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Community structure of a propanil-degrading consortium and the metabolic pathway of *Microbacterium* sp. strain T4-7

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ABSTRACT

A propanil-degrading consortium T4 was enriched from propanil polluted soil using propanil as the sole carbon and energy source. The community structure of consortium T4 was analyzed using restriction fragment length polymorphism (RFLP). Strain T4-7 isolated from consortium T4 was identified as a *Microbacterium* sp. based on physiological, biochemical characteristics and 16S rRNA gene analysis. Strain T4-7 was capable of transforming 98.94% of 100 mg L^{-1} propanil to a stable product within 10 h; the metabolite was identified as 3,4-dichloroaniline (DCA) by GC–MS. Strain T4-7 most likely converted propanil via cleavage of the amide bond to DCA and propionate and could utilize propionate as its sole carbon source for growth, but could not further degrade DCA. The propanil hydrolase from the crude enzyme preparation from strain T4-7 showed an optimal reaction temperature of 45 °C and pH of 7.0, with broad stability from 4 to 30 °C and pH 6.0–10.0. Moreover, strain T4-7 was capable of transforming 2-chlor-*N*-(2-ethyl-6-methylbenzene) acetamide (CMEPA, the intermetabolite of acetochlor) to 2,6-methyethyaniline (MEA) based on GC–MS. The enzymatic properties for CMEPA degradation were consistent with propanil. The results suggested that propanil and CMEPA may be hydrolyzed by the same amidase.

1. Introduction

Propanil (3,4-dichloropropananilide) belongs to the class of phenyl amides and is ranked in the top 20 pesticides used in the USA (Roberts et al., 2009). It is a broad spectrum, post-emergent herbicide that is widely applied in rice paddies (Santos et al., 1998). Propanil inhibits photosynthetic electron transport in broadleaf weeds producing leaf chlorosis and subsequent necrosis (Tomlin, 2009). The application of propanil in agriculture pollutes large amounts of irrigation water, which can overflow from paddy fields through the soil and contaminate surface and ground water resources (Dabrowski et al., 2002). The highest concentration of propanil reached 3600 μg L^{-1} in agricultural water (Primel et al., 2007). Notably, the allowable concentration for discharge into the water environment is 0.1 μg L^{-1} (Pesticides Framework Directive 2009/128/EC).

Propanil is a type of agricultural chemicals that exhibits toxicity for certain non-target organisms; this finding has been confirmed by a number of studies (Monteiro et al., 1996; Marques et al., 2015). Villarroel et al. (2013) investigated that exposure of *Daphnia magna* to sublethal propanil concentrations and found that they caused a decrease in its energy reserves. Pereira et al. (2007) reported that propanil was toxic to the non-target zooplankton *Daphnia* spp. at low concentrations, particularly following long-term exposure. Additionally, propanil has well-known immunotoxic effects on various compartments of the immune system (Moore et al., 1998). Sancho et al. (2009) revealed that propanil affected the intermediary metabolism of *Anguilla anguilla*; its liver TPs and g-GT activity

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decreased after propanil exposure, while its AlAT and LDH increased. Moreover, a longer recovery period was necessary to re-establish eel physiology. Cakici and Akat (2013) found that propanil induced dose-dependent histopathological changes in liver and kidney tissues and that propanil exposure might cause harmful effects to non-target organisms including humans.

In view of the widespread use of propanil and its toxicity to non-target organisms, many researchers have focused on the removal and degradation of propanil in the environment, especially via microbial degradation. Ayranci and Hoda (2004) researched the adsorption of propanil in aqueous solutions using high area activated carbon-cloth. Propanil is primarily converted to 3,4-dichloroaniline (DCA) and propionate in the natural environment, and DCA also has adverse health and ecotoxicity effects (Carvalho et al., 2010). The microbial ecology and kinetics of propanil-degrading enrichment from soils were investigated in a sequencing batch reactor (SBR) in an attempt to enhance the removal of propanil and DCA (Carvalho et al., 2010). Zhang et al., (2011) characterized the metabolic pathway of propanil degradation by *Sphingomonas* sp. Y57 and cloned the propanil hydrolase gene *prpH*. Zhang et al. (2012) isolated a *Catellibacterium nanjingense* sp. nov. Y12^T from activated sludge of a wastewater biotreatment facility; this bacterium was able to degrade approximately 90% of added propanil (100 mg L^{-1}) within 3 days of incubation. Oehmen et al. (2013) reported that propionate could stimulate the specific degradation rates of both propanil and DCA. Additionally, several bacteria and fungi capable of hydrolyzing propanil to DCA and using propionate as their sole carbon source for growth have been isolated from different environments (Lanzilotta and Pramer, 1970; Dahchour et al., 1986; Reichel et al., 1991; Correa and Steen, 1995; Zablotowicz et al., 2001).

In this study, a propanil-degrading consortium T4 was obtained by enrichment with propanil-polluted soil, and the T4 community structure was analyzed with RFLP. Strain T4-7, which could transform propanil and CMEPA, was isolated and identified from the consortium T4. The metabolic mechanism and degradation characteristics of propanil and CMEPA by the bacteria and the crude enzyme of strain T4-7 were described in this paper.

2. Materials and methods

2.1. Chemicals and media

Propanil (95.5% purity) and CMEPA (98% purity) were purchased from Jiangsu Jiannong Co., Ltd (Nanjing, China) and Qingdao Vochem Co., Ltd (Qingdao, China), respectively. Minimal salts medium (MSM, g L^{-1}, pH 7.0) consisted of the following components: K_2HPO_4 1.5, KH_2PO_4 0.5, NH_4NO_3 1.0, $MgSO_4 \cdot 7H_2O$ 0.1 and NaCl 1.0 (reagents for the analysis of purity contained trace elements). Luria-Bertani agar plates (LB, g L^{-1}, pH 7.0) consisted of the following components: tryptone 10.0, yeast extract 5.0, NaCl 10.0 and agar 18.0. All other chemicals used in this study were analytical grade or higher purity. Medium was sterilized at 121 °C for 20 min. The stock solutions of propanil and CMEPA (1%, w/v) were prepared in methanol and sterilized by membrane filtration.

2.2. Enrichment and domestication of propanil-degrading consortium T-4

Soil samples were collected from the surface layer (0–10 cm) of a nearby region with a high concentration of propanil as a waste product (Zhenjiang, Jiangsu Province, China). Five g of soil was inoculated into an Erlenmeyer flask (250 mL) containing 100 mL of MSM supplemented with 25 mg L^{-1} propanil as the sole carbon source and incubated at 30 °C and 180 rpm for 7 days. The extract was examined at 200–350 nm with an ultraviolet visible spectrophotometer (UV-2450, SHIMADZU, Japan). Ten mL of enrichment cultures showing degradation of propanil were transferred to 100 mL of fresh MSM with the initial concentration of propanil gradually increased to 100 mg L^{-1} and cultivated under the conditions described above. The enrichment consortium that exhibited an efficient degradation capability was designated T4.

2.3. Bacterial diversity analysis of consortium T4

The total bacterial cells of consortium T4 were harvested by centrifugation at 4 °C and 6000 rpm. Total genomic DNA was extracted by high-salt precipitation (Miller et al., 1988). The primer pair 27F/1492R was used to amplify 16S rRNA (Zheng et al., 2015). The purified PCR fragments were ligated into the linearized vector pMD19^T (TaKaRa Biotechnology Co., Ltd, Dalian, China) and transformed into *Escherichia coli* DH5α to construct 16S rRNA libraries (Dong et al., 2015). The 16S rRNA gene of the positive clones was amplified with the sequencing primers M13-47 and RV-M (Jia et al., 2006) in the vector pMD19^T. PCR products were digested by the restriction enzymes *Rsa* I (GT'AC) and *Hha* I (C'CGG) and analyzed by PAGE (Muckian et al., 2009; Farrelly et al., 1995; Hansen et al., 1998). The 16S rRNA genes with different restriction profiles were submitted to the Genscript Biotechnology Co., Ltd (Nanjing, China) for sequencing. The results were analyzed using the BLASTN search tools (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al., 1990).

2.4. Isolation and identification of the strain in consortium T4

The stable enrichment culture was serially diluted and spread on MSM and LB plates containing propanil to obtain more microbial species. After incubation at 30 °C for 3 days, colonies with different morphologies were purified three times on LB plates and chosen for testing in liquid MSM plus propanil to determine their degradation capabilities by high-performance liquid chromatography (HPLC) as described below. After 2 days, 4 mL of the growth medium was processed to detect propanil. One pure culture with the highest propanil-degradation efficiency was named T4-7 and selected for further study.

Phenotypic and biochemical characterization of strain T4-7 was performed according to *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994). The bacteria were identified by sequencing their partial 16S rRNA genes with the primer pair 27f and 1492r as described above. Alignment of the partial 16S rRNA sequences was performed with sequences deposited in the GenBank database, and phylogenetic analysis was performed with the software MEGA version 6.0 (Tamura et al., 2007). Distances were calculated using the Kimura two-parameter distance model. Phylogenetic trees were generated using the neighbor-joining method and evaluated by bootstrap analyses based on 1000 resamplings.

2.5. Degradation of propanil and CMEPA by strain T4-7

Strain T4-7 was pre-cultured to the stationary phase in liquid LB medium. The cells were harvested at 6000 rpm for 5 min and washed with sterilized MSM. Then the optical density of the cells at 600 nm was adjusted to 1.0 (corresponding to 1.0×10^8 cells mL^{-1}). The suspension was used as a seed inoculum for the biodegradation experiments described below.

To detect the growth of strain T4-7 and the dynamics of propanil and CMEPA degradation, a cell density of 1×10^7–1×10^8 cfu mL^{-1} was inoculated into 100 mL of MSM containing 100 mg L^{-1} of propanil or CMEPA as the sole carbon source. The cultures were incubated aerobically at 30 °C and 180 rpm on a rotary shaker. At regular intervals, 4 mL samples were taken from the cultures and