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# Characterization of an arylamidase from a newly isolated propanil-transforming strain of *Ochrobactrum* sp. PP-2

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ABSTRACT

Propanil, one of the most extensively used post-emergent contact herbicides, has also been reported to have adverse effect on environmental safety. A bacterial strain of *Ochrobactrum* sp. PP-2, which was capable of transforming propanil, was isolated from a propanil-contaminated soil collected from a chemical factory. An arylamidase gene *mah* responsible for transforming propanil to 3,4-dichloroaniline (3,4-DCA) was cloned from strain PP-2 by shotgun method and subsequently confirmed by function expression. The arylamidase Mah shares low amino acid sequence identity (27–50%) with other biochemically characterized amidases and shows less than 30% identities to other reported propanil hydrolytic enzymes. Mah was most active at pH 8 and 35 °C. Mah had a remarkable activity toward propanil ($K_m = 6.3 \pm 1.2\,\mu M$), showing the highest affinity efficiency for propanil as compared with other reported propanil hydrolytic enzymes. Our study also provides a new arylamidase for the hydrolysis of propanil.

## 1. Introduction

The use of herbicides in the agricultural system contributes greatly to the food production increase and labor source saving. However, extensive use of herbicides can also cause environmental pollution and threat human health due to their improper application. Therefore, the environmental fate of herbicides has caused great concern and removal of these pollutants from the environment is desirable (Zhang et al., 2011, 2012; Gu et al., 2013; Li et al., 2017; Pan et al., 2018; Yang et al., 2018;). Microbial degradation is considered an important mechanism in the dissipation of herbicides in the environment (Huang et al., 2017).

Propanil (3,4-dichloropropananilide) belongs to the class of phenyl amide herbicides and is ranked in the top 20 pesticides used in the USA (Roberts et al., 2009; Hou et al., 2015). As one of the most widely used post-emergent contact herbicides, propanil is applied worldwide (Santos et al., 1998; Primel et al., 2007). The functional mechanism of propanil is to inhibit the photosynthetic electron transport in broadleaf weeds, leading leaf chlorosis and subsequent necrosis (Tomlin, 2009). The application of propanil in agriculture system leads to the pollution of irrigation water, and then the surface and ground water (Dabrowski et al., 2002; Hou et al., 2015). Propanil has been reported to cause acute toxicity in a wide range of aquatic species (Crossland, 1990; Pothuluri et al., 1991; Mitsou et al., 2006; Darren et al., 2009). Propanil is also an important cause of death from acute poisoning, of which methemoglobinemia is an important symptom (Darren et al., 2009). Propanil has been detected in concentrations up to 3600 $\mu g\,L^{-1}$ in irrigation water, while the allowable concentration for propanil discharged into the aquatic environment is 0.1 $\mu g\,L^{-1}$ (Pesticides Framework Directive 2009/128/EC) (Primel et al., 2007). 3,4-Dichloroaniline (3,4-DCA) is usually the major metabolite produced from propanil transformation by microorganisms. Though 3,4-DCA still has adverse health and ecotoxicity effects, it shows lower toxicity against fish and mammals compared to its parent compound propanil (Salazar et al., 2008).

A considerable number of propanil-degrading bacteria have been isolated and characterized from different environments. Most of these isolates usually accumulate 3,4-DCA during the transformation of propanil, such as *Paracoccus* sp. FLN-7 (Zhang et al., 2012), *Paracoccus* sp. M-1 (Shen et al., 2012), *Sphingobium quisquiliarum* DC-2 (Li et al., 2013) and *Microbacterium* sp. T4–7 (Hou et al., 2015). Few strains have been shown to capable of mineralizing propanil. For example, *Sphingomonas* sp. strain Y57 can mineralize propanil and it harbors the hydrolase gene *prpH*, the aniline oxygenase gene cluster and the following catechol meta-cleavage pathway gene cluster (Zhang et al., 2011; Yan et al., 2016). Among all these reported degraders, most of them possess low transforming efficiency for propanil. Up to now, no *Ochrobactrum* species has been reported with the propanil transforming ability.

In this study, a highly effective propanil transforming strain of

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*Ochrobactrum* sp. PP-2 was isolated and characterized. A new amidase gene *mah* responsible for the initial transformation of propanil to 3,4-DCA was cloned by short-gun method using the propanil analogue 4-acetaminophenol as the screening substrate. The catalytic properties of Mah were systematically investigated, showing that the new arylamidase Mah is a good candidate for the hydrolysis of propanil.

## 2. Materials and methods

### 2.1. Chemicals and media

Unless otherwise stated, chemicals were purchased from J&K Chemical Co., Ltd. (Shanghai, China). The purities of all standard compounds exceed 98%. Molecular biology reagents were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were purchased from the Shanghai Chemical Reagent Co., Ltd., China. Luria-Bertani medium (LB) contains the following ($g\ L^{-1}$) at pH 7.0: tryptone, 10.0; NaCl, 5.0; yeast extract, 5.0. Mineral salt medium (MSM) contains the following ($g\ L^{-1}$) at pH 7.0: NaCl, 1.0; $NH_4NO_3$, 1.0; $K_2HPO_4$, 1.5; $KH_2PO_4$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2.

### 2.2. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* strains and other bacterial strains were grown in LB broth at 37 °C and 30 °C, respectively. Antibiotics were added as necessary at the following concentrations ($mg\ L^{-1}$): ampicillin (Amp), 100; kanamycin (Km), 50.

### 2.3. Isolation and identification of the propanil-transforming strain

Propanil-transforming strains were isolated by the conventional enrichment culture technique from a propanil-contaminated soil sample (Zhang et al., 2015a), which was collected from a disused pesticide factory located in Jiangsu, China. Approximately 5.0 g of soil sample was added to a 250-mL Erlenmeyer flask containing 100 mL MSM supplemented with 0.2 mM propanil and cultured at 30 °C on a rotary shaker at 160 rpm for approximately 5 days. Once the enrichment had stable propanil-transforming ability after several series of transfer, the culture was plated onto LB agar containing 0.2 mM propanil. Individual colonies of distinct morphology were selected and the propanil-transforming abilities were evaluated in liquid medium by the HPLC method as described below. One strain designated PP-2, which showed the highest transforming ability for propanil was selected. Strain PP-2 was identified based on their morphological, physiological and biochemical properties as described in *Bergey's Manual of Determinative Bacteriology* as well as its 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified with the universal primer pair 27f/1492r (Zhang et al., 2015b).

### 2.4. Transformation of propanil by strain PP-2

Cells of strain PP-2 were pre-cultured in LB medium and harvested by centrifugation at 6000 × *g* for 5 min. Cell pellets were washed twice with sterilized MSM and then inoculated into 100 mL MSM containing 0.2 mM propanil at the final concentration of $OD_{600} = 0.2$. The culture was incubated in a rotary shaker at 160 rpm at 30 °C. Samples of the suspension were taken at regular intervals to determine the concentration of propanil and the produced metabolite. Cell growth was monitored by measuring at $OD_{600}$ using a spectrophotometer (UV-2450, SHIMADZU, Japan). All these experiments were performed in triplicate.

### 2.5. Determination of propanil in liquid medium

The propanil in liquid medium was extracted with an equal volume of dichloromethane. The extract was dried by over anhydrous $Na_2SO_4$ and evaporated using a vacuum rotary evaporator at room temperature. The residual was dissolved in 500 μL of methanol and then detected by reverse-phase HPLC (600 controller, Rheodyne 7725i manual injector and 2487 Dual λ Absorbance Detector; Waters Co., Milford, MA). HPLC analyses were performed with a separation column (internal diameter, 4.6 mm; length, 250 mm) filled with Kromasil 100–5-$C_{18}$. The mobile phase was acetonitrile-water (65:35, vol/vol) with the flow rate of $1.0\ mL\ min^{-1}$. The injection volume was 20 μL and detected at the wavelength of 210 nm and 250 nm. The concentration was determined from the peak area ratio relative to its standard calibration curve.

### 2.6. Cloning of the propanil-hydrolyzing amidase gene (mah)

Genomic DNA of strain PP-2 was extracted as described by Sambrook and Russell (Sambrook and Russell, 2001). Genomic DNA from strain PP-2 was partially digested by *Sau*3AI and then the resulting 4–9 kb DNA fragments were cloned into *Bam*HI/BAP digested pUC118 ($Amp^r$, cloning vector, Takara) and introduced into *E. coli* DH5α. The library was plated onto LB agar containing 0.5 mM 4-acetaminophenol and $100\ mg\ L^{-1}$ ampicillin. The plates were incubated at 37 °C for 12 h and then stored at room temperature for 96 h. Brown colonies, due to the production of the brown compound 4-aminophenol from the hydrolysis of 4-acetaminophenol by potential amidases, were selected (Zhang et al., 2012). The selected recombinants were further tested for their abilities to hydrolyze propanil using HPLC analysis. Inserts of positive clones were sequenced by Genscript Biotechnology Co., Ltd. Nucleotide sequence and deduced amino acid sequence analyses were performed using Omiga software and ORF finder on the NCBI website. BlastN and BlastP were used for the nucleotide sequence and deduced amino acid sequence identity searches (www.ncbi.nlm.nih.gov/Blast), respectively.

### 2.7. Gene expression and purification of the recombinant Mah

To express Mah in *E. coli* using the pET29a expression system (Novagen), *mah* gene was amplified from the genomic DNA of strain PP-2 using PrimeSTAR GXL DNA polymerase (Takara). The primers for *mah* amplification were as follows: forward, 5′-accgaattcaacagaagaccaacccg-3′ (underlined is EcoR I site) and reverse, 5′-atactcgagctgactgccaaggggac-3′ (underlined is *Xho*I site). The PCR product was digested with *Eco*RI and *Xho*I, ligated into pET-29a (+) (Novagen) to generate the recombinant plasmid pET-mah, and then transformed into *E. coli* BL21 (DE3). The positive transformants were selected on LB agar containing $50\ mg\ L^{-1}$Km. The selected cells were cultured in LB broth to reach $OD_{600} = 0.6$. Then, isopropyl-*β*-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to induce the expression of Mah. After 5 h incubation at 30 °C, the cells were harvested, washed, and resuspended in Tris-HCl buffer (100 mM, pH 8.0) at 4 °C and lysed by sonication on ice. The recombinant Mah was purified with $Ni^{2+}$-NTA resin (Janknecht et al., 1991). Fractions containing recombinant Mah were pooled and concentrated using an Amicon ultrafiltration tube after dialyzed against 100 mM Tris-HCl buffer overnight at 4 °C to remove imidazole. All purification procedures were performed at 4 °C and monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bradford method (Bradford, 1976).

### 2.8. Substrate spectrum of Mah and metabolite identification

The substrate spectrum of Mah was determined by using the purified Mah extracted from *E. coli* BL21 (DE3) (pET-mah). Crude extracts from *E. coli* BL21 (DE3) harboring the empty vector were used as the negative controls. For substrate spectrum determination, 0.5 mL purified Mah was incubated with 0.2 mM of different substrates (structural analogues such as propanil, 4-acetaminophenol, linuron, diuron,