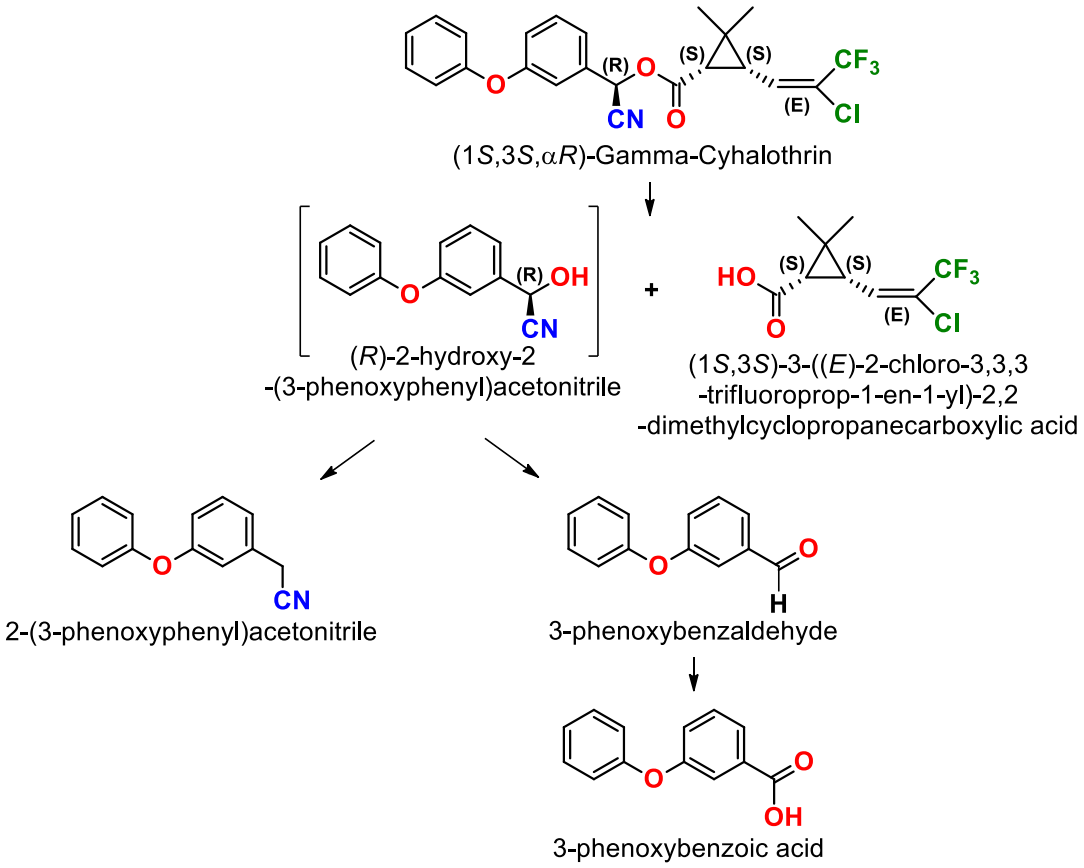


Figure 5. Proposed biodegradation pathway by cave fungi.

4. Conclusion

Among the fungi species of Gruta do Catão studied, *Aspergillus ustus CBMAI 1894* showed the highest efficiency in GCH biodegradation. However, the other two strains, *Talaromyces brunneus CBMAI 1895* and *Aspergillus* sp. CBMAI 1926, were also able to biodegrade this insecticide and showed

potential for bioremediation processes. The variable temperature affected positively and concentration of insecticide affected negativelly fungal biodegradation while pH showed no effect according to experimental design. For a better understanding of this biodegradation process, future studies on the enzymatic apparatus of these fungi should be evaluated focusing on understanding the physiology and genetics behind these microorganisms.

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