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A microbial consortium from a biomixture swiftly degrades high concentrations of carbofuran in fluidized-bed reactors

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ABSTRACT

An on-farm biopurification system (BPS) biomixture, with proven capacity to degrade carbofuran (CBF) was used as inoculum for a selective-enrichment process to obtain a bacterial consortium capable of using CBF as a sole source of carbon. Bacterial strains that comprise the consortium were identified as members of the genera *Cupriavidus*, *Achromobacter* and *Pseudomonas*. The consortium was tested in batch bioreactors, with initial CBF concentrations ranging from 50 to 200 mg L^{-1}, in which complete degradation was attained in 16–17 h. Continuous operation of the bioreactor was optimized to achieve conditions of complete removal of up to 100 mg L^{-1} of CBF at a HRT of 23 h by the microbial consortium. Cross-degradation assays showed that the consortium was also capable of degrading high concentrations of other carbamates such as aldicarb, methiocarb and methomyl in batch bioreactors. The use of this consortium for the treatment of CBF and other carbamates at reactor scale represents a potential approach for the removal of these pesticides from agricultural wastewater, as an alternative to BPS.

1. Introduction

Despite the development of new pesticide molecules, carbamates are still some of the most widely used pesticides worldwide. Carbamates are chemically defined as esters of carbamic acid [1]. These compounds are reversible inhibitors of the acetylcholinesterase enzyme, which is in charge of terminating the action of the neurotransmitter acetylcholine in synapses from vertebrates and invertebrates [2]. Carbamates are used in agriculture as insecticides, nematicides, fungicides and herbicides. More than two dozen carbamates are commercially available [3].

The majority of carbamates lack species selectivity, posing a threat to the environment and to human and animal health. Carbamates, such as CBF and aldicarb, are even considered extremely toxic following acute exposure [1]. CBF is a broad spectrum insecticide/acaricide/nematicide that has been banned from agricultural practice in several countries. In spite of this condition, in many other regions it is still used in different formulations to control pests in crops such as sugarcane, sugar beet, corn, coffee, rice, among others. It is relatively soluble in water and has the potential to contaminate different aquatic compartments, including groundwater [1].

Different methods have been developed in order to prevent pesticide contamination of environmental compartments. On-farm biopurification systems (BPS) consist on an excavation or container which accommodates a biologically active matrix (biomixture). This system is intended to collect, adsorb and degrade spills of pesticides and their diluted residues. The biomixture is normally composed of soil, a humified organic substrate, and lignocellulosic materials mixed at different ratios [4].

Pesticide depuration performance of different BPS is determined in large part by the microbial communities they harbor; consequently, careful investigation of these communities and their dynamics will allow the development of different strategies for BPS optimization [5]. BPS biomixtures with efficient CBF-degrading capacity have been recently obtained [6]; however, a detailed study of the CBF-degrading bacterial community that they host may allow the modification of these systems for increased efficiency on the removal of this or other carbamates, or the design of novel strategies and systems for pesticide residues elimination.

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Microorganisms that carry out a defined process (such as xenobiotic degradation) may sometimes be effective only when they are present in association with other organisms in a consortium. Examples of this behavior have been observed for the degradation of compounds such as pesticides, PCBs, and other halogenated organic compounds [7].

The aim of this study was to obtain, through enrichment culture, a CBF-degrading microbial consortium from a BPS biomixture with proven CBF-degrading capabilities and to evaluate its depuration capacity in different bioreactor configurations. Knowledge regarding the consortium's behavior in these conditions will allow for this microbial association to be used as an inoculum in subsequent *in situ* or *ex situ* applications.

2. Materials and methods

2.1. Chemical standards

Analytical standards CBF (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-ylmethylcarbamate, >99% purity), 3-hydroxycarbofuran (99.5%) 3-ketocarbofuran (99.5%), aldicarb (99%), methomyl (99.5%) and methiocarb (99%) were obtained from Chem Service (West Chester, PA).

2.2. Selection of a CBF-degrading microbial consortium

A selective-enrichment procedure [8] was performed to obtain a CBF-degrading microbial consortium from a BPS biomixture (coconut fiber, garden compost and soil pre-exposed to CBF (from an onion/beet field) at volumetric ratio 2:1:1) was employed as the source of degrading biomass [6]. The biomixture was aged (12 months) and spiked with CBF (50 mg kg^{-1}) four months prior to use; six grams were then inoculated in a flask containing 300 mL sterile Bushnell-Haas (BH) broth (pH = 6.5) [9] supplemented with CBF (100 mg kg^{-1}) as the only C-source. This system was incubated in orbital shaking (28 °C, 160 rpm) for 72 h (first passage of the enrichment culture; P1). After incubation, 3 mL of this suspension were transferred to 300 mL of fresh medium and incubated as previously described, for 48 h (P2). A third and a fourth passages (P3 and P4) were carried out similarly to obtain the CBF-degrading microbial consortium.

Individual strains that make up the CBF-degrading microbial community were isolated by inoculating serial dilutions of the P4 bacterial suspension on replicate tripticase soy agar (TSA) plates and BH agar plates supplemented with CBF (100 mg L^{-1}).

2.3. Degradation of CBF in a fluidized-bed reactor

A fluidized-bed bioreactor as described by Blánquez et al. [10] was employed to degrade CBF. The working volume was set at 1500 mL. Fluidized conditions were maintained by air pulses generated by an electrovalve (alternately open for 1 s and shut for 4 s). Initial pH value was 6.5, but it was not controlled during operation. The system was kept at 25 °C.

2.3.1. Batch operation

The inoculum was prepared in a flask containing 135 mL BH-medium, 6 mL from the selective-enrichment culture (P4) and CBF solution to complete 150 mL at a concentration of 25 mg L^{-1} CBF. The culture was shaken at 130 rpm for 3 d and employed as inoculum in the batch reactor. The reactor vessel was initially loaded with 1.35 L of non-sterile BH-medium, 150 mL inoculum and a CBF concentration of 30 mg L^{-1}. After total consumption of CBF, subsequent batch bioreactors were prepared at increasing initial CBF loads: 50, 100, 150 and 200 mg L^{-1}. In order to prepare each new batch, 150 mL from the previous batch were used as inoculum to 1.35 L non-sterile BH-medium.

2.3.2. Continuous operation

The non-sterilized bioreactor was initially loaded with 1.35 L BH-medium supplemented with CBF at 30 mg L^{-1} and 150 mL inoculum prepared as described in Section 2.3.1. The reactor operated in batch mode until complete consumption of CBF (28 h) and then the continuous stage was switched on with at a hydraulic residence time (HRT) of 48 h and a CBF load of 30 mg L^{-1}. The HRT and the CBF load were changed according to the system's performance during the experiment in order to maximize the degradation efficiency (maximum CBF load: 100 mg L^{-1}). The influent containing CFN was not sterilized; CFN concentration in the feed was frequently monitored.

2.4. Cross degradation of carbamates

The microbial population obtained in P4 was used to prepare the inoculum for degradation assays of different carbamates (CBF, aldicarb, methiocarb and methomyl). Fifty microliters were inoculated in 50 mL CBF solution (200 mg L^{-1}) in sterile BH-broth. After incubation (7 d, 28 °C), 5 mL were inoculated in 45 mL of fresh medium, and incubated again for 72 h. This last suspension was used as inoculum. Total bacterial population in the inoculum (4.5×10^7 CFU mL^{-1}) was determined by total heterotrophic counts in TSA plates.

Systems consisting of triplicate flasks for each carbamate were prepared and contained 2.5 mL of the inoculum and 47.5 mL of carbamate solution in BH-broth. Final concentrations were 100 mg L^{-1} for aldicarb, CBF and methomyl; and 10 mg L^{-1} for methiocarb (due to its lower solubility). Duplicate abiotic controls for each carbamate were prepared by replacing the inoculum with sterile BH-broth. All the systems were incubated in orbital shaking (28 °C, 130 rpm) in the dark for 7 or 14 d. At selected time points a 0.5 mL aliquot was withdrawn from each system and the remaining carbamate concentration was determined by HPLC/MS (Section 2.5).

2.5. Analysis of CBF and other carbamates

Centrifuged samples (3000 rpm) from the reactors and enrichment cultures were analyzed on a Dionex Ultimate 3000 HPLC (Dionex Corporation, CA) equipped with a UV-detector. Chromatographic separation was achieved in a GraceSmart RP18 column (250 mm × 4.6 mm, 5 μm), with a mobile phase of methanol-water (60:40), added isocratically at 1 mL min^{-1} [11]. Detection of CBF was done at 215 nm.

Analyses of transformation products from CBF (3-hydroxycarbofuran and 3-ketocarbofuran) were performed using an HPLC system (1200 series, Agilent Technologies, CA) coupled to a 6130 quadrupole mass spectrometer with a G1978 B multimode ion source (ESI and atmospheric pressure chemical ionization, APCI), as previously described [12]. LOD and LOQ were 0.01 mg L^{-1} and 0.052 mg L^{-1} (3-hydroxycarbofuran); 0.019 mg L^{-1} and 0.037 mg L^{-1} (3-ketocarbofuran).

Analyses of CBF, aldicarb, methiocarb and methomyl in cross degradation assays were performed directly from centrifuged samples by LC–MS/MS using ultra high performance liquid chromatography (UPLC-1290 Infinity LC, Agilent Technologies, CA) coupled to a triple quadrupole mass spectrometer (model 6460). Chromatographic separation was done at 40 °C by injecting 6 μL samples in a Poroshell 120 EC-C18 column (100 mm × 2.1 mm i.d., particle size 2.7 μm), and using acidified water (formic acid 0.1% v/v, A) and acidified methanol (formic acid 0.1% v/v, B) as mobile phases. The mobile phase flow was 0.3 mL min^{-1} at the following conditions: 30% B for 3 min, followed by a 15 min linear gradient