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Microbial Degradation of Fomesafen by a Newly Isolated Strain *Pseudomonas zeshuii* BY-1 and the Biochemical Degradation Pathway

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ABSTRACT: Fomesafen is a diphenyl ether herbicide used to control the growth of broadleaf weeds in bean fields. Although the degradation of fomesafen in soils was thought to occur primarily by microbial activity, little was known about the kinetic and metabolic behaviors of this herbicide. This paper reported the capability of the newly isolated strain *Pseudomonas zeshuii* BY-1 to use fomesafen as the sole source of carbon in pure culture for its growth. Up to 88.7% of 50 mg of L^{-1} fomesafen was degraded by this bacterium in mineral medium within 3 days. Strain BY-1 could also degrade other diphenyl ethers, including lactofen, acifluorfen, and fluoroglycofen. During the fomesafen degradation, five metabolites were detected and identified by liquid chromatography−mass spectrometry and tandem mass spectrometry. The primary degradation pathway of fomesafen might be the reduction of the nitro group to an amino group, followed by the acetylation of the amino derivative, dechlorination, and cleavage of the S−N bond. The addition of the BY-1 stain into soils treated with fomesafen resulted in a higher degradation rate than that observed in uninoculated soils, and the bacteria community in contaminated soil recovered after inoculation of the BY-1 stain. On the basis of these results, strain *P. zeshuii* BY-1 has the potential to be used in the bioremediation of fomesafen-contaminated soils.

KEYWORDS: *Diphenyl ether herbicide, fomesafen, Pseudomonas zeshuii, degradation pathway*

INTRODUCTION

Fomesafen, belonging to the members of the diphenyl ethers, has been used to control the growth of broadleaf weeds in bean fields, particularly soybean fields.[1−3] Because of its high herbicidal activity and low application rates, fomesafen has been widely used in soybean fields since its introduction in China. Because of its long-lived persistence, phytotoxicity, and negative effects on crop rotation, fomesafen has been listed as a soil contaminant in recent years.[4] Microbial degradation is the primary process affecting the persistence of herbicides in the environment. Previous studies reported that diphenyl ethers could be metabolized by bacteria, fungi, and higher plants. An aerobic fomesafen-degrading bacterium ZB-1 was isolated from soil samples and identified as a member of the genus *Lysinibacillus*.[5] Keum et al. revealed that *Sphingomonas wittichii* RW1 is capable of degrading chlomethoxyfen, nitrofen, and oxyfluorfen.[6] *Azotobacter chroococcum* could grow in medium containing oxyfluorfen as the sole carbon source.[7] Smith-Greenier and Adkins isolated six non-fermentative Gram-negative bacilli that are able to grow in minimal medium containing diclofop-methyl.[8] Liang et al. described the biotransformation of lactofen by *Brevundimonas* sp. LY-2.[9] The metabolism of fomesafen in plants has also been studied. Soybeans are able to efficiently detoxify fomesafen through glutathione *S*-transferase (GST)-mediated thiol conjugation.[10]

The pathways of microbial degradation for several diphenyl ethers have been investigated. The reaction resulted in the reduction and acetylation of the nitro group and dechlorination of fomesafen by *Lysinibacillus* sp. ZB-1.[5] Chakraborty et al. showed that *A. chroococcum* reduces the nitro group to yield an amino compound, followed by the acetylation of the amino derivative, O-dealkylation, and dechlorination.[7] In line with Chakraborty's results, Keum et al. also demonstrated that *S. wittichii* RW1 degrades nitrodiphenyl ethers through the initial reduction and N-acetylation of nitro groups, followed by ether bond cleavage.[6] The enantioselectivity of the transformation of lactofen had also been studied.[11] Liang et al. demonstrated a transformation pathway of lactofen by the bacterium, leading to the formation of 1-(carboxy)ethyl-5-(2-chloro-4-(trifluoromethyl)phenoxy)-2-nitrobenzoate and ethanol.[9]

In this study, fomesafen-degrading strain *Pseudomonas zeshuii* BY-1, isolated from agricultural soil that had been polluted with this herbicide for a long time, was tested for its capability of degrading fomesafen by BY-1 in liquid culture. The degradation of fomesafen and its main byproducts were studied in a liquid pure culture of the BY-1 strain. This paper highlights an

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important potential use of pure cultured microbial cells for the remediation of fomesafen-contaminated soils and presents a mechanism for fomesafen degradation.

MATERIALS AND METHODS

Chemicals and Media. Fomesafen (98.3% purity) was purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). Lactofen (80% purity), fluoroglycofen (95% purity), and acifluorfen (90% purity) were purchased from Qingdao Hansen Biologic Science Co., Ltd., China.

Mineral salts medium (MM) contained 1.0 g L^{-1} NH_4NO_3, 1.5 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 0.2 g L^{-1} $MgSO_4$, and 10 mL of a trace element solution at pH 7.0.[12] The Luria−Bertani (LB) medium contained 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 10 g L^{-1} NaCl at pH 7.0. Solid medium plates were prepared by adding 1.5 wt % vol^{-1} agar to the liquid media.

Isolation of the Fomesafen-Degrading Strain. A soil sample was collected from an agricultural field that had been exposed to fomesafen for at least 5 years in the city of Qiqihaer, Heilongjiang, China. Approximately 10 g of the soil sample was added to an Erlenmeyer flask (250 mL) containing 100 mL of MM containing fomesafen (50 mg L^{-1}) as the sole carbon source, and the mixture was incubated on a rotary shaker at 160 rpm for approximately 3 days. Approximately 5 mL of the enrichment culture was transferred to 100 mL of fresh MM containing 50 mg L^{-1} fomesafen every 3 days. The decrease in the fomesafen concentration was measured by high-performance liquid chromatography (HPLC) after the fourth transfer. The enrichment culture that was able to degrade fomesafen was serially diluted and spread onto MM agar plates containing 50 mg L^{-1} fomesafen. After incubation at 30 °C, colonies were picked and further purified using the streak plating method. Of several isolated bacterial strains, the pure isolate with the fomesafen-degradation efficiency, designated as BY-1, was selected for further study.

Degradation of Fomesafen and Other Diphenyl Ethers by Strain BY-1 in Liquid Culture. After growth in LB medium at 30 °C for 12 h, strain BY-1 was centrifuged at 5000 rpm for 10 min and the cell pellets were washed twice with fresh MM. After the optical density at 600 nm (OD_{600}) had been adjusted to 1.0, an inoculum (2%, vol/vol) was transferred into 100 mL of MM containing fomesafen (50 mg L^{-1}). Three samples of 100 mL cultures were incubated at 30 °C and 160 rpm on a rotary shaker. A total of 3 mL of sample was collected from the 100 mL cultures at an interval of 12 h, and the concentration of the herbicides was determined by HPLC following the protocol described below. Bacterial growth was monitored by measuring the OD_{600}.

The degradation of other diphenyl ethers by strain BY-1 was also studied. The MM was supplemented with lactofen, fluoroglycofen, or acifluorfen at 50 mg L^{-1}, and the concentration of herbicide was determined by HPLC.

To determine the effect of the initial concentration of fomesafen and the temperature on degradation, four concentrations (10.0, 50.0, 100.0, or 200.0 mg L^{-1}) in the MM and three different incubation temperature (20, 30, or 35 °C) were used.

Chemical Analysis. For fomesafen extraction from liquid culture, 3 mL of dichloromethane was added to 3 mL of sample collected from the medium. After shaking for 1 min, the dichloromethane phase was dried over anhydrous Na_2SO_4 and the solvent was removed using a stream of nitrogen at room temperature. The residues were dissolved in 200 μL of methanol. The samples were then filtered through a 0.22 μm Millipore membrane filiter. An aliquot of the solution (20 μL) was injected into a HPLC system for detection.

The fomesafen concentrations in all samples were analyzed by HPLC using a Zorbax C-18 ODS Spherex column (250 × 4.6 mm). The mobile phase was methanol/water/acetic acid (70:30:0.1, v/v/v), and the flow rate was 1.0 mL min^{-1}. The eluate was monitored by measuring the A_{230} with a Waters 2487 dual wavelength absorbance detector.

The metabolites produced during fomesafen degradation were identified by liquid chromatography−mass spectrometry (LC−MS) and tandem mass spectrometry (MS/MS). Liquid culture samples were collected when the degrading rates of fomesafen were 30, 50, and 70%.[13] Three collected samples were combined together and extracted as described above. The metabolites in the combined extracts were separated and confirmed by standard MS, ionized by electrospray with a positive polarity, and scanned in the normal mass. Characteristic fragment ions were detected with second-order MS.

The MS apparatus was a LC−mass selective detector (MSD) Trap SL system equipped with an electrospray ionization (ESI) source and was operated in the positive polarity mode (Agilent LC/Q-TOF, 6520). The ESI−MS interface was operated using a gas temperature of 350 °C and a drying gas flow of 9.0 L min^{-1}. The nebulizer nitrogen gas pressure was 45 psi. Full-scan signals were recorded within the m/z range from m/z 50 to 1000. For LC−MS, the spray voltage was 7.0 kV. The sheath and auxiliary gases were nitrogen. The sheath and auxiliary gases were adjusted to 65 and 10 arbitrary units, respectively. Auto Gain Control mode was used to optimize the injection time.

Inoculation and Degradation in Soil. The soil used in this experiment was collected from an agricultural field in Jiangsu province and has never been treated with fomesafen. Soil samples (500 g) were air-dried, sieved to 2 mm, and homogenized.[14] Fomesafen was added at a concentration of 20 mg kg^{-1} of dry soil. The treated soil was inoculated with BY-1 cells at a concentration of 4.3×10^7 colony-forming units (cfu) g^{-1} of dry soil, and control soil was inoculated with heat-killed BY-1 cells (autoclaved at 121 °C for 25 min). The inoculum was thoroughly mixed into the soils, and the moisture was adjusted to 25% (w/w of dry weight of soil) with sterile water. Every soil sample set was placed at 30 °C in the dark. After 30 days, 20 g of sub-samples was collected and analyzed immediately. All treatments were replicated 3 times.

Fomesafen extraction from soil was conducted according to a previous report.[15] Fomesafen concentrations in all samples were analyzed by HPLC.

Soil DNA Extraction and Polymerase Chain Reaction (PCR) Amplification. Total soil DNA was extracted from the soil of different treatments using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following the protocol of the manufacturer. Extracted total DNA was visualized by 1% agarose gel electrophoresis and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Finally, the extracted DNA was diluted to 10 ng mL^{-1} prior to PCR amplification.

PCR amplification was performed using 2 μL of extracted DNA as a template and the general bacterial primer PRBA338F[16] with a 40 base pair (bp) GC clamp[17] attached to its 5′ end and the PRUN518R primer.[18] The final volume of 25 μL for the PCR mixtures contained the following components: 2.5 μL of 10× buffer (Mg^{2+} free), 1.5 μL of 25 mmol mL^{-1} Mg^{2+}, 2 μL of 2.5 mmol mL^{-1} dNTP mixture, 0.3 μL of 5 units μL^{-1} Ex Taq polymerase (TaKaRa Bio, Inc., Shiga, Japan), 1 μL of each primer (10 mmol mL^{-1}, Invitrogen Corporation, Carlsbad, CA), 1 μL of DNA template, and 15.7 μL of H_2O. The PCR program used the following conditions: an initial denaturation step of 95 °C for 5 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. The PCR products were assessed by 2% agarose gel electrophoresis.

Denaturing Gradient Gel Electrophoresis (DGGE) and Cluster Analysis. DGGE was performed on PCR products described above, using 8% (wt/vol) acrylamide gels containing a linear urea-formamide gradient ranging from 40 to 60% denaturant (with 100% defined as 7 M urea and 40% formamide). Aliquots of PCR products (20 μL) were mixed with 10 μL of loading dye and run for 16 h at 80 V on a D-code universal mutation system (Bio-Rad Laboratories, Hercules, CA). The gel was visualized by silver staining and scanned with a root scanner (Epson Perfection, version 33, Seiko Epson Corporation, Japan).[19]

The DGGE images were analyzed by Quantity One Software (version 4.6.3, Bio-Rad Laboratories) for band detection and intensity. The lanes were normalized on the basis of the assumption that they contained equal amounts of total signal after background subtraction.

Cluster analysis was performed using the UPGMA algorithm (Quantity One, version 4.6.3, Bio-Rad Laboratories).

RESULTS AND DISCUSSION

Isolation and Identification. A strain designated BY-1, which could degrade 50 mg L^{-1} fomesafen in 3 days, was isolated and selected for further study. According to its physiological characteristics, 16S rRNA phylogenetic analysis, DNA−DNA hybridization, and major fatty acids analysis, strain BY-1 was identified as *P. zeshuii*.[20]

Biodegradation of Fomesafen and Other Diphenyl Ethers by Strain BY-1. The growth of strain BY-1 on fomesafen and the ability of this bacterium to degrade fomesafen are shown in Figure 1. After incubation for 3 days,

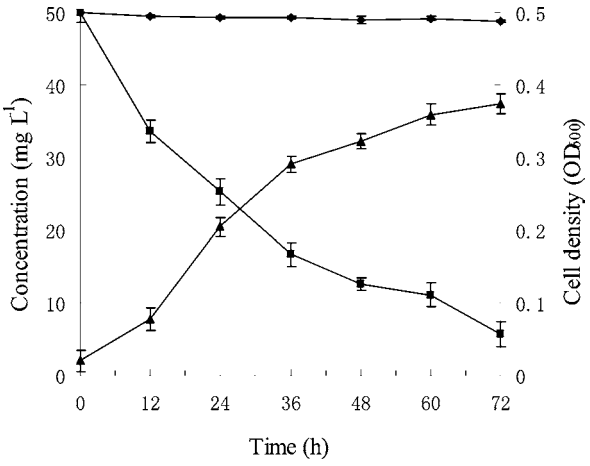

Figure 1. Decrease of fomesafen in a pure liquid culture of the *P. zeshuii* BY-1 strain: (◆) control (no bacteria), (■) degradation of fomesafen in the presence of the BY-1 strain, and (▲) cell growth. The data are represented as the mean ± standard deviation for triplicate incubations.

approximately 88.7% of the 50 mg L^{-1} fomesafen was degraded by strain BY-1 when fomesafen was used as the sole carbon source. The OD measurements at 600 nm showed a steady increase in the bacterial mass. HPLC analysis of culture media during fomesafen degradation by strain BY-1 revealed transient accumulation of five metabolites, which were subsequently identified by LC−MS and MS/MS. Analysis of the degradation of other diphenyl ethers revealed that strain BY-1 could degrade 68.9% lactofen, 53.1% acifluorfen, and 70.3% fluoroglycofen within 3 days in the MM.

Several reports on the microbial degradation of diphenyl ethers have been published. A total of 75 and 100% of 100 mg L^{-1} oxyfluorfen and nitrofen, respectively, were degraded within 7 days by *S. wittichii* RW1 in nutrient broth-supplemented M9 medium.[6] *A. chroococcum* could use oxyfluorfen as the sole carbon source and could degrade more than 60% of added oxyfluorfen at a concentration of 250 mg L^{-1} in 7 days.[7] *Sphingomonas paucimobilis* was able to use diclofop-methyl as the sole source of carbon and completely degraded 1.5 mg L^{-1} diclofop-methyl to yield diclofop acid within 54 h.[21] *Brevundimonas* sp. LY-2 degraded lactofen via the ester bond cleavage catalyzed by esterase.[8] In the current study, a newly isolated strain BY-1 could degrade fomesafen and could degrade several other diphenyl ethers.

Effect of the Concentration and Temperature on Biodegradation. To test the effect of the concentration on fomesafen degradation, the initial fomesafen concentration was varied from 10 to 200 mg L^{-1}. At a concentration of 10.0 mg L^{-1}, the residual level of fomesafen could not be detected after 3 days. At high concentrations of 100 and 200 mg L^{-1}, 58.2 and 33.4% fomesafen were degraded after 3 days, respectively (Figure 2).

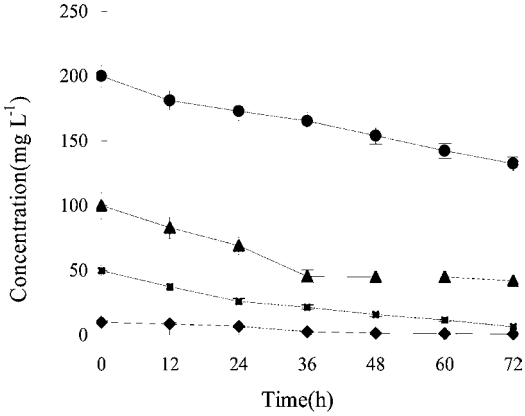

Figure 2. Effect of the initial concentration of fomesafen on its degradation by strain BY-1: (●) 200 mg L^{-1}, (▲) 100 mg L^{-1}, (■) 50 mg L^{-1}, and (◆) 10 mg L^{-1}. The data are represented as the mean ± standard deviation for triplicate incubations.

The percent degradation rates for BY-1 were 43.9, 88.4, and 60.5% after incubation for 3 days at 20, 30, and 35 °C, respectively, whereas the degradation rates for the control were 2.4, 3.8, and 5.5%, respectively. The optimal temperature for degradation by strain BY-1 was determined to be 30 °C (Figure 3).

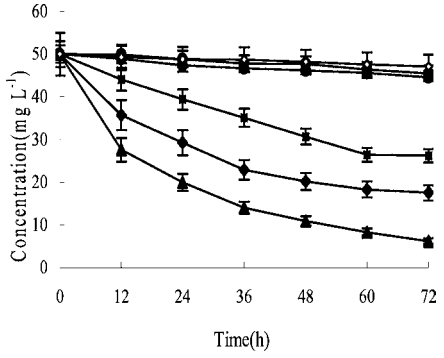

Figure 3. Effect of the temperature on fomesafen degradation by strain BY-1: (♦) uninoculated medium at 20 °C, (◇) uninoculated medium at 30 °C, (●) uninoculated medium at 35 °C, (■) inoculated medium at 20 °C, (▲) inoculated medium at 30 °C, and (◆) inoculated medium at 35 °C. The data are represented as the mean ± standard deviation for triplicate incubations.

Degradation Products and Degradation Pathway of Fomesafen. The fomesafen degradation products in the culture medium extracts were isolated and identified by LC−MS and MS/MS. In addition to fomesafen, degradation products were detected after 24 h and their concentrations reached the highest level at 36 h, followed by a decrease with longer incubation times. In standard LC−MS, prominent protonated molecular ions were observed at *m/z* 439 $[M + H]^+$, 391 $[M + H]^+$, 429 $[M + Na]^+$, 403 $[M + H]^+$, 419 $[M + H]^+$, and 343 $[M + Na]^+$, and the compounds corresponding to

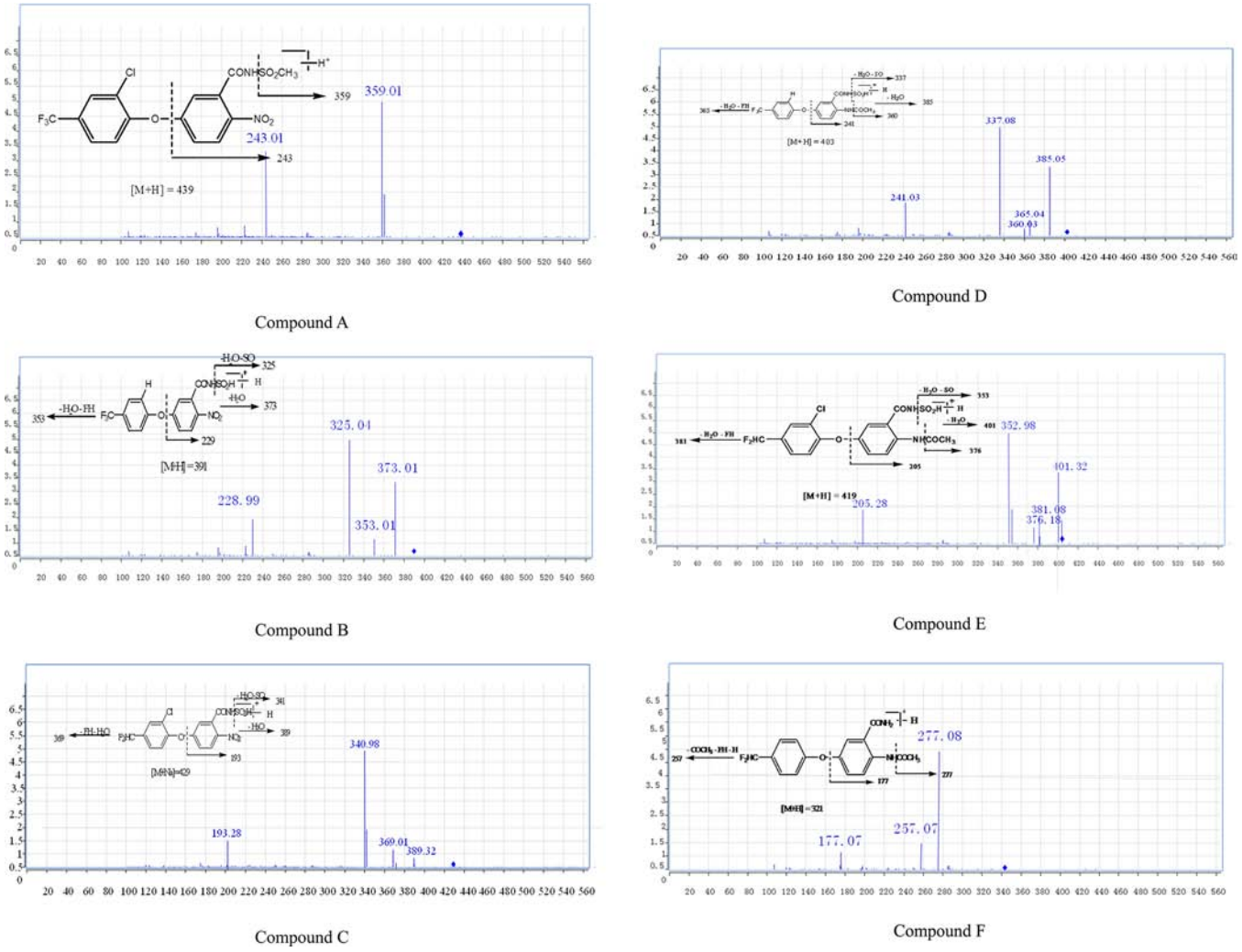

Figure 4. Mass spectrum of metabolites.

the protonated molecular ions were designated as compounds A, B, C, D, E, and F.

The *m*/*z* of compound A was 439 $[M + H]^+$, enabling the assignment of the molecular ion (M^+) at *m*/*z* 438. Compound A, with the characteristic second-order MS fragment ion peaks at *m*/*z* 243 and 359, was identified as fomesafen (Figure 4). Compound B showed a prominent protonated molecular ion at *m*/*z* 391 $[M + H]^+$ and characteristic second-order MS fragment ion peaks at *m*/*z* 229, 325, 353, and 373 (Figure 4); therefore, the molecular weight of compound B is 390. On the basis of the molecular weight and the characteristic fragment ion peaks, compound B was identified as [5-(4-trifluoromethyl-phenoxy)-2-nitro-benzoyl]-sulfinamic acid. Compound C showed a peak at *m*/*z* 429 $[M + Na]^+$, enabling the assignment of the molecular ion (M^+) at *m*/*z* 406. The characteristic second-order MS fragment ion peaks at *m*/*z* 193, 341, 369, and 389 are shown in Figure 4. Compound C was identified as [5-(2-chloro-4-difluoromethyl-phenoxy)-2-nitro-benzoyl]-sulfinamic acid. The presence of compounds B and C indicated that the first steps of fomesafen degradation by strain BY-1 were dechlorination or defluorination and cleavage of the S−N bond. Compound D showed a prominent protonated molecular ion at *m*/*z* 403 $[M + H]^+$ and was identified as {[2-acetamido-5-(4-trifuoromethyl-phenoxy)]-benzoyl}-sulfinamic acid based on its mass spectrum and characteristic fragment ion peaks at *m*/*z* 241, 337, 360, 365, and 385 (Figure 4). Compound E showed a prominent protonated molecular ion at *m*/*z* 419 $[M + H]^+$ and was identified as {[2-acetamido-5-(2-chloro-4-difulromethyl-phenoxy)]-benzoyl}-sulfinamic acid based on its mass spectrum and characteristic fragment ion peaks at *m*/*z* 205, 353, 376, 381, and 401 (Figure 4). These ions correspond to the products of the reduction of the nitro groups of compounds B and C to yield amino compounds, followed by acetylation of the amino derivative. The positive-ion chemical ionization of compound F showed a prominent molecular ion at *m*/*z* 343 $[M + Na]^+$, and the molecular weight of product F is 320. On the basis of its mass spectrum and characteristic fragment ion peaks at *m*/*z* 117, 257, and 277 (Figure 4), compound F was identified as *N*-[4-(difluoromethyl-phenoxyl)-2-formylamino-phenyl]-acetamide. The peak areas in the LC−MS analysis revealed that compound F was the main product of the biodegradation of fomesafen. The degradation pathway of fomesafen by BY-1 might involve the reduction of the nitro group to yield an amino compound, followed by acetylation of the amino derivative, dechlorination, defluorination, and cleavage of the S−N bond (Figure 5).

In this study, five metabolic products were detected and identified for the degradation of fomesafen and a more detailed degradation pathway was proposed than what has been reporated.[5] The microbial degradation of other diphenyl ethers,

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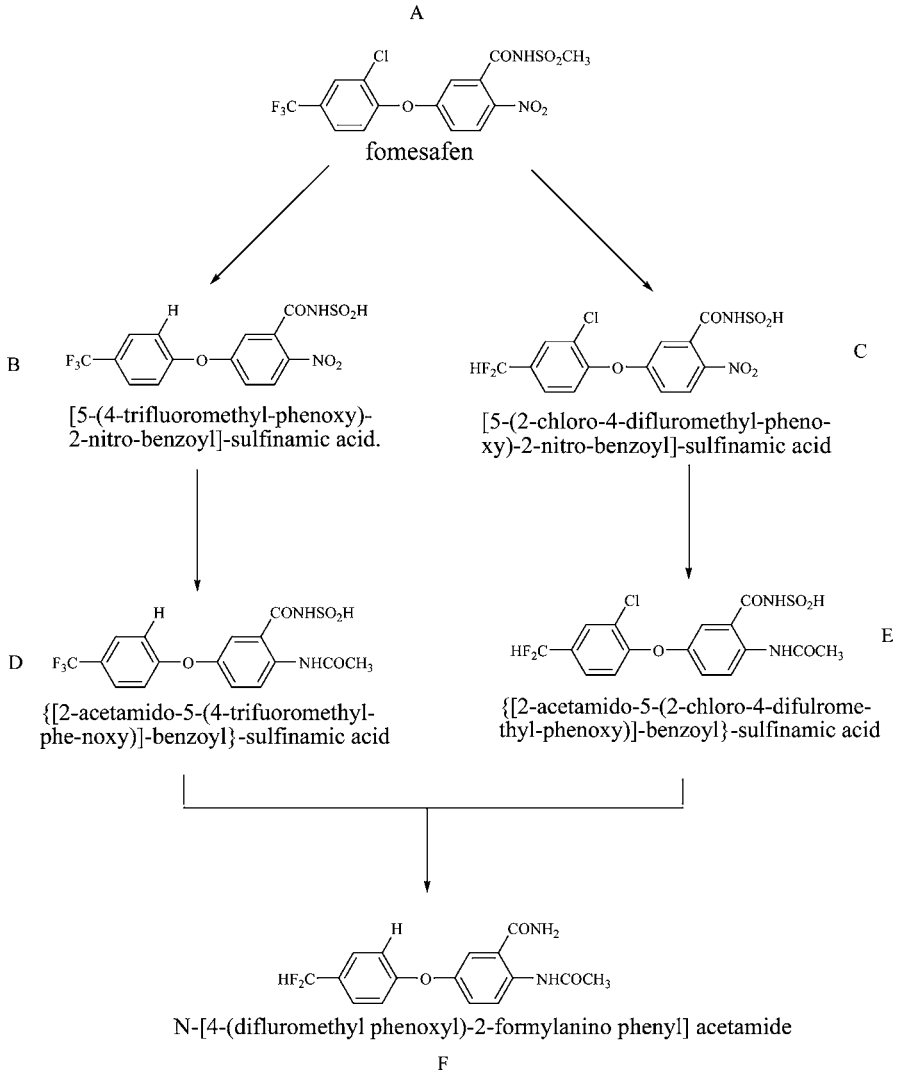

Figure 5. Proposed metabolic pathway of fomesafen degraded by BY-1.

such as oxyfluorfen, by *A. chroococcum* has been reported.[7] Keum et al. also found that nitrodiphenyl ethers could be degraded by *S. wittichii* RW1 through the initial reduction and subsequent N-acetylation of the nitro groups, followed by ether bond cleavage.[6] This study offers useful information about the biodegradation of fomesafen and presents a possible mechanism of fomesafen metabolism in a microorganism.

Effect of BY-1 on the Degradation of Fomesafen in Soil and on Bacteria Diversity. Approximately 75.6% fomesafen in soil was degraded within 30 days after inoculation with the BY-1 stain, while only 9.7% of the initially added fomesafen was degraded in uninoculated soil. The results showed that the BY-1 strain obviously enhanced the degradation of fomesafen in soil.

Cluster analyses of the bacterial communities based on the DGGE profiles of the total DNA amplified by the primer pair 338GC/518 revealed differences in the community composition of the soils between samples with or without fomesafen contamination and between samples inoculated with strain BY-1 or uninoculated (Figure 6A). Fewer bands were observed in the DGGE image of the communities from fomesafen-contaminated soil (lanes 3 and 4) than in the image for the control soil (lanes 1 and 2). When fomesafen-contaminated soil was supplemented with strain BY-1, more bands (lanes 5 and 6) were observed but not as many as in the control soil. The unweighted pair group method with arithmetic mean (UPGMA) dendrograms (Figure 6B) revealed three distinct clusters of DGGE profiles, demonstrating that there were great differences between the soil samples. Lanes 5 and 6 were closely clustered with lanes 1 and 2.

Herbicides have been used with increasing frequency, and the microbial diversity may have markedly changed following pesticide use.[22] Bioremediation is a cost-effective method to degrade toxic compounds into innocuous products. In the present study, strain BY-1 was used to degrade fomesafen in soil and the bacteria community could be recovered through the use of strain BY-1 to degrade fomesafen.

In our study, a fomesafen-degrading bacterium BY-1 was isolated from the soil sample. The strain could degrade fomesafen effectively in liquid culture and soils. Five metabolites have been identified. A high removal rate of fomesafen in soil with strain BY-1 indicates the potential of this bacterium to be useful in the bioremediation of pollution resulting from the use of fomesafen in the environment.

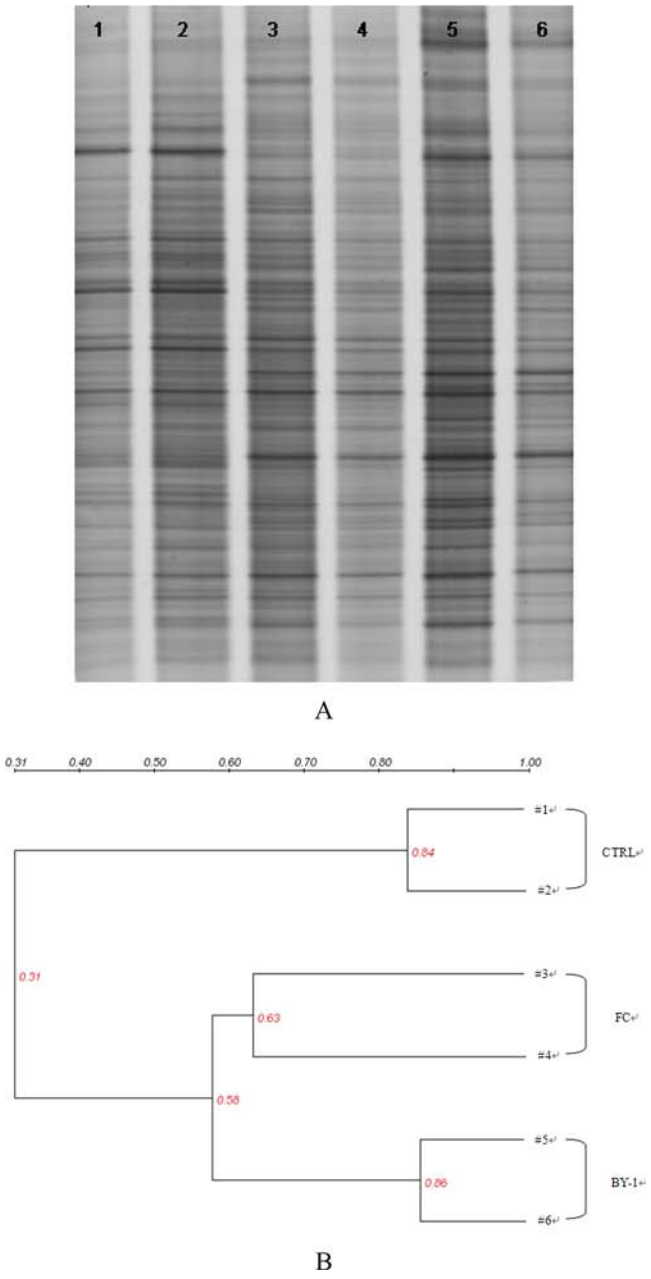

Figure 6. Effect of BY-1 on the degradation of fomesafen in soil and bacteria diversity. (A) DGGE profiles of 16S rRNA genes amplified from soil bacterial communities from replicate soil sub-samples collected from control soil (CTRL, lanes 1 and 2), formesafen-contaminated soil (FC, lanes 3 and 4), and formesafen-contaminated soil amended with strain BY-1 (BY-1, lanes 5 and 6). (B) UPGMA tree is based on a Pearson correlation UPGMA matrix between the different DGGE patterns of different samples.

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Notes

The authors declare no competing financial interest.

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