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Detoxification of diphenyl ether herbicide lactofen by *Bacillus* sp. Za and enantioselective characteristics of an esterase gene *lacE*

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HIGHLIGHTS

- Lactofen degrading strain Za belonging to the genus *Bacillus* was isolated.
- The degrading end-product aminoacifluorfen showed almost no phytotoxicity to maize.
- Gene *lacE* encoding a lactofen-hydrolyzing esterase was cloned and expressed.
- The (*S*)-(+)-lactofen was degraded faster than the (*R*)-(−)-lactofen by LacE.

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ABSTRACT

A bacterial strain Za capable of degrading diphenyl ether herbicide lactofen was isolated and identified as *Bacillus* sp. This strain could degrade 94.8% of 50 mg L^{-1} lactofen after 4 days of inoculation in flasks. It was revealed that lactofen was initially hydrolyzed to desethyl lactofen, which was further transformed to acifluorfen, followed by the reduction of the nitro group to yield aminoacifluorfen. The phytotoxicity of the transformed product aminoacifluorfen to maize was decreased significantly compared with the lactofen. A gene *lacE*, encoding an esterase responsible for lactofen hydrolysis to desethyl lactofen and acifluorfen continuously, was cloned from *Bacillus* sp. Za. The deduced amino acid belonging to the esterase family VII contained a typical Ser-His-Asp/Glu catalytic triad and the conserved motifs GXSXG. The purified recombinant protein LacE displayed maximal esterase activity at 40 °C and pH 7.0. Additionally, LacE had broad substrate specificity and was capable of hydrolyzing *p*-nitrophenyl esters. The enantioselectivity of LacE during lactofen degradation was further studied, and the results indicated that the (*S*)-(+)-lactofen was degraded faster than the (*R*)-(−)-lactofen, which could illustrate the reported phenomenon that (*S*)-(+)-lactofen was preferentially degraded in soil and sediment.

1. Introduction

Diphenyl ether herbicides have been widely used for the control of many types of weeds in agricultural fields [1]. Lactofen is a member of diphenyl ether herbicides that act by inhibiting the activity of protoporphyrinogen oxidase in the porphyrin biosynthetic pathway of broadleaf weeds, which leads to the peroxidative damage of cell membrane lipids and disruption of membrane permeability, thereby conducing to weed death [2,3]. In China, lactofen is commonly used to control broadleaf weeds in soybeans, cereal crops, potatoes, and peanuts. However, the widespread application of lactofen and their frequent occurrence in natural waters caused environmental contamination and aquatic ecosystem destruction [4,5], and it is important to evaluate the degradation of lactofen in the environment.

Research on the degradation of lactofen has focused on the plants [6], soil [7], water [8], and sediment [9]. The United States Environmental Protection Agency declared that lactofen could suffer several transformations in the environment producing desethyl lactofen and acifluorfen. In general, lactofen used in agriculture could be transformed by physical and chemical processes, but the microorganism mediated degradation is the main environmental

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degrdation approach. Up to now, several strains capable of degrading different diphenyl ether herbicides have been reported, such as *Coriolus versicolor* [10], *Azotobacter chroococcum* [11], *Sphingomonas wittichii* RW1 [12], *Mycobacterium phocaicum* MBWY-1 [13], *Pseudomonas zeshuii* BY-1 [14] and *Chryseobacterium aquifrigidense* R-21 [15]. However, only one report showed that pure microbial cultures could degrade lactofen and the transformation product was identified as desethyl lactofen [16]. To the best of our knowledge, there has been no report about the esterase genes hydrolyzing the ester bond of diphenyl ether herbicides.

Lactofen differs from other diphenyl ether herbicides in that it contains one chiral center and consists of a pair of enantiomers, named (*S*)-(+) and (*R*)-(−), with the former structure accounting for most of its herbicidal activity [17]. Enantioselective degradation in soils has been observed for various chiral pesticides, such as mecoprop, dichlorprop, metalaxyl and malathion [18–20]. The occurrence of such selective degradation involves the mediation of bacteria, enzymes, or other biological entities. The enantioselective degradation of lactofen in soil and sediment has been studied by Diao et al., they found that (*S*)-(+)-lactofen was preferentially degraded, resulting in relative enrichment of the (*R*)-(−) form [7,9]. However, the molecular mechanism of this phenomenon is still unclear now.

In this study, a potent lactofen degrading strain was isolated from contaminated soils and the degradation pathway was explored by identification of the corresponding metabolites. The phytotoxicity of lactofen and its terminal transformed product aminoacifluorfen to the sensitive plant maize was also investigated. A lactofen-hydrolyzing esterase gene *lacE*, involved in the transformation of lactofen to desethyl lactofen and acifluorfen, was cloned and the enantioselectivity of LacE during lactofen hydrolysis was also studied.

2. Materials and methods

2.1. Chemicals and media

Lactofen (99% purity) and all the *p*-nitrophenyl esters were purchased from Sigma-Aldrich Chemical Co (Shanghai, China). The stock solution of lactofen (100,000 mg L^{-1}) was prepared in methanol. Acifluorfen was purchased from Changqing Agrochemical Co (Yangzhou, China). Aminoacifluorfen was purchased from Chem Service Inc (USA). All molecular reagents were purchased from TaKaRa Co (Dalian, China). Methanol, n-hexane, and isopropanol were of pure chromatographic grade. All of the other chemicals used were of analytical grade. Mineral salt glucose medium (MSGM; pH 7.0) contained 1.0 g L^{-1} NH_4NO_3, 1.0 g L^{-1} Glucose, 1.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, and 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$. Luria-Bertani (LB; pH 7.0) medium contained 5.0 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 10 g L^{-1} NaCl. For solid medium, 15 g of agar was added per liter.

2.2. Isolation and identification of the lactofen-degrading strain

The soil used for enrichment of lactofen-degrading strain was collected from a lactofen-polluted farmland in the city of Zibo, Shandong Province, China. The soil sample (10.0 g) was inoculated into 90 mL MSGM with 50 mg L^{-1} lactofen and incubated at 30 °C for 7 days on a rotary shaker at 160 r min^{-1}. About 5 mL of enrichment culture was transferred into 95 mL fresh MSGM containing 50 mg L^{-1} lactofen and incubated for an additional 7 days. After five rounds of transfer, the enrichment culture was serially diluted, spread onto MSGM agar plates supplemented with 50 mg L^{-1} lactofen, and incubated at 30 °C for 3 days. The colonies producing visible transparent halos caused by lactofen degradation were collected, purified, and further tested by high-performance liquid chromatography (HPLC) analysis for the ability to degrade lactofen. The isolate with the highest lactofen-degradation efficiency was designated as Za and selected for further study. Strain Za was identified according to Bergey's Manual of Systematic Bacteriology [21]. The 16S rRNA gene was amplified by PCR using a set of universal primers (27F and 1492R) [22] and was sequenced by Invitrogen Biotechnology Co (Shanghai, China). The 16S rRNA gene sequence was manually aligned with reference sequences retrieved from the GenBank database following BLAST searches. A phylogenetic tree was constructed using the software package MEGA version 6.0 [23]. Bootstrapping of 1000 replicates was carried out to estimate the confidence levels of phylogenetic reconstructions.

2.3. Degradation of lactofen by strain Za in liquid culture

The cells of strain Za cultured in LB medium for 10 h were harvested by centrifugation, washed, and resuspended in MSGM. After adjusting the optical density at 600 nm (OD_{600}) to 1.0, an inoculum (5%, vol/vol) was inoculated into 50 mL of MSGM with 50 mg L^{-1} lactofen. The cultures were incubated at 30 °C and 160 r min^{-1} on a rotary shaker. Samples were collected from the cultures at 12 h intervals, and the lactofen concentration was determined by HPLC. Control experiments without inoculation were performed under the same conditions. Each treatment was performed in three replicates.

2.4. Chemical analysis

Lactofen in liquid samples was extracted with three times volume of dichloromethane. The organic layer was dried over anhydrous Na_2SO_4 and evaporated at room temperature. The residues were re-dissolved in 1 mL of methanol and analyzed by HPLC (Shimadzu LC-20A) equipped with a Kromasil 100-5C18 column (250 mm × 4.6 mm). The mobile phase was methanol-water (80:20, vol/vol) with 0.1% acetic acid at a flow rate of 1 mL min^{-1}. The UV wavelength for detection was 230 nm and the injection volume was 20 μL. The concentration of lactofen was determined from the peak area ratio relative to individual standard calibration curves. The metabolites of lactofen were analyzed by liquid chromatography-mass spectrometry (LC–MS) and tandem mass spectrometry (MS/MS). MS analyses were operated with an electrospray ionization source in the negative polarity mode.

Lactofen enantiomers were separated and quantified by HPLC with a Chiralcel OD-H column (250 mm × 4.6 mm). The mobile phase was n-hexane-isopropanol (97:3, vol/vol) at a flow rate of 1 mL min^{-1}. The UV wavelength for detection was 230 nm, and the injection volume was 20 μL.

2.5. Phytotoxicity study of degradation end-product aminoacifluorfen to maize

The residues of lactofen in contaminated agricultural soil are harmful to some crops, including maize, sugar beets and vegetables [24]. Therefore, it is important to study the mitigation phytotoxicity in the process of lactofen degradation by Za. The maize was selected as the target crop due to its sensitivity to lactofen residues. After germinated under the condition of wetness and darkness at 20 °C for 2 days, the maize seeds with the same degree of germination were chosen for the mitigation phytotoxicity experiments. The following soil pots were prepared: soil pots without lactofen or aminoacifluorfen (control), soil pots with lactofen but without aminoacifluorfen (lactofen), and soil pots with aminoacifluorfen but without lactofen (aminoacifluorfen). The different concentrations of lactofen or aminoacifluorfen in soil included in the pot experiments were 0.011, 0.022, 0.032, and 0.065 mM. Five maize