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Short Communication

Degradation of acetochlor by consortium of two bacterial strains and cloning of a novel amidase gene involved in acetochlor-degrading pathway

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HIGHLIGHTS

- Consortium comprising strains DC-2 and DE-13 could degrade acetochlor completely.
- A new biodegradation pathway of acetochlor was examined in bacterium.
- The *cmeH* gene encoding amidase involved in degradation pathway was firstly cloned.
- CmeH hydrolyzed CMEPA, propanil, fenoxaprop-p-ethyl and clodinafop-propargyl.

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ABSTRACT

Two bacterial strains *Sphingobium quisquiliarum* DC-2 and *Sphingobium baderi* DE-13 were isolated from activated sludge. Acetochlor was transformed by *S. quisquiliarum* DC-2 to a transitory intermediate 2-chloro-*N*-(2-methyl-6-ethylphenyl)acetamide (CMEPA), which was further transformed to 2-methyl-6-ethylaniline (MEA), and MEA could not be degraded by strain DC-2. *S. baderi* DE-13, incapable of degrading acetochlor, showed capability of degrading MEA to an intermediate 2-methyl-6-ethylaminophenol (MEAOH). MEAOH was further transformed to 2-methyl-6-ethylbenzoquinoneimine (MEBQI), which was mineralized by strain DE-13. A gene, *cmeH*, encoding an amidase that catalyzed the amide bond cleavage of CMEPA was cloned from strain DC-2. CmeH was expressed in *Escherichia coli* BL21 and homogenously purified using Ni-nitrilotriacetic acid affinity. CmeH efficiently hydrolyzed CMEPA and other important herbicide, such as propanil, fenoxaprop-p-ethyl and clodinafop-propargyl.

1. Introduction

Acetochlor has been widely used throughout the world as an herbicide, particularly in China. It is used for control of most annual grasses and certain broadleaf weeds (Foley et al., 2008). Residues of this herbicide have been frequently detected in soil, surface water and ground water (Dictor et al., 2008; Newcombe et al., 2005). The USEPA has classified acetochlor as a B-2 carcinogen and a probable human carcinogen (Xiao et al., 2006). Moreover, acetochlor exhibits genotoxicity, and this chemical is a suspected endocrine disruptor (Cai et al., 2007). There is great concern about the behavior and fate of acetochlor and its degradation metabolites in the environment. Several studies reported biological degradation of acetochlor resulted in the formation of two major metabolites, ethanesulphonic acid and oxanilic acid (Hladik et al., 2005). Ye found two other degradation products, hydroxyacetochlor and MEA in soils (Ye et al., 2002). Xu isolated *Pseudomonas oleovorans* that could degrade acetochlor, and found the main plausible degrading pathways involved dechlorination, hydroxylation and dehydrogenation (Xu et al., 2006). In former study, environment pollution could be degraded by two microbes (Miranda et al., 2013), but there was no report about acetochlor-degrading by consortium of two bacterial strains. The objective of this study was to isolate bacterial strains that can degrade acetochlor more effective. Experiments were also conducted to identify metabolic intermediates, and clone functional gene involved in acetochlor-degrading pathway.

2. Methods

2.1. Chemicals and medium

Acetochlor (99% purity), MEA (98% purity) were purchased from J&K Scientific Ltd. (Shanghai, China). CMEPA (98% purity) were purchased from Syntechem Co., Ltd. (Jiangsu, China). The mineral salts

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medium (MSM) and Luria–Bertani (LB) medium were used in this study (Sun et al., 2012).

2.2. Enrichment and isolation

Activated sludge was collected from an herbicide-manufacturing factory in the city of Kunshan, China. Approximately 10.0 g of sample was added to an Erlenmeyer flask (250 mL) containing 100 mL of MSM medium and acetochlor (50 mg L^{-1}). After incubation for 7 d, approximately 10 mL of enrichment culture was transferred into 100 mL of fresh MSM medium containing 50 mg L^{-1} acetochlor. The rate of acetochlor removal was determined using HPLC after the fifth transfer. The enrichment that was able to degrade acetochlor was serially diluted and spread onto MSM agar plates containing 100 mg L^{-1} acetochlor. After incubation at 30 °C, different microbial colonies appeared on plates were purified and tested acetochlor-degrading ability. Strains hold the degrading ability were identified with the methods described previously (Sun et al., 2012).

2.3. Biodegradation of acetochlor and MEA by isolated strains

Degradation of acetochlor and MEA (150 mg L^{-1}) was studied in 100 mL of sterilized MSM inoculated with 1 mL of an initial culture containing each isolated strain and their combinations in 250 mL Erlenmeyer flasks. All cultures were incubated at 30 °C and 160 rpm on a rotary shaker. At regular time intervals, sample aliquots were collected, and the concentration of the herbicide was determined by HPLC.

2.4. Chemical analysis

One milliliter of the medium was extracted with 4 mL of dichloromethane. The dichloromethane phase was dried over anhydrous Na_2SO_4, and removed using nitrogen. The residues were dissolved in 1 mL of methanol. An aliquot of the solution (20 μL) was injected into an HPLC system to determine the concentration of acetochlor. The HPLC system equipped with a Zorbax C-18 ODS Spherex column (internal diameter, 4.6 mm; length, 25 cm; Agilent). The mobile phase was methanol:water (80:20, vol vol^{-1}), and the flow rate was 0.8 mL min^{-1}. Detection of acetochlor was performed at 230 nm. The metabolites formed during degradation were identified by gas chromatography mass spectrometry (GC–MS) according to the methods described by Zhang et al. (2011).

2.5. Cloning of CMEPA-hydrolyzing amidase gene

The genomic DNA library of strain DC-2 was constructed and plated onto LB plates containing 100 mg L^{-1} ampicillin. The plates were incubated at 37 °C for approximately 12 h. The colonies were numbered serially, transferred to 96-pore plate, which was supplied with 200 μL MSMG medium containing 120 mg L^{-1} CMEPA and 100 mg L^{-1} ampicillin per pore. The functional clone holding CMEPA-hydrolyzing amidase gene screening method based on the principle that MEA could react with aminopyrine in the present of $K_3Fe(CN)_6$ to form red product in slightly acid buffer. After the plates were incubated at 37 °C for approximately 20 h, 100 μL sodium acetate-acetate acid buffer (pH 5.9), 30 μL aminopyrine solution (0.1 mol L^{-1}) and 40 μL $K_3Fe(CN)_6$ solution (0.1 mol L^{-1}) were inoculated into each pore. After the plates were incubated at 30 °C for approximately 10 min, the positive colony that showed red color, which was the indicator of the hydrolysis of CMEPA to MEA, were screened. The selected recombinants were further tested for the abilities to hydrolyze CMEPA using HPLC analysis. Inserts of positive clones were sequenced by Invitrogen Biotechnology Co., Ltd. (Shanghai, China).

2.6. Gene expression and purification of the recombinant enzyme

The primers for *cmeH* (restriction sites are underlined) were as follows: forward, 5′-GGAATTCCATATGGGTACTTCACCCCAG-3′ (*Nde*I, corresponding to sites 1–15 of the *cmeH* gene); reverse, 5′-CCGGAATTCGGCGCCCTTGAACCAC-3′ (*Eco*RI, corresponding to sites 884–900 of the *cmeH* gene). The PCR product was digested with *Nde*I and *Eco*RI, ligated into pET-29a to generate the recombinant plasmid pET-*cmeH*, and then transformed into *Escherichia coli* BL21 (DE3). The induction and purification of the recombinant *cmeH* were carried out according to the methods described by Zhang et al. (2012).

The enzymatic activities were determined in 2 mL of 50 mM sodium phosphate buffer (pH 7.5) containing 200 μM each substrate at 30 °C for 10 min. The reactions were initiated by the addition of purified CmeH (80 mg mL^{-1}) to a final concentration of 0.4 mg mL^{-1} for CMEPA, propanil, fenoxaprop-p-ethyl and clodinafop-propargyl. The reactions were terminated by adding 2 mL of dichloromethane and centrifuged at 12,000*g* for 10 min, and the supernatants were filtered through a 0.2 μm-pore-size filter. The substrate concentrations were determined by HPLC or GC analysis. One unit of enzyme activity was defined as the amount of enzyme required to hydrolysis of 1 μmol of substrate per min. The kinetic values were obtained from the Hanes–Woolf equation.

3. Results and discussion

3.1. Isolation and identification of strains

After several rounds of transfers, one colony, which could degrade acetochlor completed, had been isolated from colonies appeared on MSM agar plates containing 100 mg L^{-1} acetochlor. After purified by streaking on LB plate, one yellow strain, designed as DC-2, appeared at the 2nd day from this colony. After incubation for 3 d, another white strain designed as DE-13 appeared from the same colony. The consortium comprising strains DC-2 and DE-13 could degrade acetochlor completed. Two strains were gram negative, and the cells were non-motile, catalase and oxidase positive. The phylogenetic analysis based on 16S rRNA genes revealed that sequence of DC-2 (KC567291) was most closely related to that of *Sphingobium quisquiliarum* $P25^T$ (100.00%), and sequence of DE-13 (KC567292) was most closely related to that of *Sphingobium baderi* $LL03^T$ (98.89%) (Fig. A.1). Based on phenotypic and physiological features, 16S rRNA sequences analysis, strain DC-2 and DE-13 were identified as *S. quisquiliarum* and *S. baderi*, respectively.

3.2. Biodegradation of acetochlor and MEA by isolated strains

Acetochlor was metabolized in the culture of *S. quisquiliarum* DC-2 with acetochlor (150 mg L^{-1}) as the sole source of carbon, and complete transformation of acetochlor was achieved within 16 h. One degradation intermediate (product A) was observed at 2 h, and its concentration reached the highest level at 6 h, followed by a decrease with longer incubation times. At 16 h, this intermediate disappeared. Another intermediate (product B) appeared at 2 h, and accumulated without further transformation even after 16 h of incubation (Fig. 1a). When *S. baderi* DE-13 was inoculated into culture medium containing acetochlor or CMEPA (50 mg L^{-1}) as the sole carbon, no detectable degradation of substrate was observed. When MEA served as the sole source of carbon, *S. baderi* DE-13 could degrade this substrate rapidly (Fig. 1b). During the MEA degradation, a transitory intermediate (product C) appeared at 2 h, and its concentration reached the highest level at 8 h, followed by completely degradation. Another intermediate (product D) appeared at 6 h, and was further degraded completely. Degrada-