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Research article

Novel degradation pathway and kinetic analysis for buprofezin removal by newly isolated *Bacillus* sp.

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ABSTRACT

Given the intensive and widespread application of the pesticide, buprofezin, its environmental residues potentially pose a problem; yet little is known about buprofezin's kinetic and metabolic behaviors. In this study, a novel gram-positive strain, designated BF-5, isolated from aerobic activated sludge, was found to be capable of metabolizing buprofezin as its sole energy, carbon, and nitrogen source. Based on its physiological and biochemical characteristics, other aspects of its phenotype, and a phylogenetic analysis, strain BF-5 was identified as *Bacillus* sp. This study investigated the effect of culture conditions on bacterial growth and substrate degradation, such as pH, temperature, initial concentration, different nitrogen source, and additional nitrogen sources as co-substrates. The degradation rate parameters, q_{max}, K_s, K_i and Sm were determined to be 0.6918 h^{-1}, 105.4 mg L^{-1}, 210.5 mg L^{-1}, and 148.95 mg L^{-1} respectively. The capture of unpublished potential metabolites by gas chromatography-mass spectrometry (GC-MS) analysis has led to the proposal of a novel degradation pathway. Taken together, our results clarify buprofezin's biodegradation pathway(s) and highlight the promising potential of strain BF-5 in bioremediation of buprofezin-contaminated environments.

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1. Introduction

Buprofezin (2-*tert*-butylimino-3-isopropyl-5-phenyl-1,3,5-thiadiazinan-4-one) is an insecticide and acaricide with persistent parvicidal action against *Homopterasome Coleoptera* and *Acarina*. In addition, it is effective against *leafhoppers* in rice and potatoes, *whitefly* in citrus plants, cotton and vegetables, *Coccidae*, *Diaspididae* (scale insects) and *Pseudococcidae* (mealybugs) in citrus plants. Its insecticidal actions include both contact and gastrointestinal side effects in humans and other non-target species (Armenta et al., 2002; de Liñán Vicente, 1999). Due to its efficacy against its targets, buprofezin has been widely used in integrated pest management (IPM) programs (Gerling and Sinai, 1994; James, 2004; Nagata, 1986), initiatives that have become a source of contamination of soil, groundwater, rivers, lakes, rainwater, and air (Errami et al., 2013). Hence, buprofezin's frequency poses a potentially serious source of environmental contamination. Although a variety of decontamination methodologies exist, physical and chemical methods have been claimed as the most effective removal methods, however, secondary pollutants and high-cost and energy requirements are inevitable. By contrast, biological decontamination methods are ecologically and economically favorable, especially for the remediation of hazardous waste (Sun et al., 2016). Although numerous studies have attempted to show that natural buprofezin degradation has been largely a function of soil microorganismal action, only a few pure cultures of buprofezin-degrading bacteria have been reported (Liu et al., 2015; Chen et al., 2011; Li et al., 2011, 2012), and buprofezin's microorganismal degradative pathway has not been fully characterized.

In order to develop a technology applicable to buprofezin biodegradation, the present work describes the isolation and characterization of a buprofezin-degrading bacterium, BF-5. Effects of several parameters were investigated, including pH, temperature, and second nitrogen source as co-substrate for bacterial growth and buprofezin degradation by the isolated strain. In addition, a kinetic model of buprofezin degradation and transformation is suggested. Finally, a novel metabolic pathway is proposed for buprofezin's biodegradation by this isolated bacterium, on the basis of identified possible intermediates. This research therefore highlights a significant potential use of pure cultures of

Abbreviations: GC-MS, gas chromatography–mass spectrometry; MM, mineral salts medium; LB, Luria-Bertani; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

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microbial cells for the remediation of buprofezin-contaminated environments.

2. Materials and methods

2.1. Chemicals and media

Buprofezin (99% purity) was purchased from The Pesticide Research Institute (Shanghai, China). High-performance liquid chromatography (HPLC) gradient-grade petroleum ether, methanol, acetone, and ethyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this study were of analytical reagent grade.

Luria-Bertani (LB) medium includes the following, per liter: (10.0 g peptone (Oxoid); 5.0 g yeast extract (Oxoid); 10.0 g NaCl, pH adjusted to 7.0. Mineral salts medium (MM) includes the following, per liter: 1.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, and 1.0 g NaCl, at pH 7.0–7.2). Aliquots of a stock solution of buprofezin (prepared in HPLC- grade acetone) was added to obtain the desired working concentrations. For solid medium, agar powder was added at a concentration of 1.5%, with buprofezin added at an appropriate concentration, where necessary. All media used in this study were prepared using Milli-Q water (>18.2 MΩ) and sterilized by autoclaving at 121 °C for 25 min.

2.2. Strain isolation and characterization

Buprofezin-contaminated soil samples were collected from drainage areas at the site of the Shanghai Dongfeng Chemical Co. Ltd. (Shanghai, China). Five grams of soil sample was inoculated into 100 mL of MM containing 10 mg L^{-1} of buprofezin and incubated at 30 °C with orbital shaking (160 rpm) for 7 days. From this solution, five mL of culture was transferred to another fresh 100 mL of MM amended with 20 mg L^{-1} of buprofezin and incubated under the same conditions just mentioned. This procedure was carried out five times to increase the concentration of buprofezin up to 50 mg L^{-1}. The final enrichment culture that demonstrated buprofezin degradation was serially diluted, with an aliquot of the culture (0.1 mL) spread on MM agar plates supplemented with 50 mg L^{-1} buprofezin and incubated for 1 week at 30 °C. Bacterial colonies surrounded by transparent halos and with different morphologies were picked out, purified by repeated streaking, and tested for their buprofezin-degradation capabilities. One strain that showed the highest degradation efficiency was selected for further investigations and designated BF-5.

The isolated strain was identified based on its morphological, physiological, and biochemical properties according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). The bacterial isolate, BF-5, was further characterized using 16S rRNA gene sequencing methods. Genomic DNA was extracted by high-salt-concentration precipitation (Miller et al., 1988), and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) as described previously (Cai et al., 2013). The nucleotide sequence coding for the 16S rRNA gene of BF-5 was sequenced (Sangon, Shanghai, China) and deposited in the GenBank database under the accession number KT161957. This 16S rRNA sequence was compared to those of type strains of *Bacillus* using the BLAST search function of EzTaxon server version 2.1 (www.eztaxon.org) (Chun et al., 2007). Phylogenetic and molecular evolutionary analyses of strain BF-5 as well as construction of a neighbor joining dendrogram were performed using MEGA version 6.06 (Tamura et al., 2013).

2.3. Degradation analysis and influential factors

Strain BF-5 was precultured in 100 mL of LB medium for 48 h. Cells were harvested by centrifugation at 5000 rpm for 5 min at room temperature and washed three times with sterilized water. The optical density at 600 nm (OD_{600}) was adjusted to 1.0.

In order to supplement MM with buprofezin, a stock solution (10,000 mg L^{-1}) of buprofezin prepared in analytical grade acetone was added to an empty 50-mL Erlenmeyer flask to yield an amount equal to a desired concentration. The residual acetone was evaporated under a stream of air to leave a coat of fine buprofezin crystals at the flask bottom. An appropriate volume of MM was added to the flask to attain the desired working culture. This method was also used during growth studies and degradation kinetics studies.

For all experiments, unless otherwise specified, cells were inoculated at 2% (v/v) into 10 mL MM containing 150 mg L^{-1} buprofezin. As a control, MM inoculated with heat-killed BF-5 cells was maintained under identical conditions. Samples were withdrawn at different times and analyzed for cell biomass (based on the OD_{600} value) and residual buprofezin when necessary. All treatments were performed in triplicate.

Effects of environmental factors on bacterial growth and buprofezin degradation were investigated. The effect of temperature was first examined at seven temperatures: 20, 25, 30, 35, 37, 40 and 45 °C, at pH 8.0. These tests were followed, at the identified optimal temperature by those identifying the effect of varying pH (4.0–11.0, in increments of 1.0 pH unit).

Optimal conditions of 37 °C, pH 8.0, were utilized in assessing tests of different nitrogen sources on buprofezin degradation and bacterial growth. Five nitrogen-containing compounds, 2 g L^{-1} KNO_3, 2 g L^{-1} NH_4Cl, 2 g L^{-1} NH_4NO_3, 2 g L^{-1} $(NH_4)_2SO_4$, and 2 g L^{-1} urea, were chosen for evaluating the effects of additional nitrogen sources as co-substrates. The effects of different concentrations of $(NH_4)_2SO_4$ (1.0 g L^{-1}, 2 g L^{-1}, 5.0 g L^{-1}, and 10 g L^{-1}) were also studied. Buprofezin added as sole carbon and nitrogen source was used as control in this experimental series.

2.4. Determination of buprofezin degradation kinetics

Biodegradation experiments with different initial buprofezin concentrations were performed in 50-mL Erlenmeyer flasks with 10-mL sterile MM containing 60–600 mg L^{-1} buprofezin. The MM was incubated in triplicate at 37 °C in a shaker at 160 rpm with non-inoculated media as controls. The Andrews equation (Eq. (1)) was used to calculate the specific degradation rate (q) at different initial buprofezin concentrations (Chen et al., 2014).

$$q = \frac{q_{\max} S}{S + K_s + (S^2/K_i)} \tag{1}$$

where $q_{\max}$ is the maximum specific buprofezin degradation (per hour), K_i is the substrate inhibition constant (mg L^{-1}), K_s is the half-saturation constant (mg L^{-1}), and S is the substrate concentration (mg L^{-1}).

Although it does not appear as a term in the equation, an important parameter is Sm, the square root of $Ki \times Ks$. Sm represents a critical inhibitor concentration of the substrate that defines a turning point of the degradation rate.

2.5. Chemical analysis

Cell growth was monitored by measuring the absorbance at 600 nm using an SHIMADZU UV–Vis recording spectrophotometer (SHIMADZU Corp., Kyoto, Japan).

Buprofezin was extracted from liquid culture twice using an

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