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Biodegradation of pendimethalin by *Bacillus subtilis* Y3

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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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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

addition of pendimethalin (final concentration of 100 mg/L) to the mixture and maintained at 30°C for 3 hr, and then terminated by boiling at 100°C. The pendimethalin disappearance was monitored by HPLC, and the metabolites were identified by HPLC-MS/MS. One unit of enzyme activity was defined as the amount of enzyme required to catalyze the consumption of 1 μmol pendimethalin per min. The effects of environmental factors such as pH, temperature, metal ions, and EDTA on the pendimethalin nitroreductase activities were determined according to the method described by Wang et al. (2009).

1.6. Chemical analysis

The culture or enzyme samples were extracted with an equal volume of dichloromethane. The organic layer was dehydrated with anhydrous sodium sulfate, and dried by nitrogen gas. The residues were dissolved in methanol and filtered through a 0.22 μm Millipore membrane filter. In the HPLC analysis, a separation column (internal diameter, 4.6 mm; length, 250 mm) filled with Kromasil 100-5C18 was used. The mobile phase was a mixture of methanol and ultrapure water (85:15) with a flow rate of 0.8 mL/min. The injection volume was 20 μL, and pendimethalin was detected at 240 nm (UV-900 wavelength absorption detector).

The metabolites were detected by HPLC-MS/MS (Dionex, Thermo, USA). An LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific), with electrospray ionization (ESI) probe, was used for the mass spectrometry analysis. Data analysis was performed under positive mode ESI conditions (Ramakrishna et al., 2008). The capillary temperature was 300°C, and the source voltage was set at 4 kV. The sheath gas was set at 35 arb, auxiliary gas was set at 10 arb. Data were processed with the Xcalibur software.

1.7. Strain deposit numbers and nucleotide sequence accession numbers

Strain Y3 was deposited in the Agricultural Culture Collection of China (ACCC) and China Center for Type Culture Collection (CCTCC) under the deposit numbers ACCC 19810 and CCTCC AB 2015029, respectively. The GenBank accession number for the 16S rRNA gene sequences of strain Y3 is KP215279.

2. Results

2.1. Isolation and identification of pendimethalin-degrading strain Y3

The enrichment culture degraded about 90% of 50 mg/L pendimethalin within 2 days after three rounds of enrichments. As many as 7 different colonies were obtained, but only one strain, designated as Y3, showed the ability to degrade pendimethalin. Thus, strain Y3 was selected for further study.

Strain Y3 was a Gram-positive, spore-forming bacterium. Colonies grown on LB agar plate were white, dry, rugose, and the border was irregular. The phylogenetic analysis of 16S rRNA gene sequences indicated that strain Y3 belonged to the genus *Bacillus* and formed a subclade with *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429^T (99.9% similarity), *B. subtilis* subsp. *subtilis* NCIB 3610^T (99.4% similarity), and *B. subtilis* subsp. *spizizenii* NRRL B-23049^T (99.1% similarity) (Fig. 1). The genomic DNA G + C content of strain Y3 was 43.9 mol%. It was positive for acidification of glucose, D-mannitol, D-xylose, D-sorbitol, trehalose, cellobiose, and starch, and it was also positive for nitrate reduction, citrate utilization and the Voges–Proskauer reaction. But it was negative for dulcitol, lactose, and raffinose, indole reaction, and the methyl red test. These morphological and physiological characteristics were identical to that of *B. subtilis*. Thus, on the basis of phenotypic and phylogenetic properties, strain Y3 was identified as *B. subtilis*.

2.2. Degradation and utilization of pendimethalin by *B. subtilis* Y3

Since some previous studies reported that a concentration of 100 mg/L pendimethalin was much higher than its residue concentration in the environment and it has typically been used in biodegradation studies (Strandberg and Scott-Fordsmand, 2004), here the degradation and utilization of pendimethalin by *B. subtilis* Y3 were determined on MSM supplemented with 100 mg/L pendimethalin as the sole carbon source. The results are shown in Fig. 2. Strain Y3 could degrade about 80% of 100 mg/L pendimethalin within 1.5 days of incubation, and 99.9% pendimethalin within 2.5 days of incubation, whereas only 3.2% pendimethalin loss was detected in a control test without bacteria within 2.5 days. Simultaneously, the strain cell density in the Y3 group was increased from 1.0×10^7 to 1.83×10^8 cfu/mL. These results indicated that *B. subtilis* Y3 could efficiently degrade pendimethalin and utilize it as the sole source of carbon and energy for growth.

2.3. Metabolites of pendimethalin degradation by strain Y3

The metabolites of pendimethalin degradation were identified by HPLC-MS/MS. In the HPLC spectrum (Fig. 3), pendimethalin gave a peak with the retention time (RT) of 14.08 min, and its mass spectrum is shown in Fig. 4a. The RTs of the other three peaks (denoted as products A, B, and C, respectively) were 8.92 min, 4.85 min, and 4.50 min, respectively.

Product A was identified according to its mass spectrum characteristics. As shown in Fig. 4b, its molecular ion was at *m/z* 252.17 ($[M + H]^+$), fitting a molecular formula of $C_{13}H_{22}N_3O_2$. Furthermore, product A had characteristic base peaks at *m/z* 182.09, 164.08, 146.07, and 134.07, respectively. The peak *m/z* 182.09 was possibly generated due to the loss of C_5H_9 from $C_{13}H_{22}N_3O_2$, whereas the peaks *m/z* 164.08, *m/z* 146.07, and *m/z* 134.07 were due to the loss of $C_5H_{11}O$, $C_5H_{13}O_2$, and $C_6H_{13}O_2$, respectively. Thus, product A was identified as 6-amino pendimethalin, a nitroreduction product of pendimethalin by nitroreductase (pendimethalin nitroreductase). Similarly, products B and C were identified as 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, respectively (Fig. 4c, d).

Thus, a partial metabolic pathway of pendimethalin in strain Y3 was proposed in Fig. 5. First, one of the nitro-groups of

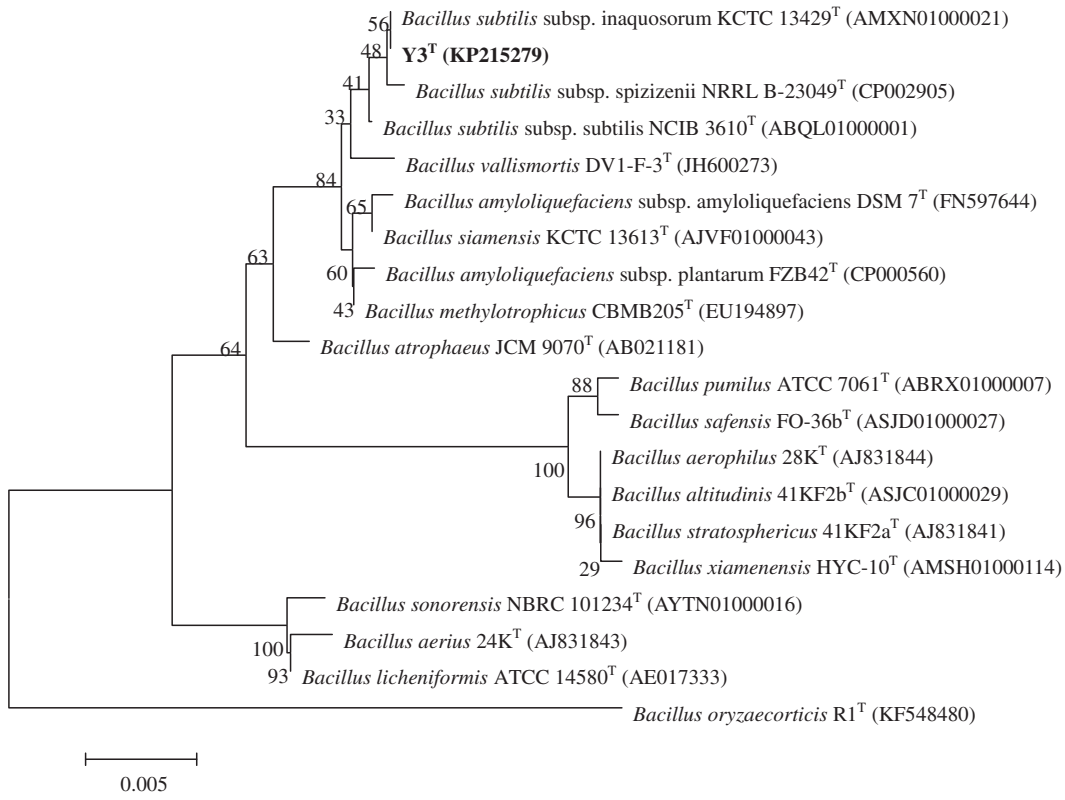

Fig. 1 – Phylogenetic tree based on the 16 s rRNA gene sequences of Y3 and related species. The GenBank accession number for each type strain used in the analysis is shown after the species name. The scale bar refers to 0.005 substitutions per nucleotide position. Bootstrap values obtained with 1000 resamplings are referred to as percentages at all branches.

pendimethalin was reduced to an amino group through nitroreduction to form 6-aminopendimethalin. The metabolite was further transformed to 5-amino-2-methyl-3-nitroso-4-(pentan-3-ylamino) benzoic acid by nitroreduction at the nitro-group connected to C-2 and carboxylation of the arylmethyl group at C-4. The latter was subsequently transformed to 8-amino-2-ethyl-5-(hydroxymethyl)-1,2-dihydroquinoxaline-6-carboxylic acid. However, the subsequent pathway is still unknown and needs further study.

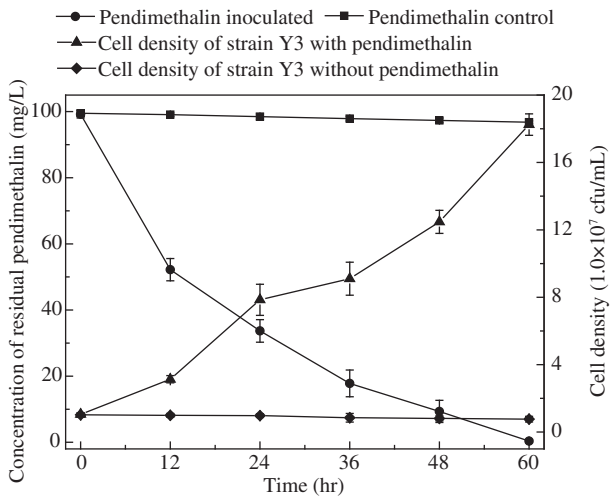

Fig. 2 – Degradation and utilization of pendimethalin during growth of strain Y3.

2.4. Enzyme activities in cell lysate

Table 1 shows that the strain Y3 cell lysate displayed strong pendimethalin nitroreductase activity when NADH/NADPH was added, suggesting that the pendimethalin nitroreductase in strain Y3 was NAD(P)H-dependent. However, weak nitroreductase activity was also detected in the control group without the exogenous NADH/NADPH. The possible reason is that a small amount of NADH/NADPH existed in the cell lysate. In addition, exogenous NADH resulted in higher nitroreductase activity than NADPH, indicating that NADH may be a preferred cofactor to NADPH for pendimethalin nitroreductase.

The effects of some environmental factors on pendimethalin nitroreductase activity were also investigated. The activity was measured from 10 to 60°C and pH 3.0 to 8.8, with the greatest activity being detected at 30°C and pH 7.5 (Fig. S1 and S2). The nitroreductase activity was severely inhibited by Hg^{2+}, Ni^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+}, Cu^{2+}, and Ag^{+}, but enhanced by Fe^{2+}, Mg^{2+}, and Ca^{2+}; whereas monovalent cations Li^{+} and Na^{+} had no obvious effect on the enzyme activity. In addition, EDTA, a divalent cation

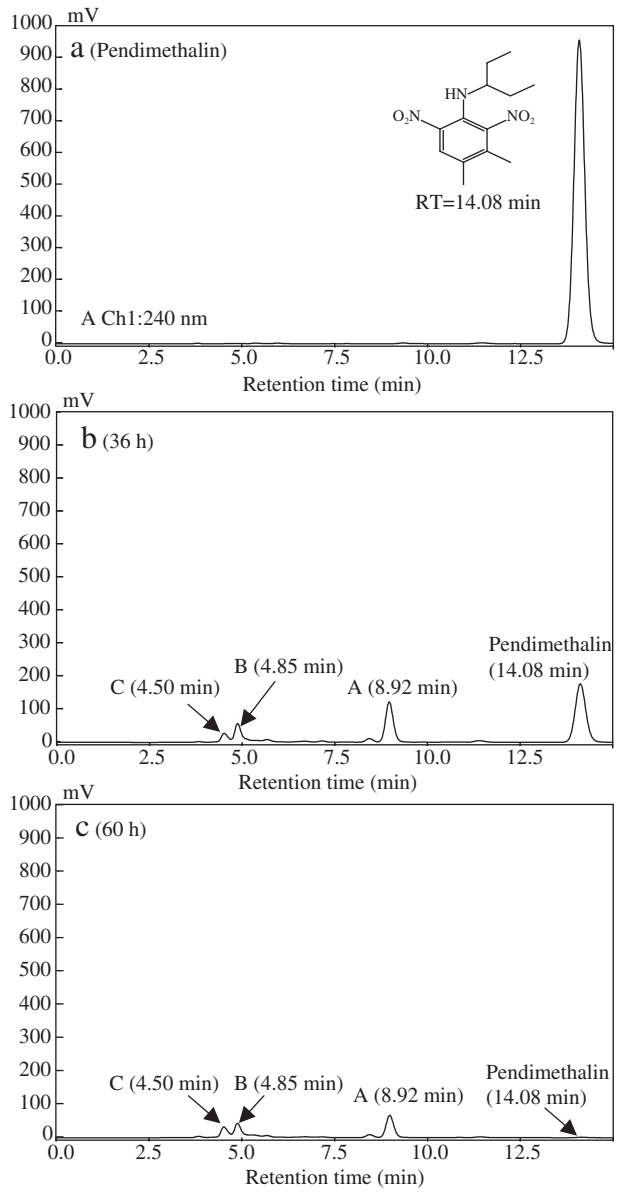

Fig. 3 – HPLC analysis of the metabolic products of pendimethalin degraded by strain Y3. (a) Authentic pendimethalin; (b and c) extract obtained from the culture at 36 hr and 60 hr, respectively.

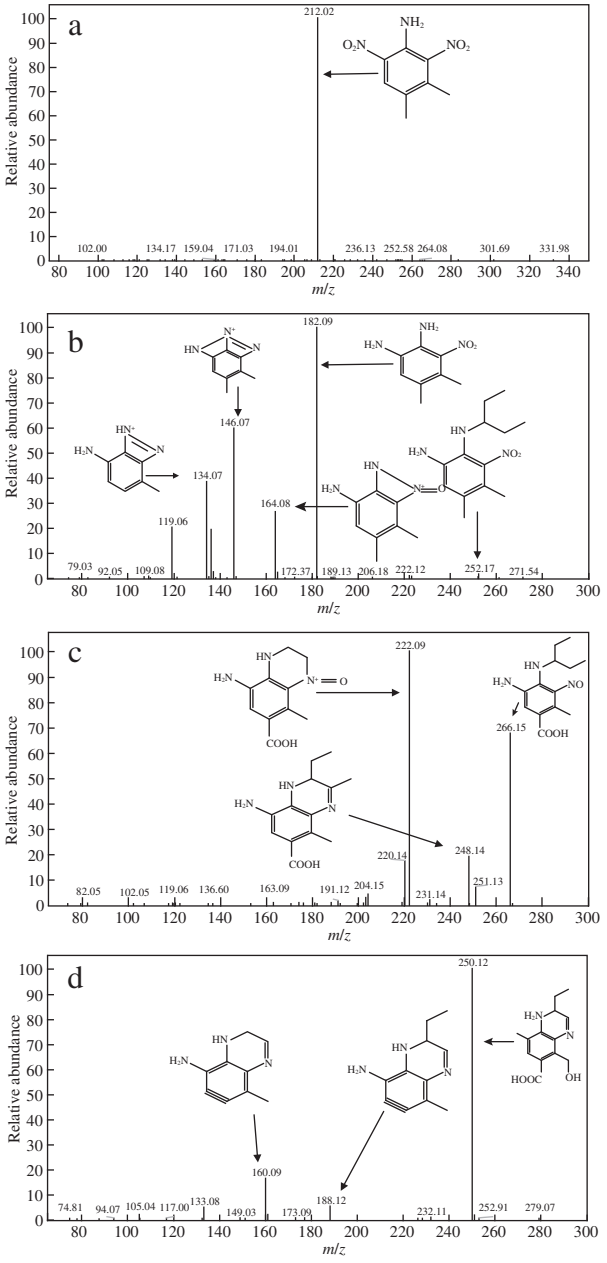

Fig. 4 – MS/MS spectrum of the metabolic products of pendimethalin degraded by strain Y3. (a) Pendimethalin at RT 14.08 min; (b) product A at RT 8.92 min; (c) product B at RT 4.85 min; (d) product C at RT 4.50 min. Products A, B, and C refer to Fig. 3. RT: retention time. MS/MS: Tandem mass spectrometry; RT: retention time.

chelating agent, severely inhibited the enzyme activity, indicating that this nitroreductase required divalent cations (Table S1).

3. Discussion

In this paper, an efficient pendimethalin-degrading strain Y3 was isolated and identified as *Bacillus* sp. Members of the genus *Bacillus* widely distributed in nature are well known for their abilities to degrade a great variety of xenobiotic pollutants. Strain Y3 could degrade 99.5% of 100 mg/kg pendimethalin within 2.5 days of incubation. Compared with other pendimethalin-degrading strains previously reported, such as *L. saksenae*, which could degrade 99.5% of 25 mg/kg pendimethalin within 10 days (Pinto et al., 2012), and *A. chroococcum*, which degraded 55% pendimethalin within 20 days (Kole et al., 1994), strain Y3 possessed very high degradation efficiency. Previously, the initial degradation pathway of pendimethalin by *A. chroococcum* was studied. *A. chroococcum* degraded pendimethalin by three different mechanisms: nitroreduction to 6-aminopendimethalin, oxidative dealkylation to 3,4-dimethyl-2,6-dinitroaniline and pentane, and arylmethyl group oxidation at C-3 to yield 2-methyl-4-nitro-5-N-(1-ethylpropy1)-6-nitrosobenzyl alcohol (Kole et al., 1994). However, the further degradation

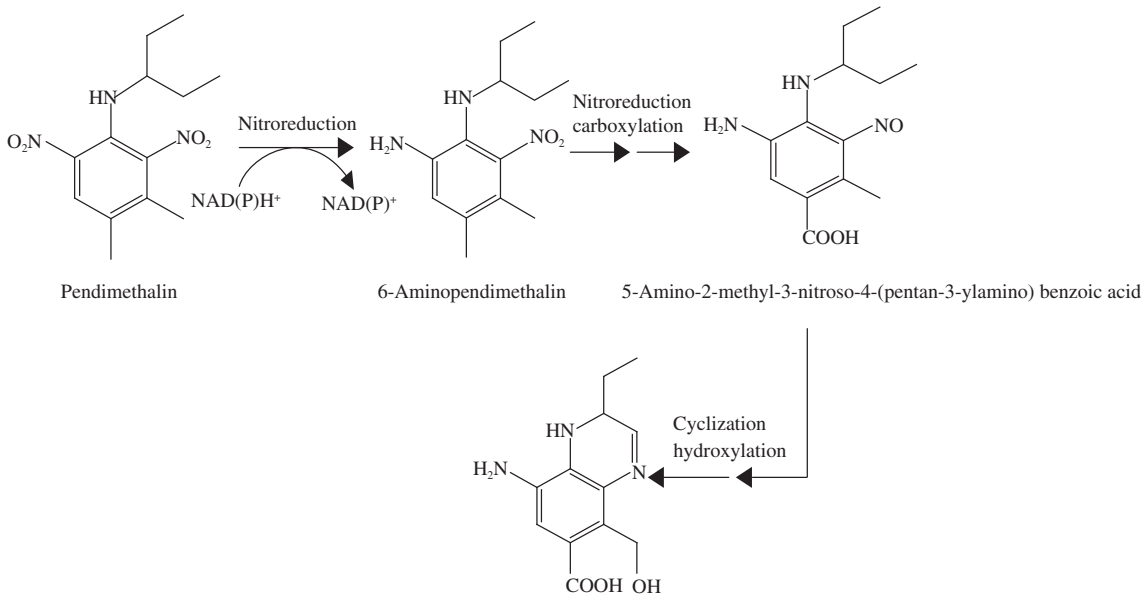

Fig. 5 – Proposed metabolic pathway of pendimethalin by strain Y3.

metabolites of 6-aminopendimethalin by this strain were still unknown. In this study, we found different phenomena: there was not only a nitroreduction pathway for *B. subtilis* Y3, but also two other downstream products 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. It is interesting that the dealkylation product 3,4-dimethyl-2,6-dinitroaniline was not detected, indicating that the dealkylation pathway did not exist in *B. subtilis* Y3. These results broaden the knowledge on the biodegradation mechanism of pendimethalin by microorganisms.

Nitroreductase is a family of the FMN-dependent superfamily, which is involved in the reduction of a variety of nitrogen-containing compounds using NAD(P)H as the reducing agent. Nitroreductase has a very broad substrate spectrum including quinines, flavins, nitroaromatic compounds, and nitroheterocyclic derivatives. It plays important roles in drug activation and detoxification, and degradation of nitrosubstituted pollutants. Thus, nitroreductases have raised great interest due to their potential applications in bioremediation, biomedicine, and biocatalysis. Strain Y3 possessed strong nitroreductase activity, catalyzing the nitro group reduction of pendimethalin to 6-aminopendimethalin, a metabolite that is almost herbicidally inactive (Kole et al., 1994), therefore this nitroreductase has potential application in bioremediation of pendimethalin-contaminated environments and construction of herbicide-resistant transgenic crops. Future research will be focused on cloning of the gene encoding nitroreductase and further study of the enzymatic characteristics.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2015.04.035.

Table 1 – Pendimethalin nitroreductase activity with different reductants.

	Pendimethalin nitroreductase activity (μmol/(min · mg))		
	Addition of NADH	Addition of NADPH	No addition of NADH or NADPH
Cell lysate	0.38 ± 0.06	0.30 ± 0.04	0.03 ± 0.01

NADH: nicotinamide adenine dinucleotide; NADPH: nicotinamide adenine dinucleotide phosphate.

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