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Article

# Biodegradation of the Pyrethroid Pesticide Esfenvalerate by a Bacterial Consortium Isolated from Brazilian Savannah

*Charlene S. dos Anjos,*[a] *Willian G. Birolli*[a] *and André L. M. Porto*[*,a]

[a]*Laboratório de Química Orgânica e Biocatálise, Instituto de Química de São Carlos, Universidade de São Paulo, Av. João Dagnone, 1100, Ed. Química Ambiental, J. Santa Angelina, 13563-120 São Carlos-SP, Brazil*

New biocatalysts for bioremediation techniques are necessary nowadays. Therefore, a bacterial consortium isolated from Brazilian Savannah was employed for biodegradation of 100 mg $L^{-1}$ esfenvalerate in liquid culture medium. The bacterial consortium (*Lysinibacillus xylanilyticus* CBMAI2085, *Bacillus cereus* CBMAI2067, *Lysinibacillus* sp. CBMAI2051 and *Bacillus* sp. CBMAI2052) biodegraded this pyrethroid efficiently. The assays were conducted in triplicate, and after 12 days, 90% of the pesticide was degraded producing 3-phenoxybenzoic acid (35.0 ± 3.1 mg $L^{-1}$) and 2-(4-chlorophenyl)-3-methylbutanoic acid (34.0 ± 2.8 mg $L^{-1}$). The bacterial consortium (52 ± 5% biodegradation) was more efficient in the biodegradation than the average of the same strains solely employed (40 ± 7% biodegradation), showing that the use of consortia is an interesting approach. However, the strain *Bacillus cereus* CBMAI2067 (67 ± 3% biodegradation) was more efficient than the bacterial consortium, showing its potential as source of carboxylesterases and proving that, in this case, the use of a unique efficient strain is more adequate.

**Keywords:** degradation, 3-phenoxybenzoic acid, microorganism, insecticide, native bacteria, Cerrado

## Introduction

Pyrethroids are synthetic insecticides derived from pyrethrins, natural pesticides found in *Chrysanthemum* plants.[1] They have been marketed since the 1970s and nowadays are characterized by several different structures. However, the most commonly used pyrethroids are dimethylcyclopropane esters with variable aromatic rings.[2]

Pyrethroids have low mammalian toxicity and environmental impact, a high efficiency against a broad spectrum of insects and require only a low dose to achieve satisfactory results.[3] This chemical class of insecticides is one of the most used pesticides worldwide for different types of crops and against several classes of insects.[1,4] However, laboratory tests have shown that pyrethroids are very toxic to fish, bees and aquatic arthropods, such as lobsters and shrimps. Therefore, pyrethroids showed effects on accidentally exposed species, including humans during application or by ingestion of contaminated food.[5]

Several bacterial strains were isolated from different environments for biodegradation processes, i.e., contaminated soil, marine environment and activated sludge.[6-10] However, the major part of the literature aiming bioremediation of pyrethroids employed bacteria isolated from contaminated soil, since it is important that the biocatalyst presents an efficient biodegradation activity and a good development in the environmental conditions. Some examples were the biodegradation of cypermethrin by *Pseudomonas aeruginosa* GF31, *Micrococcus* sp. strain CPN1 and *Bacillus* sp. SG2, fenpropathrin by *Pseudomonas aeruginosa* sp. JQ-41, deltamethrin by *Serratia marcescens* Del-1, Del-2 and permethrin by *Acinetobacter baumannii* ZH-14.[11-16]

Among the commercially available pyrethroids used as insecticides, esfenvalerate is the biologically most active stereoisomer of the pyrethroid fenvalerate. It is very effective against target species (*Anticarsia gemmatalis*, *Scaptocoris castanea* and *Atarsocoris brachiariae*), but it is also toxic to aquatic species, producing ecological imbalances.[1] Moreover, esfenvalerate half-life is relatively long (72 days) in aquatic environment at pH 7 without light.[17]

Some studies were reported[18,19] describing the biodegradation of fenvalerate, including the employment

*e-mail: almporto@iqsc.usp.br

of *Bacillus flexus* strain XJU-4 that produced 3-phenoxybenzoic acid (PBAc), protocatechuic acid and catechol, and *Bacillus licheniformis* CY-012 that generated 3-phenoxybenzylic alcohol (PBAlc), 2-(4-chlorophenyl)-3-methylbutanoic acid (ClAc), 2-(4-chlorophenyl)acetic acid, benzoic acid and phenol. In addition, esfenvalerate biodegradation by marine-derived fungi presented PBAc and ClAc as metabolites.[20] Whereas bacteria from Brazilian Savannah, sea and a tropical peat produced ClAc, PBAc, PBAlc, methyl 3-phenoxybenzoate, 3-(hydroxyphenoxy) benzoic acid, 2-(3-hydroxyphenyl)acetic acid and 3-hydroxybenzoic acid.[21]

The Brazilian Savannah is composed of variable vegetation, ranging from open fields to woodlands, being characterized by soil composition, nutrient availability and natural fire events.[22] This variability influences the microbiota, which presents great morphological, metabolic and genetic diversity and may perform several biodegradation reactions in different and harsh conditions, such as extreme temperature, acidity and oxygenation levels.[23]

Microorganisms from the Brazilian Savannah were isolated and identified because of their potential for several biotransformation reactions, including biodegradation.[21] The aim of this study was the biodegradation of esfenvalerate, which is a worldwide employed insecticide, using a bacterial consortium isolated from this important biome.

## Experimental

### Reagents, solvents and culture media

Nutrient broth and agar were purchased from Acumedia (São Paulo, SP, Brazil) and Kasvi (São José dos Pinhais, PR, Brazil). Acetonitrile and methanol high pressure liquid chromatography (HPLC) grade were acquired from Panreac (Barcelona, Spain) and ethyl acetate analytical grade was purchased from Synth (Diadema, SP, Brazil). Sodium chloride was obtained from Chemicals (Ribeirão Preto, SP, Brazil). Esfenvalerate (97%), 3-phenoxybenzoic acid (PBAc, 98%), 2-(4-chlorophenyl)-3-methylbutanoic acid (ClAc, 96%) and 3-phenoxybenzylic alcohol (98%) used as analytical standards were purchased from Sigma-Aldrich (São Paulo, Brazil). The experiments were carried out with Sumidan 150SC (15% m/v esfenvalerate), a commercial formulation donated by Iharabras (Sorocaba, SP, Brazil).

### Isolation of bacteria from Brazilian Savannah

Details of isolation and identification of microorganisms from Brazilian Savannah were reported by Meira *et al.*[21] The microorganisms used in consortium for esfenvalerate biodegradation were previously described as *Bacillus* sp. 6D (*Lysinibacillus xylanilyticus* CBMAI2085), *Bacillus* sp. 6F (*Bacillus cereus* CBMAI2067), *Bacillus* sp. 6H (*Lysinibacillus* sp. CBMAI2051) and *Bacillus* sp. 6I (*Bacillus* sp. CBMAI2052). CBMAI refers to the deposit number in the Brazilian Collection of Environmental and Industrial Microorganisms (CBMAI, code WDCM823).

### Bacterial culture in solid medium

The solid cultures were performed in Petri dishes (90 × 15 mm) with 25 mL of nutrient medium (15 g $L^{-1}$ agar, 8 g $L^{-1}$ nutrient broth, pH 7) and 100 mg $L^{-1}$ of esfenvalerate. In these Petri dishes, esfenvalerate was added during the preparation of the plates for bacterial adaption to the esfenvalerate presence as previously described.[24] After that, each bacteria (*Lysinibacillus xylanilyticus* CBMAI2085, *Bacillus cereus* CBMAI2067, *Lysinibacillus* sp. CBMAI2051, and *Bacillus* sp. CBMAI2052) was inoculated and incubated in a biochemical oxygen demand (BOD, 32 °C, 24 h).

### Biodegradation of esfenvalerate in a liquid medium by a bacterial consortium

After growth, each bacteria was singly inoculated with 1 mL of a suspension of $10^8$ colony forming units (CFU) $mL^{-1}$ (obtained from a 24 h-solid culture) in a liquid culture medium (12 mL of nutrient broth in 125 mL Erlenmeyer flasks) and subsequently incubated in orbital shaker (132 rpm, 32 °C, 24 h). Then, the bacterial suspension was transferred to a 250 mL centrifuge tube and the pellets were centrifuged (5000 rpm, 7,700 × g, CR22GIII high speed refrigerated centrifuge, Hitachi, 2 min). Subsequently, the supernatant was discarded and the pellets of each bacteria (*Lysinibacillus xylanilyticus* CBMAI2085, *Bacillus cereus* CBMAI2067, *Lysinibacillus* sp. CBMAI2051, and *Bacillus* sp. CBMAI2052) were brought together in a consortium and added in a 125 mL Erlenmeyer flask with 50 mL of saline solution (0.86%). Therefore, the biodegradation was carried out with the pesticide formulation as carbon source.

Esfenvalerate (100 mg $L^{-1}$) was diluted 20 times by the addition of 19 mL of distilled water to 1 mL of the commercial emulsifiable formulation (SUMIDAN 150SC). Then, this solution was sterilized in autoclave to prevent contamination of the bacterial culture by microorganisms present in the pesticide formulation. It is noteworthy that no thermal degradation was observed since 98.8 ± 0.6% esfenvalerate was determined and no metabolites were

observed after sterilization. Following, 668 μL of this diluted and sterile solution was added to each Erlenmeyer flask for the reaction, resulting in 100 mg $L^{-1}$ of esfenvalerate in the reaction medium.[20] Then, the flask was incubated in an orbital shaker for biodegradation (132 rpm, 32 °C, 4, 8 and 12 days). All experiments were conducted in triplicates.

Experiments without pesticide (bacterial controls) and without bacterial inoculation (abiotic controls) were carried out in triplicate during the same period of the biodegradation reactions for evaluation of the esfenvalerate stability in the experimental conditions. After the biodegradation period by the bacterial consortium, an extraction of the residual esfenvalerate and its metabolites was performed.[25]

### Extraction of esfenvalerate and its metabolites

Esfenvalerate and its metabolites were extracted after the biodegradation reaction. Initially, 50 mL of ethyl acetate (EtOAc) was added in the reaction flask (Erlenmeyer of 125 mL) and the mixture was stirred vigorously on magnetic plate for 30 min. In the next step, the mixture was transferred to a 250 mL centrifuge tube (10,000 rpm, 20 min) and centrifuged. The obtained supernatant was placed in a 250 mL beaker flask and the pH was adjusted to pH 5.0. Then a liquid-liquid extraction in three-steps with 30 mL EtOAc each was carried out. The organic phase was dried with anhydrous $Na_2SO_4$, filtered, and the solvent was evaporated under reduced pressure. The sample was suspended with methanol in a 5-mL volumetric flask and analyzed by HPLC.[25]

### Quantification of esfenvalerate and its metabolites

The residual esfenvalerate and the metabolites PBAc and ClAc were determined by HPLC analyses using an external standard method. For esfenvalerate, the solution concentrations employed were 50, 300, 550, 800, and 1050 mg $L^{-1}$ in methanol for the construction of an analytical curve. Two standard curves were obtained for each compound, PBAc and ClAc: one standard curve with standard solutions of 5, 12, 19, 26, 33 mg $L^{-1}$, and an additional curve with standard solutions of 50, 80, 135, 190 and 250 mg $L^{-1}$ in methanol (Supplementary Information (SI) section).

The separation was performed using a column Shim-pack CLC-ODS (4.6 mm × 25 cm, 5 μm of particle size) in reverse phase with water and acetonitrile with 0.5% of formic acid, as described in a previous study.[20] This method was validated for bacteria in another paper.[25]

### Metabolites identification by gas chromatography coupled to mass spectrometry (GC-MS)

The identification of metabolites resulted from the esfenvalerate biodegradation was carried out by gas chromatography coupled to mass spectrometry in a Shimadzu GC2010plus chromatograph with a mass spectrometer Shimadzu MS2010plus in electron ionization mode (70 eV) with a DB5 column (J&W Scientific, 30 m × 0.25 mm × 0.25 μm) as already described.[20] The identified compounds were confirmed with authentic standards.

## Results and Discussion

The biodegradation of esfenvalerate was performed using a bacterial consortium composed of the strains *Lysinibacillus xylanilyticus* CBMAI2085, *Bacillus cereus* CBMAI2067, *Lysinibacillus* sp. CBMAI2051, and *Bacillus* sp. CBMAI2052, which were isolated from a Brazilian Savannah area. The pesticide concentration decreased from the initial 100 mg $L^{-1}$ to 10.6 ± 4.0 mg $L^{-1}$ after 12 days, producing 35.0 ± 3.1 mg $L^{-1}$ PBAc and 34.0 ± 2.8 mg $L^{-1}$ ClAc (Table 1). Therefore, about 20 mg $L^{-1}$ (20% of the initial esfenvalerate concentration) was mineralized or biotransformed into unidentified metabolites.

**Table 1.** Quantification of esfenvalerate (initial concentration of 100 mg $L^{-1}$), 3-phenoxybenzoic acid (PBAc) and 2-(4-chlorophenyl)-3-methylbutanoic (ClAc) after biodegradation by a bacterial consortium (32 °C, 130 rpm, 0-12 days)

| Reaction time / days | c / (mg $L^{-1}$) | | | |
|---|---|---|---|---|
| | Esfenvalerate | PBAc | ClAc | PBAld |
| Abiotic control[a] | 99 ± 2 | NQ | ND | 1.1 ± 0.1 |
| Killed-cells control[a] | 99 ± 0.3 | 0.3 ± 0.2 | 1.0 ± 0.5 | 0.3 ± 0.0 |
| 4 | 60.8 ± 6.8 | 6.0 ± 1.8 | 6.9 ± 2.3 | ND |
| 8 | 22.9 ± 2.6 | 16.2 ± 3.4 | 16.7 ± 2.3 | ND |
| 12 | 10.6 ± 4.0 | 35.0 ± 3.1 | 34.0 ± 2.8 | ND |

[a]The reactions of the sterilized killed-cells control and abiotic control were performed in triplicates in the period of 4, 8 and 12 days. c: concentration determined by high-performance liquid chromatography (HPLC); PBAc: 3-phenoxybenzoic acid; ClAc: 2-(4-chlorophenyl)-3-methylbutanoic; PBAld: 3-phenoxybenzaldehyde; NQ: compound PBAc was detected, however, its concentration was below the limit of quantification of 0.1 mg $L^{-1}$ and was not determined; ND: not detected, below limit of detection of 0.1 mg $L^{-1}$ for ClAc and 0.03 mg $L^{-1}$ for PBAld. Data is presented as value ± standard deviation.

The esfenvalerate biodegradation over time was presented in Figure 1. The esfenvalerate concentration was reduced and the production of the metabolites PBAc

and ClAc was determined. The presence of the metabolite 3-phenoxybenzylic alcohol was also evaluated, but this compound was not detected (limit of detection of 0.02 mg $L^{-1}$) in the biodegradation experiments and neither in the control samples. These results showed that 3-PBAc was the main metabolite and its biotransformation should be evaluated in following studies.

The rate of esfenvalerate biodegradation was approximately constant at 10.1 mg $L^{-1}$ $day^{-1}$ from 0 to 8 days and, from 8 to 12 days the reaction was slower with a biodegradation rate of 3.1 mg $L^{-1}$ $day^{-1}$. The degradation kinetics for esfenvalerate was evaluated and a first-order kinetic was observed, obtaining the regression $C_t = 114e^{-0.196t}$ (where $C_t$ is the residual concentration of esfenvalerate in mg $L^{-1}$ and t is the degradation period in days), the first-order constant k was 0.196 $day^{-1}$, half-life ($t_{1/2}$) = 3.6 days and a correlation coefficient ($R^2$) of 0.989.

The stability of esfenvalerate was described in the literature,[17] the hydrolysis at pH 7 is very discrete as observed in our work. However, esfenvalerate presents a $t_{1/2}$ of 129 days at pH 5 and 65 days at pH 9 in abiotic conditions.[17] In our study, $t_{1/2}$ was about 3.6 days showing the efficiency of the biodegradation by the employed bacterial consortium.

A comparison between this employed consortium and the results available in the literature for the same strains solely employed was performed for reactions of 5 days (Figure 2).[21] The biodegradation by the bacterial consortium (48.4 ± 5.4 mg $L^{-1}$ of residual esfenvalerate) was more efficient than the average biodegradation of the separated strains (59.6 ± 7.4 mg $L^{-1}$ of residual esfenvalerate). However, the consortium did not biodegrade more than the most efficient strain *Bacillus cereus* CBMAI2067 (33.4 ± 3.2 mg $L^{-1}$ of residual esfenvalerate) solely employed in the same biocatalyst amount and conditions.

It is noteworthy that the consortium biodegradation experiments were performed during 4, 8 and 12 days, thus a second order polynomial estimation was carried out to estimate the residual esfenvalerate concentration for 5 days biodegradation (residual esfenvalerate (mg $L^{-1}$) = $0.483T^2 - 13.75T + 105.02$, where T = time in days).

A comparison with the reported literature is difficult because different biodegradation conditions and pyrethroids were investigated. For example, *Rhodopseudomonas* sp. PSB07-8 biodegraded only 23% of esfenvalerate (7 days, 20 mg $L^{-1}$ initial concentration, photosynthetic bacterium medium (PSB) medium, 30 °C, pH 7.0), *Pseudomonas aeruginosa* sp. JQ-41 biodegraded 89% of fenpropathrin (7 days, 100 mg $L^{-1}$ initial concentration, mineral salt medium, 30 °C, pH 7.0) and *Brevibacterium aureum* DG-12 biodegraded 80% of bifenthrin (5 days, 50 mg $L^{-1}$ initial concentration, mineral salt medium, 27 °C, pH 7.8) in different studies reported in the literature.[14,26,27]

The use of a microbial consortium to remove chemical compounds can be more effective than the use of a single strain, as reported in the literature[28] for lambda-cyhalothrin by bacteria. Therefore, motivated by the importance of consortia, we efficiently performed a biodegradation study of esfenvalerate by a bacterial consortium isolated from Brazilian Savannah.

The bacterial consortium was more efficient than the average of the strains solely employed, showing synergistic effects by the bacterial strains. However, the consortium did not biodegrade more than the most efficient strain

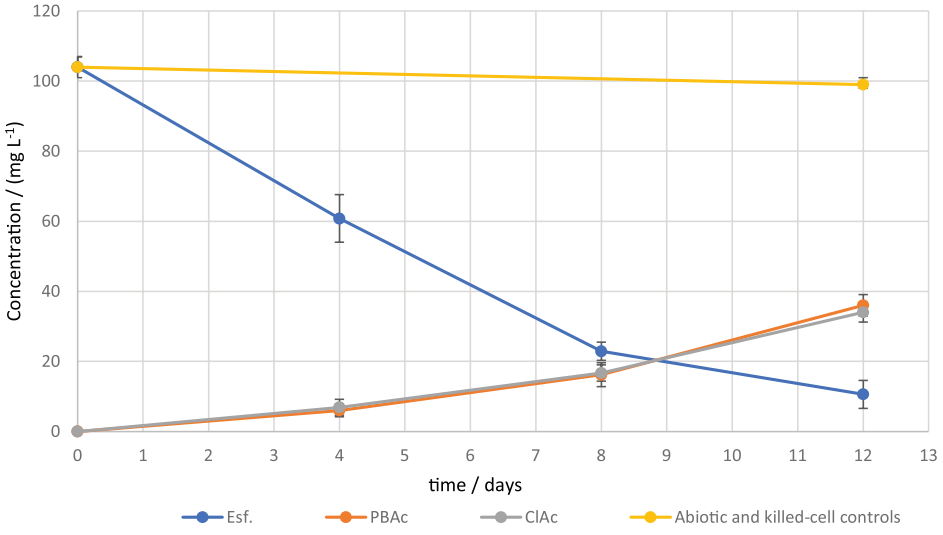


**Figure 1.** Biodegradation of esfenvalerate (100 mg $L^{-1}$) and production of 3-phenoxybenzoic acid (PBAc) and 2-(4-chlorophenyl)-3-methylbutanoic acid (ClAc) using a bacterial consortium from Brazilian Savannah (32 °C, 130 rpm, from 0 to 12 days).

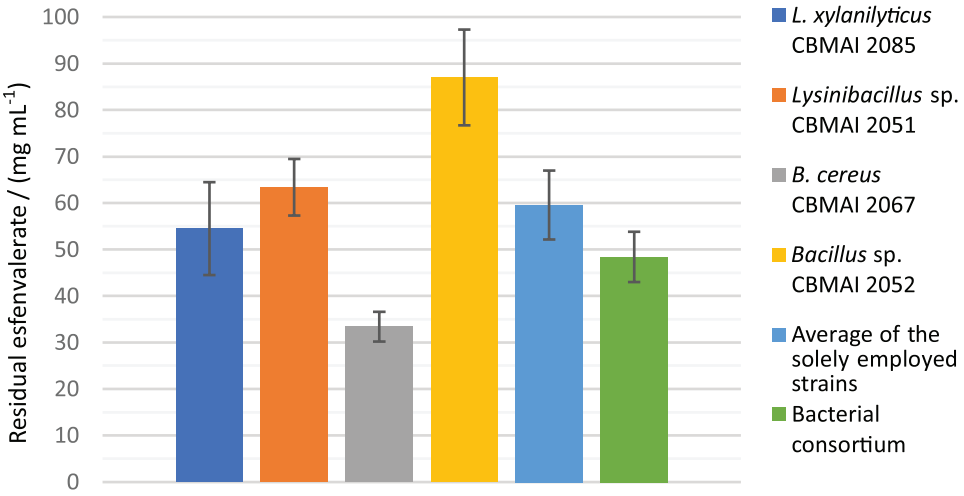


**Figure 2.** Biodegradation of esfenvalerate by isolated strains and the bacterial consortium obtained from Brazilian Savannah (32 °C, 130 rpm, 5 days).

*Bacillus cereus* CBMAI2067 employed in the same amount and conditions. Suggesting that for this case the employment of a unique effective strain was more adequate than the employed consortium.

Esfenvalerate is a pyrethroid pesticide that possesses an ester bond that can be hydrolyzed, the enzymes carboxylesterases catalyze the hydrolysis of esters to produce alcohols and carboxylic acids.[29] The hydrolysis of esfenvalerate via carboxylesterases from the employed bacterial consortium produced ClAc, which was quantified by HPLC analyses. However, 2-hydroxy-2-(3-phenoxyphenyl) acetonitrile was not detected probably because of its quickly conversion into 3-PBAc.

After the ester bond break, 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile can be biotransformed into 3-phenoxybenzaldehyde. Thus, enzymes named oxynitrilases (hydroxynitrile lyases) that catalyze the production of aldehydes or ketones by the cleavage of cyanohydrins might be involved in the reaction.[30]

The conversion of aldehydes into carboxylic acids can be carried out by aldehyde dehydrogenases present in the bacterial metabolism.[31] Hence, it was possible to suggest the conversion of 3-phenoxybenzaldehyde to 3-phenoxybenzoic acid by the employed consortium, as observed in other studies.[24,25] Therefore, a partial biodegradation pathway was proposed (Figure 3).

It is important to note that the absence of the metabolites 2-hydroxy-2-(3-phenoxyphenyl)acetonitrile and 3-phenoxybenzaldehyde was confirmed by GC-MS. There was a signal referring to a small concentration of 3-phenoxybenzaldehyde in the biodegradation analyses by GC-MS, but the same concentration was observed in the analysis of a pure standard of esfenvalerate at the same determined concentration of the sample. Therefore, this compound was generated by thermal instability of esfenvalerate at the analysis, as described in previous studies[8,20,25,28] with pyrethroids.

The biodegradation of esfenvalerate generated the metabolite PBAc, which is a toxic xenobiotic constantly detected in human urine.[32] This compound was also reported as a persistent and recalcitrant contaminant in urban and natural environments.[33] Thus, the biodegradation of this compound is important and might be studied, as reported recently in the literature[34] for fungi and bacteria.

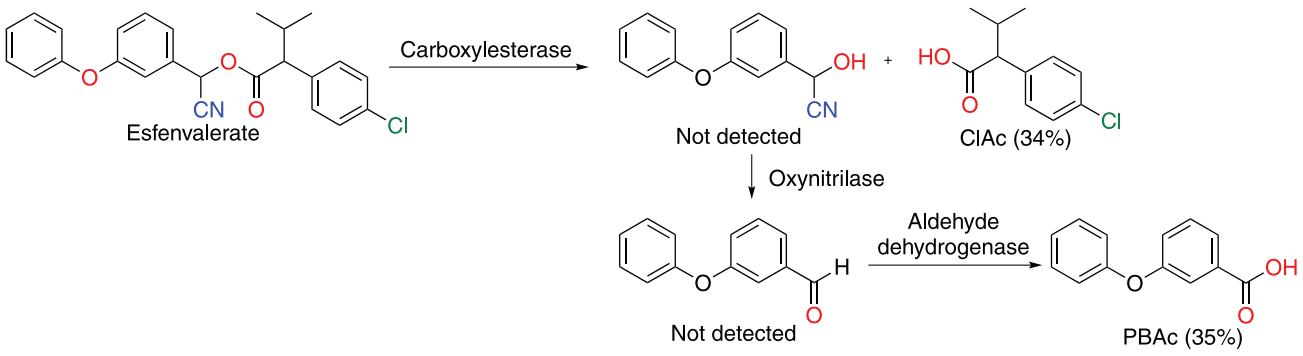


**Figure 3.** Partial biodegradation pathway of esfenvalerate by a bacterial consortium isolated from Brazilian Savannah.

## Conclusions

A bacterial consortium isolated from Brazilian Savannah was efficient in the biodegradation of the pyrethroid insecticide esfenvalerate. Therefore, the strains *Lysinibacillus xylanilyticus* CBMAI2085, *Bacillus cereus* CBMAI2067, *Lysinibacillus* sp. CBMAI2051, and *Bacillus* sp. CBMAI2052 were promising microorganisms for esfenvalerate biodegradation. The bacterial consortium was more efficient in the biodegradation than the average of the same strains solely employed, showing that the use of consortia is an interesting approach. However, the strain *Bacillus cereus* CBMAI2067 was more efficient than the bacterial consortium, showing its potential as source of carboxylesterases. In addition, this study showed the ability of native bacteria for biodegradation of pyrethroid pesticides.

## Supplementary Information

Supplementary information (analytical curves of the esfenvalerate pesticide and the metabolites 3-phenoxybenzoic acid and 2-(4-chlorophenyl)-3-methyl-butanoic acid, table of quantifications of the pesticide and its metabolites, and chromatograms obtained by HPLC-UV) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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