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Biodegradation of bensulphuron-methyl by a novel *Penicillium pinophilum* strain, BP-H-02

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ABSTRACT

A fungal strain able to rapidly degrade bensulphuron-methyl (BSM), called BP-H-02, was isolated for the first time from soil that had been contaminated with BSM for several years. BP-H-02 can use BSM as the sole carbon and energy source for growth in a mineral salt medium. Based on morphological and internal transcribed spacer (ITS) analysis, BP-H-02 was identified as *Penicillium pinophilum*. Under optimal conditions (pH 6.5, temperature 30 °C and 200 mg/L VSSinoculum), more than 87% of the initially added BSM (50 mg/L) was degraded after 60 h. Metabolites were identified as 2-amino-4,6-dimethoxypyrimidine and 1-(4,6-dimethoxypyrimidin-2-yl) urea by liquid chromatography–mass spectrometry (LC–MS), and a possible degradation pathway was deduced. In a soil bioremediation experiment, inoculation of soil with BP-H-02 promoted the degradation of BSM more effectively than did the control. These results revealed that BP-H-02 can biodegrade bensulphuron-methyl efficiently and could potentially be used to bioremediate sulphonylurea herbicides contamination.

1. Introduction

The sulphonylureas are a class of widely used herbicides that have high herbicidal activity, good crop selectivity, low application rates, broad-spectrum activity, and low acute mammalian toxicity [1,2]. They are much more potent than other classes of herbicides, resulting in application rates that are 1/100 of the rates of higher use alternatives [3]. Their mechanism of action is based on the inhibition of acetolactate synthase (ALS) in plants and some microorganisms, which is the key enzyme responsible for the biosynthesis of three-branched-chain amino acids (Valine, Leucine, and Isoleucine) [2,4]. These characteristics make the sulphonylureas an ideal substitute for older herbicides in weed control [5]. Bensulphuron-methyl (2-[[[[[(4,6-dimethoxypyrimidin-2yl) amino] carbonyl] amino] sulphonyl] methyl] benzoate) (BSM) is a representative sulphonylurea herbicide that is widely used [6]. Although BSM shares many of the advantages of the other sulphonylurea herbicides, some research has shown that its residues can cause environment and health damage. Hay [7] found that BSM has phytotoxicity to succeeding crops. It poisoned phytoplankton and submerged macrophytes [8,9] and caused acute toxic effects on *Pseudomonas putida* [10]. Soybean suspension-cultured cells treated with bensulphuron-methyl also showed growth inhibition [11]. Moreover, long-term exposure to BSM can cause some chronic diseases in human and animals [2]. Ding et al., [12] found that the interaction between BSM and human serum albumin (HSA) induced a conformational change in the protein. BSM is mobile and can widely contaminate waters and soils through surface runoff [13]. Moreover, BSM's low volatility, photodegradation and long persistence (over 100 days) in certain pedo-climatic conditions (dry climates, alkaline soils) has raised increasing concerns about the risk of contamination of nearby aquatic systems or damage to subsequent crops in rotation [14]. Therefore, it is important to develop strategies to decrease and eliminate BSM residues in the environment.

Microbial degradation of contaminants involves the use of living microorganisms to detoxify and degrade hazardous materials, which is generally considered to be an effective and safe way to remove contaminants from environment. It has been widely applied to degrade pollutants such as pesticides [15], plastic [16], petroleum [17], and surface-active agents [18], and it might also be useful to degrade BSM. Brusa et al. [19] found that biological degradation of BSM could take place under anaerobic conditions. Xie et al. [20] reported that repeated applications of BSM could accelerate degradation of the herbicide, but they could not isolate microbes that were able to degrade BSM. Zhu et al. [21] isolated a strain of *Brevibacterium* sp. that is capable of degrading BSM. In

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sterile soil to which 50 mg/L BSM was added, *Bacillus megaterium* degraded 94.3% of the BSM in 42 days [22]. However, there are no reports of fungi that can degrade BSM effectively.

The aim of this study was to investigate the potential for microbiological degradation of BSM. The microbe was collected and isolated from soil samples of a rice field, which has been applied BSM for several years; we hypothesised that there would be microorganisms present in this field that would be able to metabolise the xenobiotic [19]. A series of studies were carried out, including strain identification by morphological and molecular analysis, determination of optimal degradation conditions by single-factor optimisation tests, identification of metabolites and deduction of the metabolic fate by liquid chromatography–mass spectrometry (LC–MS), and a soil bioremediation experiment.

2. Methods

2.1. Chemicals

Bensulphuron-methyl (99% purity) was purchased from Sigma–Aldrich Chemical Co., Inc. All microbe culture media components were of at least analytical grade. All organic solvents were for residue analysis. The soil used for the isolation of degrading microbes was collected from soils that had been contaminated by sulphonylurea herbicides for several years in Baojing county, Hunan province, China.

2.2. Enrichment and isolation of fungal strains

The degrading fungi were isolated using an enrichment culture technique. The microorganisms used in the present study were isolated from BSM-contaminated soils. The mineral salt medium (MSM) (per litre distilled water) contained: 1 g K_2HPO_4, 2 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4$, and 1.0 g KH_2PO_4. The final pH was adjusted to 7.0. Soil samples were cultivated in 250 mL Erlenmeyer flasks in MSM (2.5 g soil, per 50 mL MSM) supplemented with 50 mg/L BSM as the sole source of carbon and energy. Cultivation was carried out for 7 days at 28 °C with shaking. The enrichment cultivation was carried out under the same conditions and repeated five times totally for 5 weeks, with different concentrations (50 mg/L, 200 mg/L and 500 mg/L) of BSM in the media. The microorganisms present in the enrichment culture were isolated on 1.5% agar MSM (AMSM).

2.3. Inoculum and growth curves

The fungal biomass is measured by volatile suspended solids (VSS). Volatile solids are those solids lost on ignition (heating to 550 °C). They are useful to the treatment plant operator because they give a rough approximation of the amount of organic matter present in the solid fraction of wastewater, activated sludge and industrial wastes. They are also used to measure fungal biomass usually [23].

Spores were collected using a cotton swab from 7-day-old cultures and suspended in sterile saline containing 0.1% Tween 80. The suspensions were adjusted to an initial inoculum 50 mg/L VSS for weight loss after ignition at 550 °C. After inoculation, the VSS were measured from 0 to 7 days at the interval of 1 day by culturing.

2.4. Identification of BP-H-02

The BP-H-02 strain was identified by reference to Bergey's Manual of Determinative Bacteriology [24]. A partial ITS sequence was amplified using PCR with the following primers: 5′-TCCGTAGGTGAACCTGCGG-3′ as forward and 5′-TCCTCCGCTTATTGATATGC-3′ as the reverse [25,26]. Taq DNA polymerase was purchased from TaKaRa Biotechnology, Dalian Co., Ltd., China. PCR reactions were carried out under the following conditions: 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 45 s at 56 °C and 90 s at 72 °C; plus an additional 10 min cycle at 72 °C. PCR fragments were ligated into the linear vector pMD20-T purchased from TaKaRa Biotechnology after purification by agarose gel electrophoresis and transformed into competent *Escherichia coli* DH5α cells. The recombinant plasmid in positive clones was extracted and used as the template for direct sequencing of ITS fragment using an automatic sequencer. The partial ITS sequence was compared to known sequences found in the GenBank database using Blast (http://www.ncbi.nlm.nih.gov/BLAST/). The robustness of the tree topology was assessed by bootstrap analysis, with 1000 resembling replicates.

2.5. Optimal conditions for degrading BSM by BP-H-02

BP-H-02 was incubated in liquid medium containing 50 mg/L BSM at 30 °C and 180 rpm on a rotary shaker. The VSS of 50 mg/L BP-H-02 was inoculated for BSM biodegradation studies. The residual pesticide concentration and VSS of BP-H-02 were determined at 24 h intervals. To determine the optimal conditions for degrading BSM by the strain BP-H-02, a single-factor optimisation test was designed under different growth conditions, including the inoculum amount (VSS 10–250 mg/L), temperature (20–40 °C) and pH (5.0–10.0). Three replicates within each treatment were utilised with non-inoculated samples as a control. The residual pesticide concentration in the single-factor optimisation test was determined after 7 days.

2.6. Analysis

Samples were extracted and analysed on an Agilent 1100 HPLC equipped with a ternary gradient pump, programmable variable-wavelength UV detector, column oven, electric sample valve and C_{18} reversed-phase column (Hypersil ODS2, 5 μm × 4.6 mm × 250 mm). The mobile phase used was a 50:50 mixture of acetonitrile (phase A) and water containing 0.5% acetic acid (phase B). The sample injection volume was 10 μL. The flow rate of the mobile phase was 1 mL/min and the wavelength was 234 nm. The retention time for BSM was 5.5 min. The HPLC analysis was reproducible with repeated injections of the same sample ($n = 3$).

The metabolites were analysed and identified by liquid chromatography-mass spectrometry (LC-MS). The procedure was performed on a Hypersil ODS column (250 mm × 4.0 mm i.d., 5 μm) using acetonitrile-water containing 0.5% formic acid (40:60, v/v) as the mobile phase at a flow rate of 1 mL/min. The column temperature was set at 25 °C, and the injection volume was 10 μL. MS detection was performed on a triple quadrupole analyser equipped with an ESI source in the positive ion mode. Two ions at m/z 156 and 196 corresponding to the $[M+H]^+$ ions of the BSM products were detected. A possible degradation pathway was deduced according to the results and some previous reports [27–29]

2.7. Soil bioremediation experiment

The soil samples were collected from the top 0–10 cm from a farm in South China Agricultural University, Guangzhou, China, that had never been treated with BSM. Detailed properties of the soil are showed in Table 1. The samples were dried at room temperature and sieved to 5 mm. The soil samples were sterilised as described by Zhang et al. [30]. BSM was added to soil samples (1000 g) to give concentrations of 25 mg/kg, 50 mg/kg, and 100 mg/kg soil. The soil was inoculated with 200 mg VSS BP-H-02/kg soil. In the soil bioremediation experiments, both fresh and sterile soils were inoculated

Table 1
General properties of the soil used in the experiment.

Silt (%)	48.92
Clay (%)	8.65
Soil moisture (%)	13
pH (H_2O)	6.8
Bulk density (g/cm^3)	1.6
C:N	18.5
Total P (g/kg)	15
Total C (g/kg)	20
Total N (g/kg)	1.08

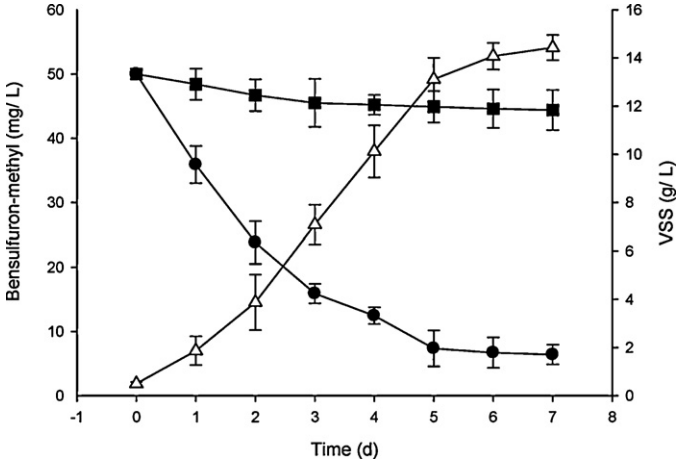

Fig. 1. Degradation of BSM (●), the control (■) and growth curves (△) of BP-H-02. Error bars represent the standard deviation of the mean.

with BP-H-02. Both soils without BP-H-02 were used as a control. The samples were incubated in the dark at 30 °C for 60 h, and the concentration of BSM in the soils was determined at 12 h intervals. Soil moisture content was maintained at 40% water-holding capacity by regular addition of distilled water if necessary. The moisture content was measured with soil humiture recorder ZDR-20j (Hangzhou Zeda Instruments Co, Ltd, China).

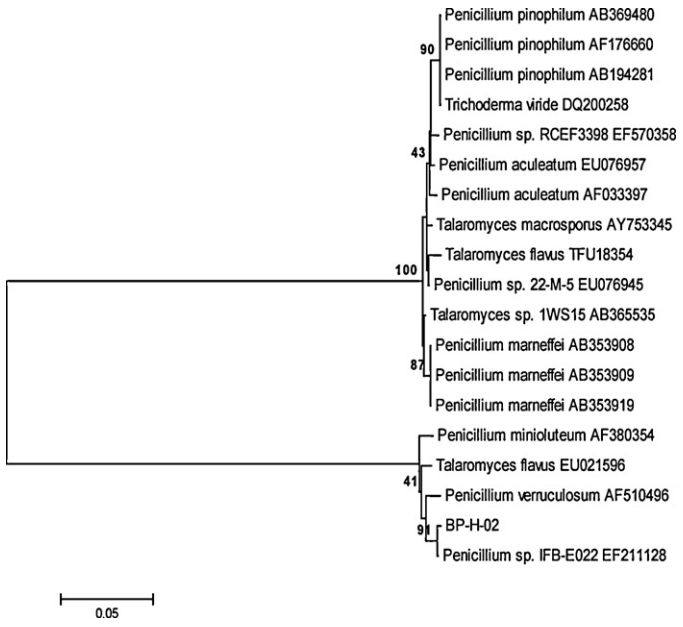

Fig. 2. Phylogenetic analysis of BP-H-02 and related species by the neighbor-joining approach. The scale bars represent 0.05 substitutions per site.

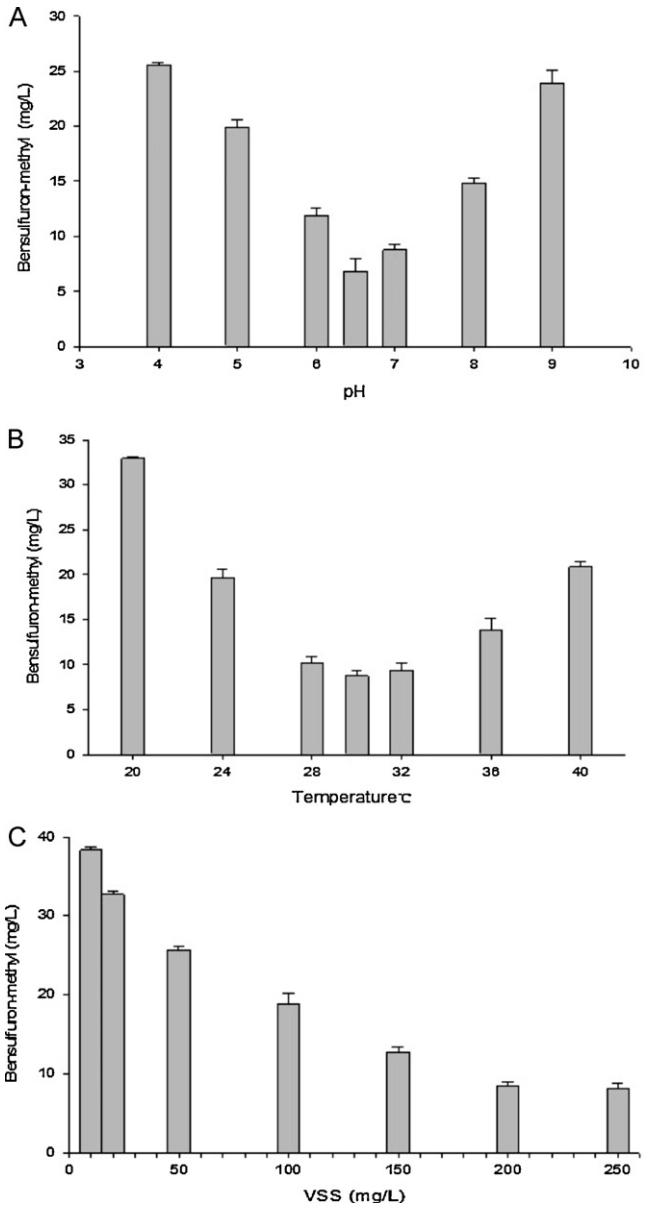

Fig. 3. Removal of BSM in different culture conditions: pH (a); temperature (b); inoculum amount (c). Error bars represent the standard deviation of the mean.

3. Results and discussion

3.1. Isolation of bensulphuron-methyl degrading fungi

During the enrichment culture, 6 different fungi strains grown on AMSM that effectively degraded 500 mg/L of BSM were obtained by the streaking plate method. They were named BP-H-01 to -06. The degradation experiment showed that after 7-day treatment, BP-H-01-06 degraded 50 mg/L BSM by 83.2%, 85.3%, 63.6%, 51.0%, 49.7% and 48.2%, respectively. BP-H-02 possessed the highest degradation capacity among all of the strains and was able to use BSM as its sole carbon source. Therefore, BP-H-02 was selected for further studies.

BP-H-02 was able to degrade BSM effectively. Under the original condition (pH 7.0, temperature 30 °C and 50 mg/L VSS inoculum), BP-H-02 degraded 85.3% of 50 mg/L BSM within 5 days (Fig. 1). Furthermore, BSM was completely degraded after 10 days. BP-H-02 is capable of using BSM as its sole carbon and energy source in SMS and grew rapidly in MSM supplemented with 50 mg/L of BSM when

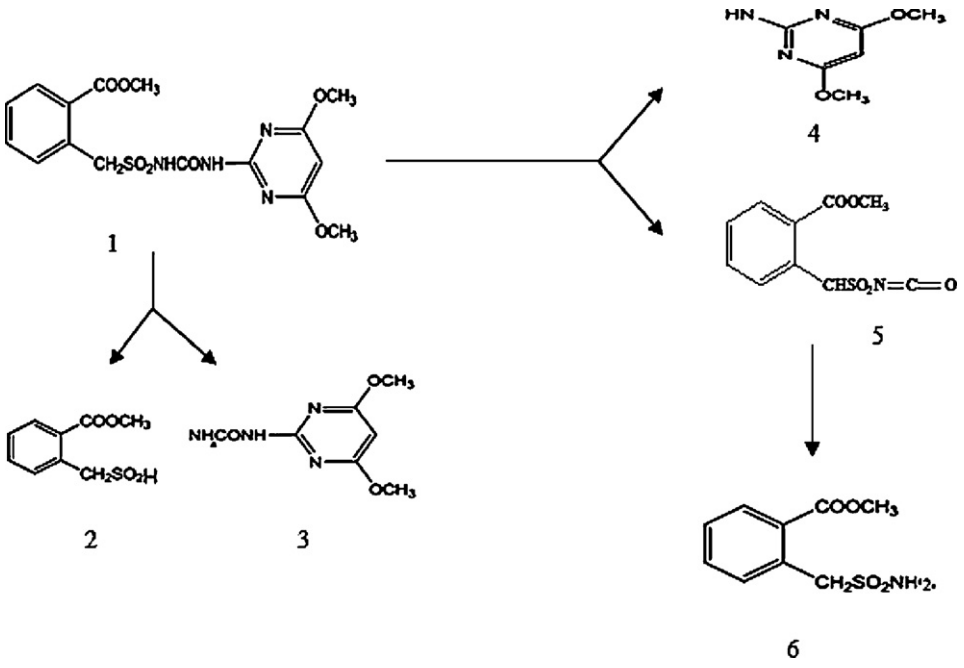

Fig. 4. Proposed metabolic pathways of bensulphuron-methyl by BP-H-02 in soil. 1, BSM; 2,2-methylformate-benzenemethl-sulphoacid; 3,1-(4,6-dimethoxypyrimidin-2-yl)urea; 4,2-amino-4,6-dimethoxypyrimidine; 5,2-methylformate-benzenemethlsulfonyl-isocyanate 6,2-methylformate-benzenemethl-sulfonamide.

the initial VSS was 50 mg/L (Fig. 1). After 5 days of incubation, the growth curve of BP-H-02 flattened.

3.2. Identification of the BP-H-02 strain

3.2.1. Morphology analysis

On AMSM plates, BP-H-02 forms colonies approximately 22 mm in diameter after 4 days of incubation at 28 °C. The colonies are circular, center raised, sulcate and light orange on the reverse side. The wrinkle and the colour of colonies deepen with time. Under the microscope, conidiophores showed aerial and superficial mycelium. Stipes are septate, smooth, thick walled. Penicilli is strictly biverticillate. Metulae and phialides are in verticils, smooth. Conidia showed spheroidal to subspherioid, smooth, thick walled, which are about 3.3 μm in diameter.

3.2.2. Sequence analysis of ITS

The ITS gene from BP-H-02 was amplified, and a 542 bp fragment (GenBank Accession No. JQ003471) was completely sequenced. The ITS sequence analysis showed that BP-H-02 shares 99% identity with *Talaromyces flavus* (GenBank Accession No. EU021596) and 98% identity with *Penicillium pinophilum* (GenBank Accession No. AB194281). To identify the phylogeny of BP-H-02, strains from different genera were chosen to construct a phylogenetic tree based on ITS sequences using MEGA 4.0 (Fig. 2). Phylogenetic tree showed that BP-H-02 exited highest identity with *Penicillium sp.* (GenBank Accession No. EF211128). However, the morphology clearly showed it was similar to *P. pinophilum*. So, combining morphological characterization with ITS gene analyses, BP-H-02 was identified as *P. pinophilum* (phylum Deuteromycotina, class Hyphomycetes, order Moniliales, family Moniliaceae).

3.3. Optimal conditions for degrading BSM by BP-H-02

To acquire better biodegradation, the initial concentration of BSM was maintained at 50 mg/L in the optimisation tests that investigated pH, temperature and inoculum amount. The pH is an important factor determining the degradation ability of microorganisms capable of degrading pesticides [31]. Different pHs (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) were tested in the optimisation experiment. After 7 days of treatment, there were only 25.6, 19.9, 11.85, 8.75, 14.8 and 23.95 mg/L of BSM left, respectively (Fig. 3a). The optimal initial pH value for degradation was between 6.0 and 7.0. Therefore, the initial pH value was adjusted to 6.5, at which only 6.85 mg/L of BSM remained after 7 days. Our results demonstrated that BP-H-02 can degrade BSM effectively in neutral and mildly acidic pH conditions. This is an important feature for an organism that is to be employed for bioremediation in variable environments.

Temperature obviously affects microbes degrading hazardous materials [32]. The degradation of BSM by BP-H-02 was determined under incubation temperatures ranging from 20 °C to 40 °C at the interval 4 °C After 7 days of cultivation, BSM was degraded by BP-H-02 to a different extent at different temperatures (Fig. 3b). The concentrations of BSM were 32.9 mg/L and 19.6 mg/L at 20 °C and 24 °C and were 13.8 mg/L and 20.85 mg/L at 36 °C and 40 °C. Only 10.25 mg/L and 9.35 mg/L of BSM remained, after cultivating at 28 °C and 32 °C, respectively. These results indicated that temperature was important to the degradation of BSM by BP-H-02. Temperatures both too high (36 °C, 40 °C) and too low (20 °C, 24 °C) inhibited degradation. It is possible that some key enzyme(s) responsible for BSM degradation have their optimum enzymatic activity over this temperature range [33]. In order to determine the preferable temperature, 30 °C was used for the degradation experiment. BSM was degraded up to 82.5% (only 8.75 mg/L of BSM remained) within 7 days. Therefore, 30 °C was found to be the optimal temperature.

The inoculum amount is another important factor that significantly influences the efficient biodegradation of pesticides [34]. A series of experiments were designed to investigate the influence of strain inoculum amount over the range of 10–250 mg/L of VSS. After 7 days, the remaining concentrations of BSM with the different inoculum amounts (10, 20, 50, 100, 150, 200, and 250 mg/L of VSS) were 23.4%, 34.6%, 48.7%, 62.3%, 74.5%, 83.2%, and 83.8%, respectively (Fig. 3c). The peak area of BSM in samples with 250 mg/L of

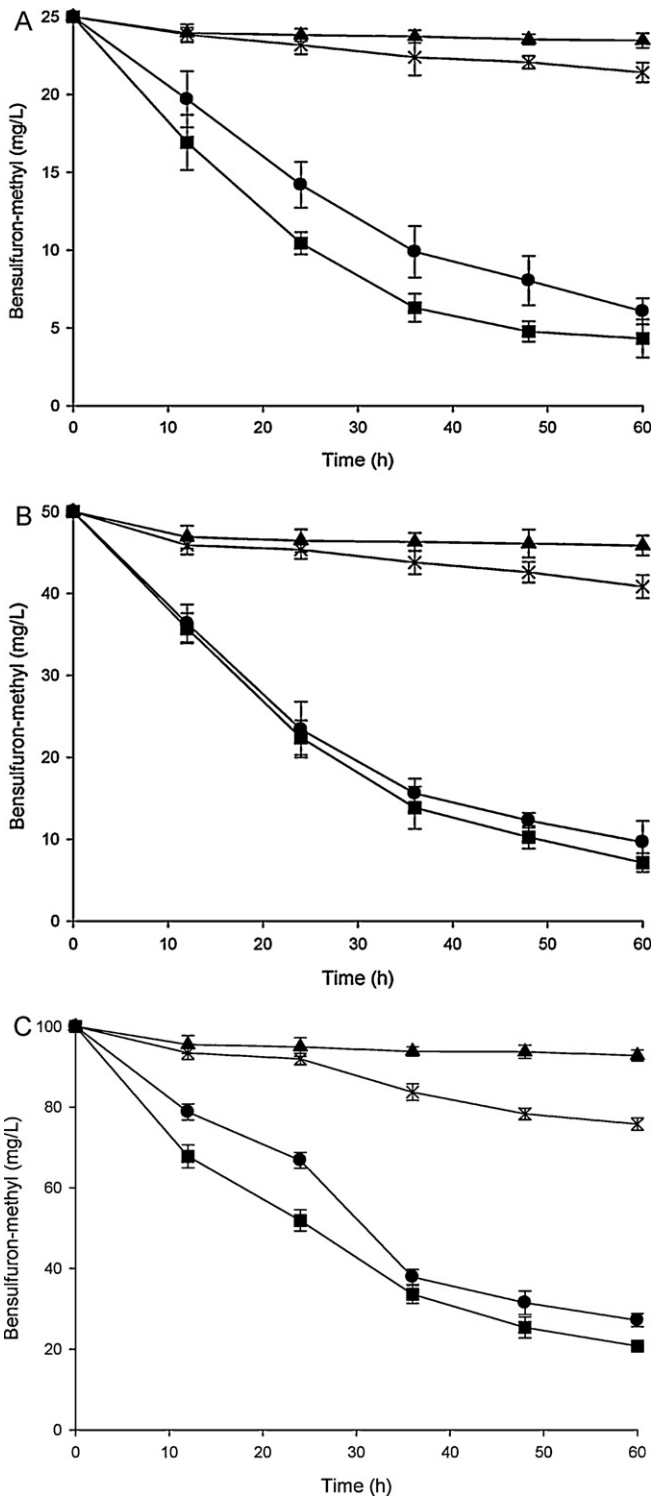

Fig. 5. Removal of BSM in inoculated and uninoculated soil microcosms. 25 mg/L BSM (a); 50 mg/L BSM (b); 100 mg/L BSM (c). Sterilised soil (▲); fresh soil (×); sterilised soil + BP-H-02 (■); fresh soil + BP-H-02 (●). Error bars represent the standard deviation of the mean.

VSS was larger than that of the other inoculum concentrations for BSM. However, there was no significant difference in the amount of degradation between 200 mg/L of VSS and 250 mg/L of VSS. Therefore, 200 mg/L of VSS was considered to be the most appropriate inoculum amount.

From these results, it was concluded that the optimal conditions were pH 6.5, 30 °C, and 200 mg/L of VSS for BSM degradation by BP-H-02. Under these optimal conditions, 87.3% of the BSM was degraded after 7 days in the verification experiment.

3.4. Degradation products

LC–MS analysis of an ethyl acetate extract of the soil revealed that the identified metabolite had a molecular weight and fragmentation pattern identical to those of 2-amino-4, 6-dimethoxypyrimidine (AdMP), which is consistent with previous findings [35]. In addition, a fragment with m/z of 196 occurred in mass spectra performed in the ESI^+ scan mode. Its structure could possibly be 1-(4,6-dimethoxypyrimidin-2-yl) urea. The chemical bond broken to form the degradation product is similar to that described in a previous study [29].

Using the results of the LC–MS analysis and the chemical properties of BSM described in related reports [29,35,36], we propose the metabolic pathway for BSM biodegradation shown in Fig. 4. There were two pathways. After the cleavage of the Urea bridge, products (2) and (3) were emerged. Another possibility was that products (4) and (5) generated. However, products (5) are not stable. It may turn to products (6).

Ye et al. [29] reported that the metsulphuron-methyl metabolites 2-amino-4-methoxyl-6-methyl-1,3,5-triazine and 2-methylformate-benzenesulphonamide had no phytotoxicity. Because sulphonylurea herbicides have similar structures and properties, 2-amino-4,6-dimethoxypyrimidine (4) and 2-methylformate-benzenemethl-sulphonamide (6) analogues were hypothesised to be non-phytotoxic metabolites. Because products (4) and (6) had no phytotoxicity, we predict that products (2) and (3) should also lack biological activity against succeeding crops.

3.5. Soil bioremediation experiment

The experiment demonstrated that BP-H-02 in medium can rapidly degrade BSM under co-culture conditions. In addition, the removal of bensulphuron-methyl in soil samples with different treatments, including sterilised soil, fresh soil, sterilised soil + BP-H-02, and fresh soil + BP-H-02, are shown in Fig. 5. Within 60 h, BP-H-02 degraded 75.7%, 80.7% and 72.8% of the 25 mg/L, 50 mg/L, and 100 mg/L of BSM, respectively, added to fresh soil; in contrast, the control (fresh soil) degraded 14.3%, 18.3% and 24.2%, respectively, of the same levels of BSM. In the sterile soil with BP-H-02 added, the degradation percentages were 82.7%, 85.7% and 79.2% within 60 h, while those of the control (sterilised soil) were 6.1%, 8.3% and 7.2%. The degradation in non-sterilised soil was better than in sterilised soil at all concentrations, suggesting that indigenous microbes in non-sterilised soil may play a role in the BSM degradation. The degradation of BSM in sterilised soil inoculated with BP-H-02 was superior to that in fresh soil inoculated with BP-H-02, suggesting that indigenous microbes in the soil may be antagonists of BP-H-02. Our results show that the development of BP-H-02 as a bioremediation agent for sulphonylurea-herbicide-contaminated environments is worthy of further investigation.

4. Conclusion

In the present study, a new strain of fungi, BP-H-02, was isolated that effectively degraded BSM. Based on morphological and ITS analyses, BP-H-02 was identified to be *P. pinophilum*. BP-H-02 was able to use BSM as its sole carbon and energy source for growth in MSM. Its metabolites have no phytotoxicity, which suggests that there will not be secondary toxicity in the environment. These results provide important information for the application of biological remediation in BSM-contaminated environments after the application of strain improvement techniques.

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