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JES JOURNAL OF ENVIRONMENTAL SCIENCES www.jesc.ac.cn

Biodegradation of pendimethalin by *Bacillus subtilis* Y3

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ARTICLE INFO

Article history:
Received 9 February 2015
Revised 22 April 2015
Accepted 24 April 2015
Available online 3 September 2015

Keywords:
Pendimethalin
Biodegradation
Bacillus sp. Y3
Metabolic pathway
Nitroreductase

ABSTRACT

A bacterium strain Y3, capable of efficiently degrading pendimethalin, was isolated from activated sludge and identified as *Bacillus subtilis* according to its phenotypic features and 16S rRNA phylogenetic analysis. This strain could grow on pendimethalin as a sole carbon source and degrade 99.5% of 100 mg/L pendimethalin within 2.5 days in batch liquid culture, demonstrating a greater efficiency than any other reported strains. Three metabolic products, 6-aminopendimethalin, 5-amino-2-methyl-3-nitroso-4-(pentan-3-ylamino) benzoic acid, and 8-amino-2-ethyl-5-(hydroxymethyl)-1,2-dihydroquinoxaline-6-carboxylic acid, were identified by HPLC-MS/MS, and a new microbial degradation pathway was proposed. A nitroreductase catalyzing nitroreduction of pendimethalin to 6-aminopendimethalin was detected in the cell lysate of strain Y3. The cofactor was nicotinamide adenine dinucleotide phosphate (NADPH) or more preferably nicotinamide adenine dinucleotide (NADH). The optimal temperature and pH for the nitroreductase were 30°C and 7.5, respectively. Hg^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+} Cu^{2+}, Ag^{+}, and EDTA severely inhibited the nitroreductase activity, whereas Fe^{2+}, Mg^{2+}, and Ca^{2+} enhanced it. This study provides an efficient pendimethalin-degrading microorganism and broadens the knowledge of the microbial degradation pathway of pendimethalin.

Introduction

Pendimethalin [N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine], a selective pre-emergence dinitroaniline herbicide, is widely used to control a variety of annual grasses and broadleaf weeds (Kole et al., 1994; Ramakrishna et al., 2008). Its primary mode of action is to prevent cell division and cell elongation (Ma et al., 2006). Presently, it is the third largest herbicide and the largest selective herbicide in the world. Pendimethalin is moderately persistent in soil environments, with a half-life of about 60 days under tropical field conditions. Its widespread use and persistence lead to its frequent detection in soil and water (Barbash and Resek, 1996; Barriuso et al., 1997; Capel et al., 1998; Larson et al., 1999; Racke, 2000). Despite its low acute toxicity, pendimethalin is still a potential toxic. It is highly toxic to aquatic and terrestrial invertebrates (Kamrin, 1997) and increases the risk of thyroid

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http://dx.doi.org/10.1016/j.jes.2015.04.035
1001-0742/

follicular cell adenomas in rats. Presently, it has been classified as a possible human carcinogen (Hou et al., 2004). Therefore, it is essential and necessary to study its health and environmental effects.

Normally, pendimethalin is removed from the environment abiotically and biotically, including by volatilization, photodegradation, and biodegradation (Moza et al., 1992; Piutti et al., 2002; Venkata Mohan et al., 2007; Zhang et al., 2000). Microbial degradation plays a very important role in the fate of pendimethalin in the environment. Many bacteria and fungi strains capable of degrading pendimethalin have been isolated, and the metabolism pathways were characterized (Pinto et al., 2012). Kole et al. (1994) reported that *Azotobacter chroococcum* could degrade 55% of 25 mg/L pendimethalin in 20 days. Pinto et al. (2012) isolated a fungi strain *Lecanicillium saksenae* from a loamy sand soil, which could remove 99.5% of 25 mg/kg pendimethalin within 10 days of incubation. However, little is known about the physiological, biochemical, and genetic mechanisms of pendimethalin biodegradation.

In this study, an efficient pendimethalin-degrading strain Y3 was isolated from activated sludge collected from an herbicide-manufacturing factory by enrichment culture, and identified as *Bacillus* sp. The strain could utilize pendimethalin as the sole carbon source and degrade 99.5% of 100 mg/L pendimethalin within 2.5 days of incubation. The degradation pathway of pendimethalin by strain Y3 was further proposed on the basis of metabolite identification. Moreover, the preliminary characteristics of the pendimethalin nitroreductase were also investigated because of its crucial role in catalyzing the nitroreduction of pendimethalin.

1. Materials and methods

1.1. Chemicals and medium

Pendimethalin (97%) was a generous gift provided by Rosi Chemical Co., Ltd., Zhejiang Province, China. High-performance liquid chromatography (HPLC)-grade formic acid and acetonitrile, and all other analytical grade chemicals were purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). Taq deoxyribonucleic acid (DNA) polymerase was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Songon Biotech Co., Ltd. (Shanghai, China). The Luria-Bertani (LB) medium and mineral salts medium (MSM) used in this study were prepared as described by Chen et al. (2014).

1.2. Enrichment, isolation, and identification of pendimethalin-degrading bacteria

The activated sludge used as initial inoculant was collected from an herbicide-manufacturing factory in Jiangsu Province, China. To isolate pendimethalin-degrading strains, a conventional enrichment was carried out according to the method of Nie et al. (2011) with some modifications. Pendimethalin was added into 100 mL of MSM at a final concentration of 50 mg/L as the sole carbon source. After three rounds of enrichment, 0.1-mL aliquots of serial tenfold dilutions were spread onto LB agar. The plates were incubated at 30°C for 2 days. Bacteria colonies were purified by streaking on LB agar, and checked for pendimethalin-degrading abilities.

The pendimethalin-degrading isolates were characterized and identified by their phenotypic characteristics, as well as phylogenetic analysis of the 16S ribosomal ribonucleic acid (rRNA) gene sequence. The 16S rRNA gene sequence was PCR amplified using a set of universal primers, 5′-AGAGTTTGATCCTGGCTCAG-3′ (*Escherichia coli* bases 8-27) and 5′-TACCTTGTTACGACTT-3′ (*E. coli* bases 1507-1492) (Lane, 1991). The PCR product was sequenced by an automatic sequencer (Applied Biosystems, model 3730). Pairwise sequence similarity was calculated at the EzTaxon server (Chun et al., 2007). Phylogenetic analysis was performed by using the software package MEGA version 5.0 (Tamura et al., 2011) according to the method of Nie et al. (2011). The G + C content of the DNA was determined by using reversed-phase HPLC according to Mesbah et al. (1989).

1.3. Degradation of pendimethalin by the isolated strain

To prepare the seed culture, the isolate was preincubated in LB broth at 30°C on a rotary shaker at 150 r/min for 10 hr. Cells were collected at the mid-exponential phase by centrifugation. After being washed twice with fresh MSM, the cells were resuspended in fresh MSM with an adjusted density of 1.0×10^9 cfu/mL. An aliquot of the seed culture (1%, V/V) was added into 20 mL of MSM supplemented with 100 mg/L pendimethalin as the carbon source (in a 50 mL Erlenmeyer flask). The cultures were aerobically incubated on a rotary shaker at 150 r/min, 30°C. The biomass of strain Y3 and the biodegradation of pendimethalin were evaluated every 12 hr. Bacterial growth was monitored based on the colony forming units (cfu/mL). The concentration of pendimethalin was determined by HPLC, and the metabolites were identified by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) as described below. The uninoculated control experiments were carried out under the same conditions. Each treatment was done in triplicate.

1.4. Preparation of cell-free extract

The pendimethalin-degrading strain was aerobically cultured in LB broth or in MSM supplemented with 100 mg/L pendimethalin at 30°C. The cells were harvested at the mid-exponential phase, and washed twice with 100 mM Tris–HCl buffer (pH 7.5). The pellet was resuspended in the same buffer to an OD_{600} of 5.0, sonicated (Auto Science, UH-650B ultrasonic processor, 40% intensity) for 10 min and centrifuged at 12,000 r/min for 30 min to remove undisrupted cells and cell debris. The supernatant was then passed through a 0.22 μm pore-size Millipore membrane. All the procedures were performed at 4°C. Protein concentration was quantified by the method of Bradford (1976).

1.5. Assays of enzyme activity

The activity of the nitroreductase catalyzing the nitroreduction of pendimethalin (named pendimethalin nitroreductase) in the cell lysate was determined in 1 mL of a mixture containing 100 mmol/L Tris–HCl buffer (pH 7.5), 100 μL cell lysate and 0.5 mmol/L NADH or NADPH. The reaction was initiated by