



Biodegradation of Pendimethalin by *Paracoccus* sp. P13

Haiyan Ni¹ · Na Li² · Jiguo Qiu² · Qing Chen³ · Jian He²

Received: 20 December 2017 / Accepted: 17 April 2018 / Published online: 19 April 2018
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Abstract

In this study, a bacterial strain P13 capable of degrading pendimethalin was isolated from the soil of a fruit garden. Based on observed cellular morphology and physiology characteristics and a phylogenetic analysis of 16S rRNA gene sequences, strain P13 was identified as a member of the genus *Paracoccus*. Strain P13 grew on pendimethalin as the sole carbon source, and could degrade 100 mg/L pendimethalin within 2 days and 200 mg/L pendimethalin within 5 days. Pendimethalin degradation was proposed to be initiated by oxidation ring cleavage to yield 1,3-dinitro-2-(pentan-3-ylamino)butane-1,4-diol, an alkane organic compound that was identified by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS), which then underwent a series of enzymatic reactions to produce CO₂ and H₂O. The optimal pH and temperature for pendimethalin degradation by strain P13 were 7.0 and 30 °C, respectively. This study identified the bacterial strain *Paracoccus* sp. P13, which degraded pendimethalin with a relatively high efficiency, and presents a previously unreported microbial pendimethalin degradation pathway.

Introduction

Currently, increases in food quantity and quality rely heavily on the use of pesticides. Pendimethalin [*N*-(1-ethylpropyl)-2,6-dinitro-3,4-xylylidine], is a selective preemergence dinitroaniline herbicide, which is commonly used to control most annual grasses and certain broadleaf weeds for many crops, such as cotton, soybeans, wheat, rice, and maize. Its

mode of action is inhibiting plant cell division and cell wall formation [9, 20]. Pendimethalin has a relatively low volatility [16, 21], and is moderately persistent with a field half-life of approximately 90 days and does not undergo rapid microbial degradation [23]. Due to its widespread use, pendimethalin has been detected as a contaminant in soil, ground water, and surface water [14, 24]. Pendimethalin is a possible human carcinogen and shows high toxicity to terrestrial and aquatic invertebrates [1, 3]. Furthermore, pendimethalin can bioaccumulate and concentrate to 7000 times its initial concentration [17], and the US Environmental Protection Agency (EPA) has classified pendimethalin as a persistent, bioaccumulative, and toxic substance [18]. Thus, a better understanding of the degradative fate of pendimethalin in soil is needed.

Biodegradation is the most important route by which pendimethalin is degraded in the environment [20, 22, 25]. Numerous pendimethalin-degrading microorganisms have been isolated, including *Bacillus* sp. Y3 [12], *Bacillus lehen-sis* XJU [11], *Pseudomonas aeruginosa*, *Bacillus mycoides*, *Bacillus cereus* [19], *Pseudomonas putida* E15 [2], *Bacillus circulans* [10], *Fusarium oxysporum* [8], and *Azotobacter chroococcum* [7]. Generally, pendimethalin degradation is initiated by three different mechanisms, nitroreduction, oxidative *N*-dealkylation, and cyclization [7]. In this study, we described a *Paracoccus* sp. bacterium P13 that was observed to degrade pendimethalin, which was determined

Haiyan Ni and Na Li contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00284-018-1494-0>) contains supplementary material, which is available to authorized users.

✉ Qing Chen
chenqing8686@126.com

✉ Jian He
hejian@njau.edu.cn
Haiyan Ni
nihaiyan16@163.com

¹ College of Life Science, Jiangxi Normal University, Nanchang 330022, Jiangxi, China

² College of Life Sciences, Laboratory Centre of Life Sciences, Nanjing Agricultural University, Nanjing 210095, Jiangsu, China

³ College of Life Sciences, Zaozhuang University, Zaozhuang 277160, Shandong, China

to be initiated through oxidation ring cleavage on the basis of metabolite identification.

Materials and Methods

Chemicals and Medium

Pendimethalin (97% purity) was provided by Rosi Chemical Co., Ltd. (Zhejiang Province, China). Methanol and acetonitrile were of chromatographic grade (Sigma-Aldrich, Shanghai, China), dichloromethanol and other organic reagents were of analytical grade (Shou-De, Nanjing, China). Pendimethalin was first dissolved in methanol and then filtered through a 0.22 μm Millipore membrane filter before use.

Mineral salts medium (MSM) consisted of (in g/L) 1.0 NH_4NO_3 , 0.5 NaCl, 1.5 K_2HPO_4 , 0.5 KH_2PO_4 , and 0.2 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, supplemented with pendimethalin as the sole carbon source. The Luria–Bertani (LB) broth contained (in g/L) 10.0 tryptone, 5.0 yeast extract, and 10.0 NaCl. Agar was added at 15.0 g/L for solid medium. Both media were sterilized at 121 $^\circ\text{C}$ for 30 min before use.

Enrichment, Isolation, and Identification of Pendimethalin-Degrading Strains

A conventional enrichment culture method was performed to isolate pendimethalin-degrading bacteria. Ten gram soil samples, collected from the surface layer (0–10 cm) of a fruit garden in Shandong Province, China, was added to an Erlenmeyer flask with 100 mL of sterile deionized water and shaken for 1 h at 30 $^\circ\text{C}$ and 150 r/min. Next, 5 mL of the soil suspension was added to a 250-mL Erlenmeyer flask containing 95 mL fresh MSM supplemented with 100 mg/L pendimethalin as the sole carbon source. The mixture was incubated on a rotary shaker at 30 $^\circ\text{C}$ and 150 r/min in the dark for 5 days. Subsequently, 5 mL of the initial enrichment culture was transferred into 95 mL of fresh MSM with 100 mg/L pendimethalin, and incubated for another 5 days under the same conditions described above. The transfer was repeated three times.

Serial gradient dilutions (1.0×10^{-1} – 1.0×10^{-7}) of the final enrichment culture were prepared, and then 100 μL of each dilution was spread on MSM agar plates supplemented with 200 mg/L pendimethalin. The plates were incubated at 30 $^\circ\text{C}$ for 5 days and monitored for the appearance of bacterial colonies. Single colonies were picked and further purified by repeated streaking onto LB broth agar plates supplemented with 200 mg/L pendimethalin.

Each isolate was then tested for the ability to degrade pendimethalin. Isolates that were effective at degrading pendimethalin were characterized and identified by morphological, physiological, and biochemical characteristics as well

as by 16S rRNA gene sequences analysis according to the method described by Nie et al. [13].

Cell Growth and Pendimethalin Degradation

The isolates were pregrown in baffled Erlenmeyer flasks containing fresh LB broth, which were incubated overnight at 30 $^\circ\text{C}$ on a rotary shaker at 150 r/min. The contents were harvested by centrifugation at 5000 r/min for 10 min and then washed twice with fresh MSM. The cell density was adjusted to 1.0×10^8 CFU/mL and served as seed cultures. An aliquot of seed culture (1%, V/V) was transferred into 20 mL MSM supplemented with 200 mg/L pendimethalin in a 50-mL Erlenmeyer flask. The degradation of pendimethalin by free-living cells of strain P13 was carried out aerobically at 30 $^\circ\text{C}$ on a rotary shaker at 150 r/min in the dark. Control experiments without free cells or pendimethalin were performed under the same conditions. Each treatment was performed three times. The enrichment cultures were sampled at 24 h intervals to calculate cell growth and residual pendimethalin. Bacteria growth was monitored by measuring the colony-forming units (CFU/mL), and the residual concentration of pendimethalin was confirmed by ultra-high performance liquid chromatography (UHPLC) as described below.

Chemicals Analysis

The culture medium samples were partitioned with an equal volume of dichloromethanol. The organic extracts were passed through anhydrous sodium sulfate and evaporated to dryness under a gentle nitrogen stream (40 $^\circ\text{C}$). Finally, all the samples were redissolved in methanol, filtered through 0.22 μm Millipore membrane filters, and then used for the following analysis.

The structural identification of pendimethalin metabolites in samples was performed by LC–MS. The LC–MS system consisted of a UHPLC (Dionex, Thermo, USA) coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific). The UHPLC column utilized was a Hyper-sil GOLD C18 column (100 mm \times 2.10 mm, 3 μm particle sizes, Thermo Fisher Scientific). The UHPLC mobile phase consisted of 0.02% of formic acid in water (buffer A) and 100% acetonitrile (buffer B). Separation was carried out over 30 min at a flow rate of 0.2 mL/min under the following conditions: 0–4 min, 30% B; 4–20 min, 30–95% B; 20–24.5 min, 95% B; 24.5–25 min, 95–30% B; and 25–30 min, 30% B. The injection volume was 5 μL , and the detection wavelength was set at 240 nm for pendimethalin (UV-900 wavelength absorption detector). An LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) probe, was used for the mass spectral analysis of pendimethalin and its metabolites.

The capillary temperature was set at 300 °C, and the source voltage was set at 4 kV. The sheath gas was set at 35 arb, and the Aux Gas was set at 10 arb. The data analysis was performed under positive mode ESI conditions [15]. Data were processed using Xcalibur.

Effect of pH and Temperature on Pendimethalin Degradation by Strain P13

To determine the effect of pH and temperature on pendimethalin degradation by strain P13, 20 mL MSM containing 200 mg/L pendimethalin was used as the pendimethalin-degrading medium, and inoculated by a bacterial seed culture to an initial cell density of 1.0×10^6 CFU/mL. To determine the optimum pH, experiments were performed at pH values of 4.0–9.0. To determine the effect of temperature, the pendimethalin-degrading medium with the optimum pH was incubated at 20–45 °C. The effect of pH and temperature on pendimethalin degradation by strain P13 was examined using an incubation period of 5 days on a rotary shaker at 30 °C and 150 r/min. Residual pendimethalin under different conditions was measured by UHPLC, and the amount of degraded pendimethalin was calculated.

Strain Deposition and Nucleotide Sequence Accession Numbers

Strain P13 was deposited at the China Center for Type Culture Collection (CCTCC) under the deposition number CCTCC AB 2015071. The GenBank accession number for the strain P13 16S rRNA gene sequence is KP215280.

Results and Discussion

Strain Isolation and Identification

After four rounds of selective enrichment, the enrichment mixture degraded approximately 99.9% of the initial 100 mg/L pendimethalin within 5 days, and 13 morphologically different microorganisms were isolated from the enrichment samples. All 13 strains were individually cultured in MSM and LB broth supplemented with 200 mg/L pendimethalin as the carbon source to assess pendimethalin degradation. The results suggested that only 3 of the 13 strains were able to grow in both media and degrade pendimethalin. One strain (P13) exhibited the highest degradation rate (99.8%) compared with the other two strains (degradation rates of 57 and 46%) and was chosen for further study.

Strain P13 was identified by morphological and physiological characteristics and a phylogenetic analysis of 16S rRNA gene sequences. Strain P13 was determined to be a gram-negative, non-spore-forming, and non-motile

bacterium. Colonies grown on LB and MSM agar media were pale yellow, wet, convex, rod-shaped, and glossy with a smooth surface and neat edges. In addition, this microorganism tested positive for nitrate reduction, oxidase and catalase, and for the acidification of L-arabinose, D-glucose, D-mannose, and D-fructose. Strain P13 tested negative for urease, indole reaction, methyl red, and Voges–Proskauer tests, hydrolysis of gelatin and starch, and for the acidification of maltose, sucrose, lactose, and trehalose. The obtained 16S rRNA gene sequence of strain P13, nearly complete, was 1336 bp in length. The sequence was deposited under the accession number KP215280 in GenBank. Phylogenetic analysis of 16S rRNA gene sequences suggested that strain P13 grouped among *Paracoccus* species and was most closely related to *Paracoccus huijuniae* strain FLN-7^T (similarity = 100%), forming a subclade with *P. huijuniae* FLN-7^T (100% similarity) and *P. aminovorans* DSM 8537 (99.18% similarity) (Fig. S1). Thus, in light of our results, strain P13 was identified as a *Paracoccus* sp. and was deposited at the China Center for Type Culture Collection (CCTCC) under the deposition number CCTCC AB 2015071.

Enrichment culturing is a useful and efficient method to isolate bacterial strains that are able to degrade different pesticides with high efficiency, and the addition of pendimethalin provides a selective pressure for the evolution of microorganisms that could degrade this xenobiotic compound. *Paracoccus* species are widely distributed in soils and involved in the degradation of various xenobiotics and pollutants. Numerous *Paracoccus* sp. have been isolated, for example, *Paracoccus* sp. FLN-7 has been reported to degrade diflufenzuron [26], *Paracoccus* sp. M-1 has been shown to degrade monocrotophos [5], and *Paracoccus* sp. QCT6 has been reported to degrade metribuzin [4]. This is the first report of a *Paracoccus* sp. that can use pendimethalin as the carbon source for cell growth and degrade it. Further study of this genus may provide a better understanding of the ecological diversity of these organisms and their usefulness for biodegradation.

Utilization and Degradation of Pendimethalin by *Paracoccus* sp. P13

The observed growth of *Paracoccus* sp. P13 in liquid MSM containing 200 mg/L pendimethalin and its capacity to degrade pendimethalin are presented in Fig. 1. The growth curve showed a steady increase in P13 population, and the cell density increased from 1.0×10^6 to 3.42×10^7 CFU/mL within 5 days. In the meantime, UHPLC analysis showed a substantial reduction of the pendimethalin concentration in the medium, as 50% of the initial 200 mg/L pendimethalin was degraded by strain P13 within 2 days, and the degradation rate reached 99.8% within 5 days. No cell growth was observed when strain P13 was incubated in the cultures

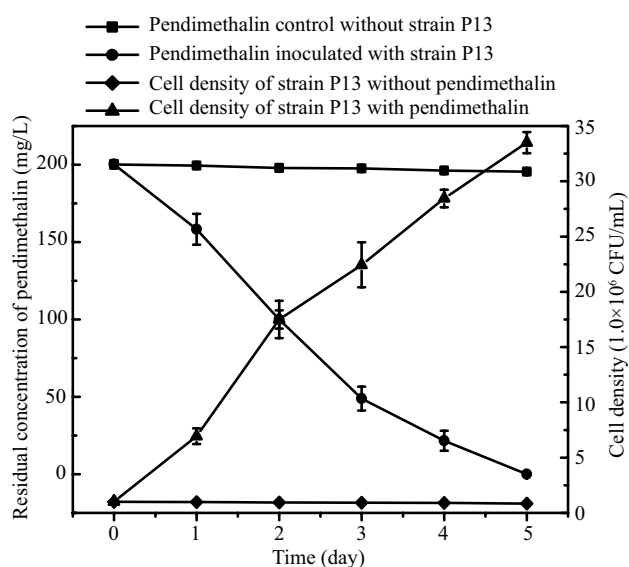


Fig. 1 Utilization and degradation of pendimethalin during cell growth of strain P13 in MSM with pendimethalin as the sole carbon source

without pendimethalin, and no significant change in the pendimethalin concentration was observed in uninoculated medium. The consumption of pendimethalin supported the cell growth of strain P13, suggesting that this microorganism could degrade pendimethalin and utilize it as a carbon source for growth.

In this study, *Paracoccus* sp. P13 was observed to degrade 100 mg/L pendimethalin within 2 days and 200 mg/L pendimethalin within 5 days. *A. chroococcum* was previously shown to degrade 45 and 55% of 25 µg/mL pendimethalin within 10 and 20 days [7], respectively. *Lecanicillium sakseanae* degraded 99.5% of 25 mg/L pendimethalin within 10 days [14]. *P. aeruginosa*, *B. mycoides*, and *B. cereus* could degrade 80.75, 71.75, and 80.25% of an initial 400 mg/L pendimethalin within 30 days, respectively [19]. *Bacillus* sp. Y3 degraded 99.5% of 100 mg/L pendimethalin within 2.5 days [12]. Compared with these pendimethalin-degrading strains, strain P13 possessed a relatively high degradation efficiency, suggesting that this microorganism may have a greater potential in eliminating pendimethalin residue pollution.

Metabolites of Pendimethalin Degradation by Strain P13

Identification of the target compound in samples was based on determining HPLC retention times (RTs) compared to that of a standard, and the identification of pendimethalin metabolites was performed by UHPLC–MS/MS. By injecting a standard sample and comparing the RT, a peak with a RT of 18.64 min was detected as pendimethalin, which had

a molecular peak $[M + H]^+$ at m/z 282.1448 and a base peak $[M + H]^+$ at m/z 212.1212 due to oxidation *N*-dealkylation. In the tested sample, only one metabolite was detected with a RT of 3.29 min (Fig. 2). The MS/MS spectrum of this metabolite indicated that it had a molecular peak $[M + H]^+$ at m/z 266.1354, in agreement with the molecular formula of $C_9H_{19}N_3O_6$. The fragmentation pattern showed the base peak $[M + H]^+$ was at m/z 166.0024, which was due to the removal of *N*-alkyl group and nitroreduction of one nitro group. According to the above analysis, the metabolite was identified as 1,3-dinitro-2-(pentan-3-ylamino)butane-1,4-diol. Therefore, the proposed biochemical pathway of pendimethalin degradation in strain P13 was figured out (Fig. 3): pendimethalin first underwent oxidative ring cleavage to form 1,3-dinitro-2-(pentan-3-ylamino)butane-1,4-diol, with a subsequent series of enzymatic reactions resulting in the production of CO_2 and H_2O .

Previous reports have indicated that some microorganisms can degrade pendimethalin, however the degradation pathway of pendimethalin was diverse. In *A. chroococcum*, three mechanisms were involved in pendimethalin degradation: nitroreduction to form 6-aminopendimethalin, oxidation *N*-dealkylation to produce 3,4-dimethyl-2,6-dinitroaniline, and arylmethyl group oxidation to yield [6-methyl-3,4-dinitro-3-(pentanyl-3-amino)phenyl]methanol [7]. 6-Aminopendimethalin and 3,4-dimethyl-2,6-dinitroaniline were identified as pendimethalin degradation products by *B. circulans* and *B. lehensis* XJU [10, 11]. Pendimethalin was observed to be initially degraded by nitroreduction, and three metabolites, 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 [12]. Pendimethalin degradation involved nitroreduction of both nitro groups and demethylation of one methyl group, and the metabolite was identified as *N*-(1-ethylpropyl)-3-methyl-2,6-diaminobenzene in *P. aeruginosa* [19]. *N*-dealkylation and nitroreduction are the very common pathways of microbial transformation of pendimethalin and other dinitroaniline compounds, which directed to detoxify their herbicidal activities [7]. Sequentially, the metabolites are cleaved by the ortho- or meta-cleavage pathway as that used for catechol in microorganisms [6]. In this study, *Paracoccus* sp. P13 was determined to initially degrade pendimethalin by oxidative ring cleavage to produce the alkane metabolite 1,3-dinitro-2-(pentan-3-ylamino)butane-1,4-diol, whereas previously identified metabolites of pendimethalin biodegradation were all aromatic products. This indicated that a different process involving unique degrading enzymes is responsible for the biodegradation of this dinitroaniline herbicide in *Paracoccus* sp. P13. Further studies with additional molecular biological techniques are needed to better understand the details of pendimethalin degradation by strain P13.

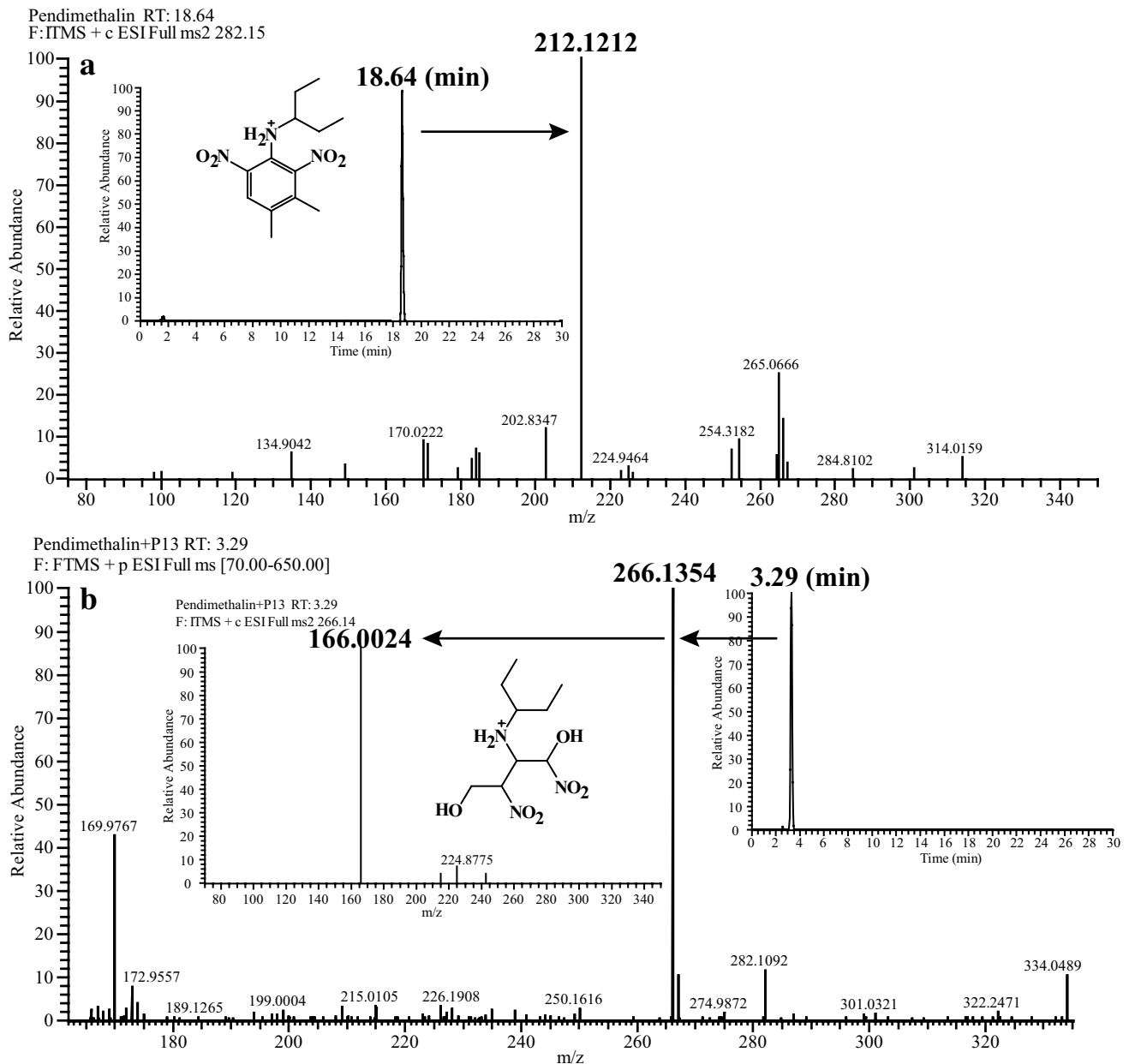


Fig. 2 UHPLC–MS/MS analysis of the metabolite of pendimethalin degradation by strain P13. **a** UHPLC–MS/MS analysis of pendimethalin, RT=18.64 min, the molecular peak $[M+H]^+$ was at m/z 282.1448, and the base peak $[M+H]^+$ was at m/z 212.1212; **b**

UHPLC–MS/MS analysis of the metabolic product, RT=3.29 min, the molecular peak $[M+H]^+$ was at m/z 266.1354, and the base peak $[M+H]^+$ at m/z 166.0024

Effect of pH and Temperature on Pendimethalin Degradation by Strain P13

Factors such as pH and temperature can influence the growth of microorganisms, and hence influence their ability to degrade pesticides. The effect of pH on pendimethalin degradation by strain P13 is shown in Fig. 4. The maximum degradation rate was observed at pH 7.0, and strain P13 could degrade >75% of 200 mg/L pendimethalin at pH

values of 6.0–8.0. The degradation rate was significantly decreased under strong acid conditions (pH values below 4.0). The optimum temperature for pendimethalin degradation was determined to be 30 °C (Fig. 5). More than 80% of 200 mg/L pendimethalin was degraded at 25–35 °C, and pendimethalin degradation was inhibited at 20 and 40 °C.

Microorganisms are a major component of the natural environment and play a considerable role in the biodegradation of herbicides. Additional studies of the biodegradation

Fig. 3 Proposed biodegradation pathway of pendimethalin in strain P13

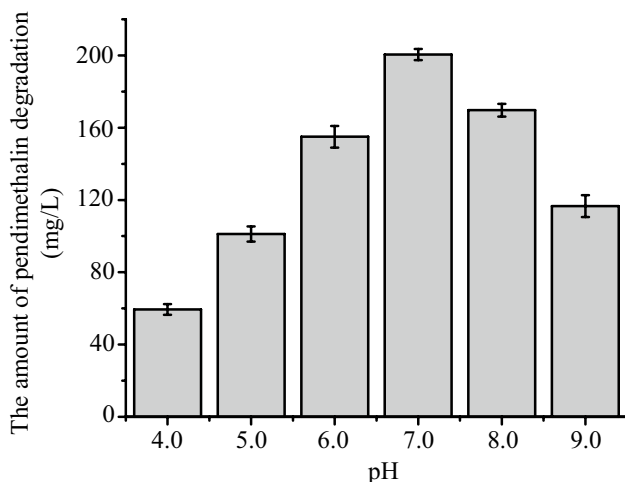
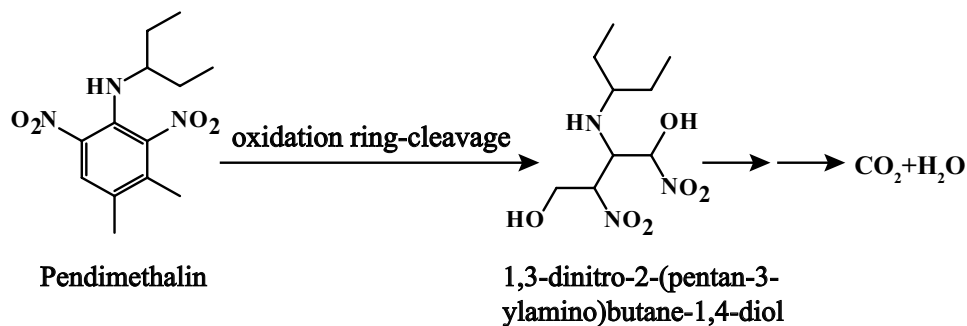


Fig. 4 Effect of pH on pendimethalin degradation by strain P13

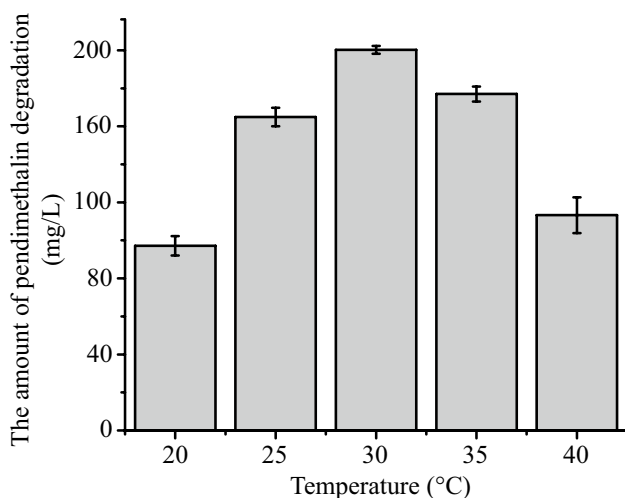


Fig. 5 Effect of temperature on pendimethalin degradation by strain P13

characteristics in *Paracoccus* sp. P13 will further our understanding of how microorganisms are involved in the biodegradation of dinitroaniline herbicides.

Conclusion

Pendimethalin is a widely used herbicide with a high herbicidal efficiency. The dinitroaniline herbicide pendimethalin is very stable and persists in the environment. Pendimethalin exhibits high toxicity to terrestrial and aquatic invertebrates and is a possible human carcinogen. Microbial metabolism is the most important factor in the degradation of pendimethalin in soil. Strain P13 was isolated from the soil of a fruit garden and was characterized as a *Paracoccus* sp. that could degrade approximately 100 and 200 mg/L pendimethalin after 2 and 5 days of incubation, respectively. An alkane metabolite, 1,3-dinitro-2-(pentan-3-ylamino)butane-1,4-diol, was identified as a product of P13-mediated pendimethalin degradation by UHPLC–MS/MS. This finding suggested that there may be a new degradation mechanism of pendimethalin in strain P13 that differs from those that are commonly observed, the details of which will need to be further studied.

Acknowledgements This work was financially supported by the National Natural Science Foundation of China (31760031 and 31600080), the Natural Science Foundation of Jiangxi Province (20171BAB214002), and the Natural Science Foundation of Shandong Province (ZR2016CB29).

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