Screening, Characterization, and Application of *Shigella flexneri* FB5 in Fomesafen-Contaminated Soil

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Abstract

Based on fomesafen residue toxicity in soil, the strain FB5, which can utilize fomesafen as the sole carbon source, was isolated from a fomesafen-contaminated soybean field in Heilongjiang province, China. It was found to degrade 88.32% of 500mg/L fomesafen within 96h. Combined with morphological, physiological, biochemical characteristics and 16S rDNA sequence, the strain was identified as *Shigella flexneri*. After evaluating the effects of environmental factors such as temperature, fomesafen concentration, and primary pH, the optimum growth conditions of the strain were obtained. A remarkable remediation ability of the strain to fomesafen–polluted soils was demonstrated by a sensitive crop bioassay in pot soil. This will provide a new suitable candidate for fomesafen biodegradation and a workable pathway for solving the hazards of fomesafen residues in agricultural soils.

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Keywords: fomesafen; biodegradation; *Shigella flexneri*; soil remediation;

1. Introduction

Fomesafen was one of the diphenyl ether herbicides explored by Zeneca (England), which found that the recommended dose of Fomesafen is 250–375g/hm\textsuperscript{2} [1]. This herbicide plays an important role in the removal of broadleaf weeds in soybean, fruit trees, and rubber estate fields [2]. Along with the succession and multiplication of weeds in soybean farmlands in recent years, fomesafen has been used extensively in...
China. The number of fomesafen production companies increased annually [3]. However, because of the prolonged use of fomesafen over the years, this herbicide has caused adverse effects, such as a decline or absence of harvest of some aftercrops, even hazards to agricultural adjustment and security due to the high residue in soil [1, 4]. Therefore, the effective removal of fomesafen residue toxicity is a critical problem that should be resolved immediately.

Several diphenyl ether degrading strains have been reported, such as *Coriolus versicolor* [5], *Azotobacter chroococcum* [6], *Sphingomonas wittichii* RW1 [7] and so on [8]. Also as one of the same kind herbicide, only few reports on fomesafen-degrading strains are available. Only two such strains, namely, *Aspergillus niger* S7 (a fungus) [9] and *Lysinibacillus* sp. ZB–1 (a bacterium) [10], were reported only recently. In recent years, researchers have obtained certain remediation effects, e.g. *Pseudomonas putida* and *Bacillus subtilis* to crude oil [11], *Phlebia brevispora* to pesticides [12], seven isolated strains to heavy metals [13], and *Rhodococcus jialingiae* djl-6-2 to germicide pollutants [14]. However, few studies have reported on the remediation of diphenyl ether herbicide pollution. The experiments were performed to study the degradation of chlornitrofen [15] and diphenyl ethers [16] using *Phlebia brevispora* and *Lentinus tigrinus*, respectively. Of the two fomesafen-degrading strains described above, the aftercrop remediation trials has not been reported yet. Hence, obtaining highly efficient fomesafen-degrading strains and developing an available method to apply this strain to polluted soils are necessary.

In the present study, the *Shigella flexneri* strain FB5 capable of degrading fomesafen was isolated and identified, and a sensitive plant test was successfully performed in pot soil. This strain will provide a good microbial resource and theoretical basis for the bioremediation of fomesafen-contaminated soils.

2. Materials and Method

2.1. Materials

The soil sample used for microbe isolation was collected from a fomesafen-contaminated soybean field in Heilongjiang Province, China. The average concentration of fomesafen residue was approximately 4.86mg/kg. The soil used for the sensitive plant test was collected from the same field not yet exposed to fomesafen, and sterilized for use. Both soil samples were filtered through 40 mesh, detected by high-performance liquid chromatography.

The seeds of aftercrops were purchased from the Heilongjiang Agriculture Academy.

The mineral salt medium (MSM) contained (g/L) NaCl 1.0, NH₄NO₃ 1.0, K₂HPO₄ 1.5, KH₂PO₄ 0.5, MgSO₄·7H₂O 0.1. The MSM was sterilized at 121°C for 15 min. An amount of fomesafen was added as the sole carbon source at 50–60°C.

The reagents used mainly were fomesafen standard (99% purity; Sigma–Aldrich Co.), fomesafen (25% water aqua; Dalian Songliang Chemical Industry Co. Ltd), methanol (chromatographic pure; Shandong Yuwang Industrial Co. Ltd.), and glacial acetic acid (analytical reagent; Tianjin Guangfu Fine Chemical Research Institute). The common reagents were purchased from Sangon Biotech (Shanghai) Co., Ltd.; polymerase chain reaction (PCR) and electrophoresis reagents were purchased from Takara Biotechnology (Dalian) Co., Ltd. The PCR oligo synthesis and sequence determination was performed by Invitrogen Life Technologies.

The instruments used in the present study were high-performance liquid chromatograph (HPLC) (CBM–102; Shimadzu Corporation), ultraviolet–visible spectrophotometer (TU–1810; Beijing Purkinje General Instrument Co., Ltd.), scanning electron microscope (SEM) (S–3400N; Hitachi, Ltd.), transmission electron microscope (TEM) (H7650; Hitachi, Ltd.), PCR Thermocycler (TPSG; Biometra Analytik Jena Company), and gel-imaging system (Imager HP; Alpha Innotech Company), high-speed freezing centrifuge (CF16RXII; Hitachi, Ltd.).
2.2. Isolation and degradation of the microbes

The soil sample was put in a 100mg/L fomesafen MSM, incubated at 30°C, and shaken at 180rpm, and then transferred into a fresh MSM, which improved 100mg/L of fomesafen every 7d. The enrichment culture was spread onto an MSM agar plate containing 500mg/L fomesafen [17]. Then good colonies were selected and purified as target strains.

The target strain \( A_{600}=1.0, 1\% \) was inoculated into 500mg/L fomesafen MSM (pH 7.0), incubated at 30°C, and shaken at 180rpm. A culture without inoculating the strain was set as control. Three replicates were performed. The curves of the strain growth and fomesafen degradation was drawn by sampling every 12h after incubation. The fomesafen in the liquid culture was extracted by dichloromethane, diluted by methanol, and detected by HPLC. The chromatographic conditions were as follows: chromatographic column, Waters DELTA PAK C\(_{18}\) (300mm × 3.9mm, 15μm); column temperature, 30°C; mobile phase, methanol, acetic acid, and water =60:0.5:40; detection wave, 280nm; fluid speed, 1.0ml/min; and injection volume, 20µl. Fomesafen degradation percentage was calculated using formula (1).

\[
\text{Fomesafen degradation}\% = 1 - \left( \frac{\text{The treated residue}}{\text{The control residue}} \right) \times 100. \quad (1)
\]

2.3. Identification of the strain

The morphological features of the strain FB5 in LB were observed using optical microscopes, SEM, and TEM. Physiological and biochemical reactions of the strain were performed in accordance with Bergey's Manual of Systematic Bacteriology [18]. The concentration of antibiotic used in the present study was 50μg/mL. 16S rDNA was amplified from the genome DNA by PCR using bacterial universal primers [10]. The segment was linked to the pMD 18-T Vector at 16°C overnight, and then transferred into an Escherichia coli DH5α competent cell prepared using CaCl\(_2\) [19]. After incubation at 37°C for 18h, the positive colony was selected by PCR and restriction analysis, and sent for sequencing. The sequence obtained was blasted with the sequences submitted to GenBank. Then, the phylogenetic tree of the strain was constructed to analyze its homology to other strains by the Neighbor-Joining of MEGA 5.03.

2.4. Effects of environmental factors on strain growth

The strain was made into a suspension of \( A_{600}=1.0 \). A total of 1% volume ratio suspension was inoculated into 500mg/L fomesafen MSM (pH 7.0), and incubated at 28, 30, 35, 37, and 40°C; 1% suspension were inoculated into 250, 500, 1,000, 2,000, and 4,000mg/L fomesafen MSM, incubated at 37°C; 1% suspension were inoculated into pH 5.0, 6.0, 7.0, 8.0, and 10.0 in the fomesafen MSM, also incubated at 37°C. All above were shaken at 180rpm. The effects of temperature, fomesafen concentration and primary pH on strain growth were observed by detecting \( A_{600} \) after incubation for 96h. A culture without the strain was set as the control. The experiments were performed in three replicates.

2.5. Bioassay trials of the strain in pot soil

Sensitive aftercrops (maize and sorghum) were chosen as plant indicators. The full seeds were selected and soaked to force sprouting under a constant temperature incubator for 48h [20,21]. The following soil pots were prepared, three replicates each: a pot without fomesafen or the strain (Blank); a pot without fomesafen but with the strain (CK1); a pot with fomesafen but without the strain (CK2); and a pot with fomesafen and the strain (treatment). The seeds with strong radicle were selected and sown at the same time after the soil was treated with the strain for 5, 10, 15, 20, 25, and 30d. Based on the residue
concentration of the soil sample used for microbes isolation, the concentration of fomesafen in the control and treatment was 5mg/kg soil. The inoculation amount of the strain was $10^9$ cfu/kg soil. After incubation at 27–30°C in a light room for 5d, the biomass (dry weight and root length) of the plant, were recorded. The remediation efficiency of the strain was statistically analyzed using Excel and SPSS [22].

3. Results and discussion

3.1. Selection and dynamics curve of strain FB5

The strain FB5 capable of utilizing fomesafen for growth was isolated from the soil sample. It could degrade 88.32% of 500mg/L fomesafen within 96h with good repeatability, as shown by HPLC. Based on the growth curve, FB5 was at the lag phase in the fomesafen MSM before 24h, and then it entered the log phase thereafter and began to decline after 36h, when the peak and the strain may be at the most active point. Other hand, seen from the degradation curve, FB5 increased sharply after incubation for 24h, and the optimum period of degradation was between 24 and 72h. Subsequently, a stable stage was achieved between 72 and 96h, during which fomesafen was almost exhausted. The reduction in biomass may be due to the death of partial cells caused by the lack of elements necessary for growth (Fig. 1).

![Fig. 1. Curves of strain FB5 growth and fomesafen degradation.](image)

3.2. Identification of strain FB5

The shape and size of FB5 cell were observed using an SEM by magnifying it 12,000-fold (Fig. 2(a)). The separated cell was observed as axiolitic, fimbriae without flagellum, and some separating cells were dumbbell-shaped, using a TEM, 30,000-fold (Fig. 2(b)). The morphological, physiological, and biochemical features, as well as 30 indices, of the strain FB5 are presented in Table 1.

![Fig. 2. Micrographs of fomesafen-degrading strain FB5.](image)

(a) Scanning electron micrograph (12,000×); (b) Electron transmitted micrograph (30,000×)
Table 1. Morphological, physiological, and biochemical features of strain FB5

| Index                  | State       | Index                  | State       | Index                  | State       |
|------------------------|-------------|------------------------|-------------|------------------------|-------------|
| Colonial Shape         | Round       | Sorbitol               | –           | Tetracycline           | –           |
| Colonial Color         | Milk white  | Lactose                | –           | Kanamycin              | –           |
| Cellular Shape         | Rod         | Sucrose                | –           | Ampicillin             | +           |
| Cellular size (μm)     | (0.5–0.7)×(1.2–1.6) | Gelatin liquefaction   | –           | Cefoperazone sodium    | +           |
| Methyl red             | –           | Amylohydrolysis        | –           | Chloramphenicol        | +           |
| Salicin                | –           | V.P. test              | –           | Amoxicillin            | +           |
| G+/G–                  | –           | Esculin soluble        | –           | Ceftriazone sodium     | –           |
| Flagella               | –           | H₂S production         | –           | Streptomycin           | +           |
| Spore                  | –           | Urease                 | –           | Cefotaxime sodium      | +           |
| Pellicle               | –           | Oxidase                | –           | Spectinomycin          | +           |

+, positive growth; –, no growth.

The 16S rDNA sequence (1541bp) was found 99% similar to genus Shigella by blasting. The accession number HQ701686 in GenBank and No.4410 in CGMCC were assigned to the strain. Based on the features in Table 1, and the homology analysis of its phylogenetic tree (Fig. 3), FB5 was identified as Shigella flexneri; this genus is most commonly seen in clinical pathogenicity [23–25] and drug resistance research [25–28]. However, the strain was never reported among the fomesafen-degrading strains.

Fig. 3. Phylogenetic tree of strain FB5. The numbers in parentheses represent the accession number of the sequences in GenBank. The number at each node is the percentage supported by bootstrapping 100 times. Bar, 0.02% sequence divergence.

3.3. Effects of temperature, fomesafen concentration, and primary pH on strain FB5 growth

FB5 was able to grow in fomesafen MSM at 28–40°C and peaked at 37°C (Fig. 4(a)). Different growth of FB5 were observed in 250–4000mg/L of fomesafen MSM, and the higher was in 500mg/L (Fig. 4(b)). The biomass of the strain at primary pH 7 was higher than that of other pH values (Fig. 4(c)).
Compared with the only two fomesafen-degrading strains (Aspergillus niger S7 [9] and Lysinibacillus sp. ZB-1 [10] aforementioned), FB5 apparently occupies a more dominant position: as its sole carbon source, FB5 could degrade 88.32% of 500mg/L fomesafen within 96h and it was at log phase from 24 to 36h (Fig.1). Besides, it could grow in a broader conditions of fomesafen concentrations (250–4000mg/L), temperature (28–40°C) and primary pH (5.0–10.0). So FB5 has stronger environmental adaptation ability. This attribute will provide a new strain resource for the bioremediation of fomesafen.

3.4. Remediation of strain FB5 to fomesafen-contaminated soil

To better reflect the real remediation activity of the strain, we inoculated FB5 to treat the pot soil in the present study. The average dry weight and root length of a plant (maize and sorghum) after the polluted soil was treated with FB5 for different durations are shown in Fig.5. All the CK2 was significantly lower than those of the Blank, respectively. The dry weight and root length of maize, as well as the root length of sorghum, exhibited good remediation after the polluted soil was treated with FB5 for 20d, and were already recovered to the Blank level. However, the dry weight of sorghum did not reach the Blank level until treated for 30d. This result may be attributed to the fomesafen in the soil inhibiting root growth and water absorption, further affecting dry weight and so on. In comparison, there was no significant difference in each index between every CK1 and its corresponding Blank. These results indicate that although strain FB5 was applied independently to the soil without fomesafen, it could not cause disease damage to the two types of aftercrops.

4. Conclusions

A highly efficient fomesafen degrading strain FB5 was isolated from a soybean field suffered fomesafen in Heilongjiang province, China. As the sole carbon source, it could degrade 88.32% of 500mg/L fomesafen within 96h, and it was identified as Shigella flexneri. The optimum growth conditions were as follows: 35–37°C, 500mg/L fomesafen, and primary pH6.0–7.0. Bioassay trials indicated that strain FB5 could significantly remediate the biomass of aftercrops on the soil with 5mg/kg fomesafen, and no disease or damage was observed. Therefore, strain FB5 owned significant potential in bioremediation of fomesafen residues in soils, and further studies are needed. If success, it will provide a workable pathway for agricultural contaminated soils. Moreover, the genes, enzymes, and metabolic products of strain FB5 related to its ability to degrade fomesafen also must be investigated in the future.
Fig. 5. Effect of strain FB5 on maize and sorghum growth indices. Multiple comparisons by Duncan. Capital and small letters in the same column stand for 0.01 and 0.05 significant levels, respectively. (a) Dry weight; (b) Root length

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