BIODEGRADATION OF 4-CHLORO-2-METHYLPHENOXYACETIC ACID BY ENDOPHYTIC FUNGUS *PHOMOPSIS* SP. IN LIQUID MEDIUM AND SOIL

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Abstract. An endophytic fungus strain, newly isolated from *Psidium guajava* (common guava), could effectively degrade 4-chloro-2-methylphenoxyacetic acid (MCPA). According to the analysis of its phenotypic feature and ITS rDNA gene sequence, the isolated strain was identified as *Phomopsis* sp. and named E41. The influencing factors including medium pH, temperature, and initial concentration of MCPA affecting the degradation of MCPA by this strain were optimized in this study. The maximum biodegradation rate of 86.89% was obtained after inoculation for 7 days under the optimal conditions with MCPA concentration of 50 mg/L, temperature 30 °C and a pH of 6. The metabolite 4-chloro-2-methylphenol was detected by using gas chromatography-mass spectrometry (GC-MS). In the bioremediation experiment, the biodegradation of MCPA was significantly enhanced in all soils with the presence of the strain E41. These results suggested that the strain E41 may have potential value for the bioremediation of MCPA-contaminated environment.
Keywords: *phenoxy acid herbicide, Psidium guajava, microbe, decompose, bioremediation*

Introduction

4-chloro-2-methylphenoxyacetic acid (MCPA), a phenoxy acid herbicide, was registered for the control of annual and perennial post-emergence broad-leaf weeds in agricultural field. MCPA is relatively soluble in water, weakly sorbed by soil, where it is highly mobile in soil, which gives rise to high leaching risk into surface water and groundwater (Cabrera et al., 2011; Önneby et al., 2014). MCPA has been frequently detected in surface water, groundwater and well water in many countries. Some studies have reported that this phenoxy acid herbicide could cause endocrine disturbance for humans and animals although it has relatively slight toxicity (Bukowska et al., 2008; Salvo et al., 2015). For these reasons, the European Community and US Environmental Protection Agency have classified MCPA as priority pollutant (Sanchis et al., 2014). Therefore, it is necessary to develop effective ways to accelerate the decrease and elimination of MCPA residue in environment.

Previous studies have shown that degradation by microbes is regarded as an effective method in the removal of MCPA residue for the environment (Verma et al., 2014; Wu et al., 2017). However, only a few bacteria and fungi were isolated to utilize MCPA as sole carbon source for growth due to its antibacterial properties (Tan et al., 2013; Xiang et al., 2020; Zhang et al., 2020). It is essential to screen more excellent MCPA-degrading microbes with high environmental tolerance. The endophytic microbes as a valuable microbial resource reside in internal tissues of living plants without causing apparent symptoms of infection and have mutualism effect on plants. It has been demonstrated that endophytes play a crucial role in host plant for the adaptation to the polluted environment (Khan et al., 2014). Many endophytic microbes have been successfully isolated from different plants with pollutant-degrading activities (Sun et al., 2014a,b; Zhu et al., 2016). Recently, several endophytic bacteria have been reported to degrade herbicides including simazine, glyphosate and quinclorac (Ozawa et al., 2004; Liu et al., 2014). Up to now, no MCPA-degrading endophytic microbe has been reported.

In this work, an endophytic fungus with the ability to degrade MCPA was isolated from leaves of *Psidium guajava* grown in Nanning city of south China and then was identified by morphological and molecular analysis. The effect factors on degradation efficiency were also optimized by single-factor optimization test. The metabolic fate of MCPA was deducted according to the identification of metabolites by gas chromatography-mass spectrometry (GC-MS). The soil bioremediation experiment was carried out to evaluate its potential in the remediation of MCPA-contaminated soil. To our knowledge, it is the first report on the biodegradation of MCPA by endophyte isolated from *Psidium guajava*.

Materials and methods

Chemicals and solutions

MCPA (98% purity) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile and methanol were HPLC grade (Fisher Scientific Company, USA) and other reagents were analytical grade (National Pharmaceutical Group Corporation, China). Unless otherwise stated, deionized water was used in all of the experiments.

The mineral salt medium (MSM) was composed of (g/L) NH_4NO_3 1.0, K_2HPO_4 1.5, KH_2PO_4 0.5, NaCl 1.0, $MgSO_4·7H_2O$ 0.1, and $FeSO_4$ 0.025. The potato dextrose (PD) medium was prepared by containing 200 g/L potato extract and 20 g/L D-glucose. The composition of Luria–Bertani (LB) medium contained (g/L): yeast extract 5, tryptone 10, NaCl 10. Solid media were prepared by adding 1.5% (w/v) agar into the above liquid media.

Psidium guajava was collected at July when the leaf was fully unfolded and grew well nearby warehouse of the factory producing MCPA in Nanning city of south China (22°35′15.29″N, 108°13′1.26″E) for at least 5 years. The stems and leaves were placed into labelled plastic bags, then immediately transported to the laboratory for use.

Isolation of MCPA-degrading endophytic fungi

The surface of samples (stems and leaves) was sterilized by immersing into 75% ethanol for 2–3 min, and 0.1% mercuric chloride for 5 min. Then the sterilized samples were washed three times with sterile water for 2 min and cut into pieces (approximately

1×2 cm) with a sterilized blade. To verify whether the sterilization process was successful, aliquots of the final washing sterile water were spread onto LB agar plate and incubated at 28 ºC. The sterilization process was considered successful if no colony was found on agar plate after inoculation.

After sterilization, 1 g samples were ground with 10 mL sterile water using a sterilized mortar and pestle, and then the samples were stepwise diluted to 10^{-2} and 10^{-3}. 100 μL diluted solution was spread onto MSM agar plate containing 500 mg/L MCPA and then incubated at 28 ºC. Isolates exhibiting distinct colonial morphologies were isolated and purified by repeated streaking on PD agar plate.

Identification of endophytic fungus

According to the results of screening experiment, a strain named E41 was selected for further analysis. After strain E41 was incubated on PD agar plate at 28 ºC for 7 days, the morphological feature was observed. The ITS sequence of strain E41 was extracted, purified and amplified by polymerase chain reaction (PCR) with universal fungal primers of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Song et al., 2013). The PCR condition was as follows: denaturation at 95 ºC for 5 min, 35 cycles with each consisting of 95 ºC for 30 s, 55 ºC for 30 s and 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The PCR product was verified and purified by agarose gel electrophoresis, and then sequenced in Shanghai Invitrogen Biological Technology Co., Ltd. The sequence was compared with the published sequences in GenBank using BLAST program on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The partial ITS gene sequences of the isolate and related fungi were then aligned and processed to produce an unrooted phylogenetic tree using the neighbor-joining method with MEGA software package (Tamura et al., 2011).

Biodegradation of MCPA in MSM

A MCPA-degrading strain designated as E41 was used for inoculum preparation and cultured in a 250-mL Erlenmeyer flask containing 100 mL PD medium supplemented with 50 mg/L MCPA. After incubation for 3 days, the strain was collected by centrifugation at 12000 rpm for 2 min and washed three times with MSM to remove the remaining PD medium. Then, the biomass (0.55 g dr w/L) was seeded into 100 mL MSM supplemented with 50 mg/L MCPA as the only carbon source.

In order to study the optimal degradation condition of MCPA by this strain, single-factor test was designed under different conditions including pH (4–9), temperature (20–40 ºC), and initial concentration of MCPA (25–100 mg/L). All samples in triplicate were incubated at 28 ºC in a rotary shaker (150 rpm).

Samples were periodically collected to determine MCPA concentration and fungal biomass. 5 mL culture solution was collected, followed by the addition of 5 mL acetonitrile and 2 g NaCl. After shaking at 150 rpm for 2 h, 2 mL supernatant was taken, added with 0.2 g $MgSO_4$, vortexed for 1 min, and centrifuged at 4000 rpm for 2 min. The solution was then filtered through an organic membrane prior to the analysis of MPCA residue by high-performance liquid chromatograph (HPLC). The biomass of strain E41 was measured by dry weight of mycelium (in grams per liter).

Determination of degradation products

During the biodegradation experiment in MSM under the optimal conditions, 10 mL culture solution was collected and centrifuged at 12000 rpm for 2 min to remove the proteins. The supernatant was mixed with 30 mL acetonitrile to extract the degradation products. Then 8 g NaCl was added to make the mixed solution separate into two layers. Finally, the upper layer was taken, dewatered, dried and re-dissolved before the detection by GC-MS (Zertal et al., 2004).

The degradation products of MCPA were detected by an Agilent 7890N/5975 GC-MS equipped with HP-5MS capillary column (30 m×0.25 mm i.d., 0.25 μm film). The temperature program was as follows: the column was held initially at 40 °C for 5 min, raised with 10 °C/min to 200 °C, held at 200 °C for 1 min, ramped with 10 °C/min to 280 °C, and then held at 280 °C for 8 min. Analyses were performed in splitless mode at 220 ºC. The transfer line temperature was set at 250 ºC, and the ion source was operated at 70 eV and 250 ºC. Full scans were obtained by scanning from 100 to 500 m/z. Helium was used as a carrier gas with a flow rate of 1.5 mL/min.

Bioremediation of MCPA in soil

The bioremediation experiment with this fungus strain was carried out in soils. Soil sample was sterilized three times by autoclaving for 1 h at 121 ºC. The stock solution of MCPA was added to 500 g sterile soil with a final concentration of 2.5 mg/kg. After mixing and solvent evaporation, the microbial suspension was introduced into the soil with the final biomass of approximately 1 g per 100 g soil. In order to study the dissipation rate of MCPA under abiotic condition, the sample of sterile soil without the added fungus was kept as control. The water content in soil samples was adjusted to 50% of the maximum water-holding capacity by the addition of sterile deionized water. All soil samples were incubated at 30 ºC in a dark thermostatic chamber. During the incubation period, sterile deionized water was added to the soil treatments to compensate for any water loss exceeding 5% of the initial amount added. Periodically, samples of soil treatments were aseptically removed for the determination of MCPA concentration.

Soil samples were mixed with 40 mL acetonitrile for 6 h by shaking at 150 rpm, added with 5 g NaCl, and vortexed for 1 min. After centrifugation at 4000 rpm for 5 min, 30 mL supernatant was taken, added with 3 g $MgSO_4$, vortexed for 1 min, and centrifuged at 4000 rpm for 5 min. Then, 20 mL supernatant was collected, transferred into a 50-mL rotating steam bottle, rotated to dryness at 40 °C, and dissolved in 2 mL acetonitrile. Finally, the solution was filtered through an organic membrane before the determination of MCPA concentration by HPLC.

Determination of MCPA

The MCPA residue was detected by an Agilent 1260 HPLC equipped with a UV detector and ZORBAX Eclipse XDB C_{18} column (250 mm×4.6 mm i.d., 5 μm). A mixture of methanol and water with 0.2% acetic acid (60:40, v/v) was used as mobile phase with a flow rate of 1 mL/min. The detection wavelength was 230 nm and the injection volume was 20 μL with column temperature at 30 ºC. The degradation rate was calculated by regression using a first-order reaction model (*equation 1*):

$$C_t = C_0 \times e^{-kt} \qquad\qquad (Eq.1)$$

where C_0 (mg/L) is the initial concentration of MCPA, C_t (mg/L) is the MCPA concentration at time t (d) and k is the degradation rate (day^{-1}). The half-life was calculated as *equation (2)* (Tortella et al., 2010):

$$t_{1/2} = \ln(2)/k \qquad \qquad (Eq.2)$$

Statistical analysis

The data were calculated using Excel 2010 and then analyzed with the SPSS 22.0 software and Origin 9.0 software. The statistical significance of differences was performed with the one-way analysis of variance (ANOVA) by Tukey's test. Differences were considered significant at p values < 0.05.

Results and Discussion

Isolation and identification of fungus

As the result of the screening process, thirteen fungus strains were isolated and purified from *Psidium guajava* by streaking plate method. Among the isolated strains, primary experiments showed that the degradation rates of MCPA were below 30% by 12 fungus strains. Thus, a strain named E41 was selected for further detailed research due to its relatively high MCPA-degrading ability. This strain could utilize MCPA as the sole carbon source and energy source for its growth in MSM.

Morphological feature was used to identify strain E41. On PD agar plate, strain E41 was full of the whole plate at the seventh day with the growth rate of 0.64 cm/d. After incubation for 7 days at 28 °C (*Fig. 1*), the colony approximately 5 cm in semidiameter was dark brown with white villous on both sides, and grew radially with similar annual wheel-like boundaries. The thickness of the center was basically the same as that of the edge, and the edge of the colony was in an irregular shape.
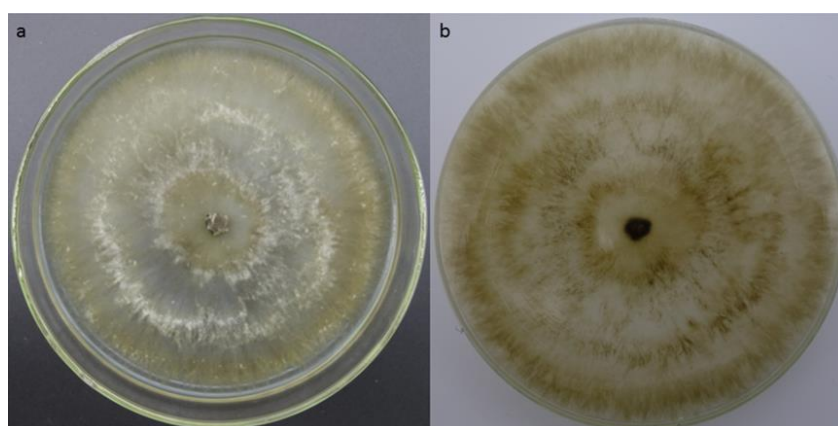

Figure 1. Colonies on PD agar plate in 9-cm petri dish after incubation for 7 days, the front side (a), and the reverse side (b)

Due to the lack of sporulation, identification of strain E41 was further performed by the analysis of ITS sequence. The ITS gene sequence with 555 bps was deposited in GenBank database under accession number KY797680. Analysis of partial ITS rDNA

sequence showed that strain E41 was closely related to the species of fungus *Phomopsis* sp. (GenBank Accession No. GU066614.1, GU066605.1, KX953471.1, GU066629.1, and GU066607.1). To identify the phylogeny of strain E41, strains from different genera were chosen to construct a phylogenetic tree based on ITS sequence using MEGA 6.0 (*Fig. 2*). Phylogenetic tree showed that strain E41 exhibited 99% similarity to *Phomopsis* sp. (GenBank Accession No. GU066614.1).
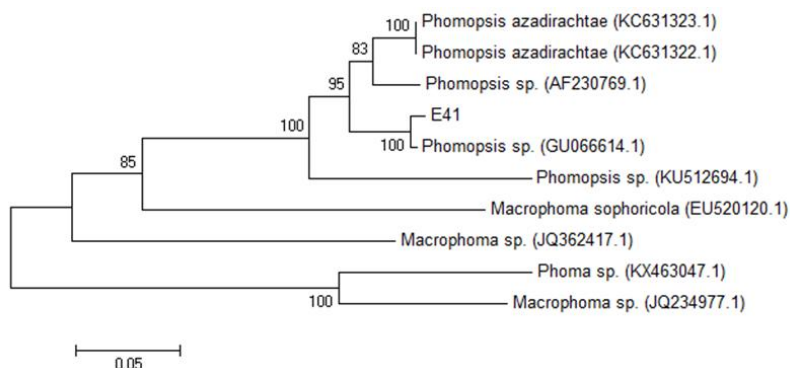

Figure 2. Phylogenetic tree of strain E41 and related by the neighbor-joining approach. Bootstrap values obtained with 1000 repetitions are indicated as percentage at all branches

According to the results of phenotypic feature and ITS gene sequence, strain E41 was identified as *Phomopsis* sp. Previous reports have demonstrated that members of endophyte *Phomopsis* sp. have the potential ability to degrade organic pollutants such as 4-hydroxybenzoic acid (Chen et al., 2011), N-heterocyclic indole (Chen et al., 2013a), ferulic acid (Xie and Dai, 2015), and soil resveratrol (Wang et al., 2019) and have the potential application to the ecological remediation of long-term cropping soil (Chen et al., 2013b). At present, many bacteria and fungi capable of degrading phenoxy acid herbicide have been isolated from various environments (Wu et al., 2017; Xiao et al., 2017; Zhang et al., 2020; Sheng et al., 2020). However, there are few reports on the degradation of MCPA by endophytic fungi. This study was the first report about MCPA-degrading endophytic fungus and expanded the application range of endophytic fungus in the field of environmental pollution remediation.

Biodegradation of MCPA in MSM

The effect of pH values on the degradation of MCPA by strain E41 was investigated and the result was shown in *Fig. 3a*. Observed from *Fig. 3a*, strain E41 could degrade MCPA in a range of pH 4–9 with the biodegradation rate from 60.69% to 89.21%. Many studies also demonstrated that biodegradation of organic pollutants was depended on the initial pH (Wang and Xie, 2012; Lin et al., 2014; Yu et al., 2015). Compared with *Cupriavidus campinensis* BJ71 and *Achromobacter* sp. LZ35, strain E41 showed a wider pH range to phenoxy acid herbicide (Han et al., 2015; Xia et al., 2017). The biodegradation rate of MCPA reached the maximum value at optimal pH 6. Meanwhile, the degradation rate of MCPA by strain E41 was not affected significantly in the pH range of 5–7. These results showed that strain E41 was more efficient to degrade MCPA in weak acid and neutral conditions. The similar pH condition was previously reported on the biodegradation of phenoxy acid herbicide (Greer et al., 1990; Byunghoon et al., 2003; Wu et al., 2017).
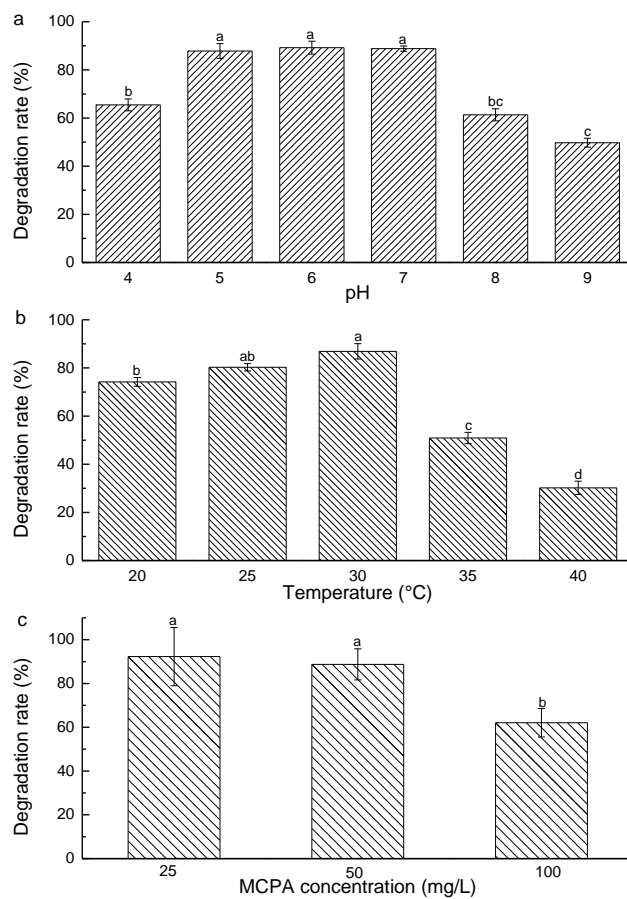
Figure 3. *The effect of different conditions on MCPA degradation by strain E41. (a) Effect of pH on the removal of MCPA, (b) effect of temperature on the removal of MCPA, (c) Effect of initial concentration on the removal of MCPA. The columns marked by different letters indicate significantly different from each other. Error bars represent the standard deviation of three replicates*

Temperature was an important factor on the biodegradation of organic pollutant in MSM (Yu et al., 2005; Song et al., 2013; Liu et al., 2016). The degradation rate of MCPA by strain E41 was investigated at the temperature from 20 to 40 °C and the result was illustrated in *Fig. 3b*. The biodegradation rates at 20, 25, 30, 35, and 40 °C were 74.19, 80.29, 86.89, 50.92, and 30.21% after incubation with strain E41 for 7 days, respectively. The degradation rate of MCPA gradually increased at 20–30 °C, but significantly decreased at 40 °C. The optimal temperature was 30 °C for the degradation of MCPA by strain E41. The temperature condition of strain E41 for MCPA degradation were similar to those previous findings on the biodegradation of phenoxy acid herbicide (Han et al., 2015; Samir et al., 2015; Xia et al., 2017; Xiao et al., 2017).

The biodegradation experiments were carried out with the initial MCPA concentration from 25 to 100 mg/L at 30 °C and pH 6, while the corresponding degradation rates were 92.31, 88.75, and 62.08% in MSM with strain E41, respectively (*Fig. 3c*). This result indicated that the initial concentration of MCPA above 100 mg/L could inhibit the degradation capacity of strain E41. This finding was consistent with the report of Chen et al. (2011), who demonstrated that 4-hydroxybenzoic acid in high concentrations could inhibit the growth of endophytic fungus *Phomopsis liquidambari* in vitro. Similarly, Wu

et al. (2017) reported that the degradation of 2,4-dichlorophenoxyacetic acid by *Cupriavidus gilardii* T-1 was inhibited with increasing concentrations.

The MCPA residue and biomass of strain E41 under optimal conditions were periodically determined during degradation process (*Fig. 4*). Observed from *Fig. 4*, the MCPA residue was gradually decreasing and the fungal biomass was contrarily increasing with the increase of incubation time. The degradation rate of MCPA was up to 99.80% after incubation with strain E41 for 14 days. Meanwhile, the degradation of MCPA was associated with a concomitant increase in fungal biomass up to 4.19 g dry wt/L in MSM culture. This result indicated that strain E41 could utilize MCPA as the sole carbon source for its growth and effectively degrade MCPA. In previous report, ninety fungus strains belonging to ten taxonomic groups were used to degrade phenoxy acid herbicides in liquid medium. However, only two of the species attained a dissipation potential of about 50% within 5 days (Vroumsia et al., 2005). In this study, strain E41 showed a potential ability to degrade MCPA herbicide.
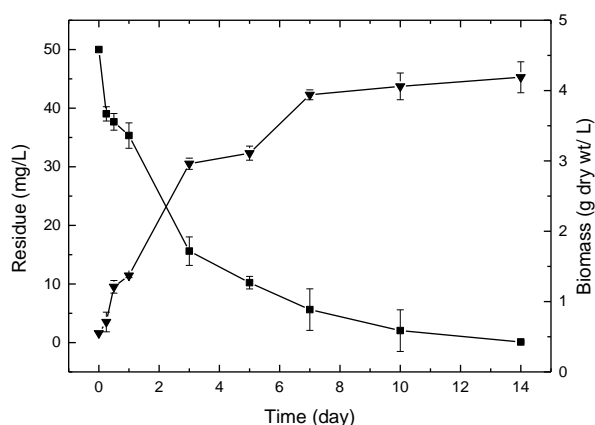

Figure 4. The degradation kinetics of MCPA (square) and biomass of strain E41 (triangle) in MSM. Error bars represent the standard deviation of three replicates

Degradation products and pathway of MCPA

The degradation products of MCPA in MSM culture were extracted and identified by GC-MS. A metabolite was observed with retention time of 15.77 min in *Fig. 5a*. By comparison with the National Institute of Standards and Technology (NIST, USA) library database, the metabolite was identified as 4-chloro-2-methylphenol and its mass spectrogram was illustrated in *Fig. 5b* with characteristic fragment ion peaks at m/z 142.0, 107.0 and 77.0. The metabolite was also reported in direct photolysis of MCPA (Zertal et al., 1999; Costa et al., 2013). In previous report, MCPA degraded by most bacteria was demonstrated to be initiated by the oxidative cleavage of the ether bond producing 4-chloro-2-methylphenol, subsequent hydroxylated to catechol, and finally underwent ring-cleavage (Häggblom, 1992). Ring hydroxylation was also an important metabolic route in the biodegradation of MCPA by fungi. *Aspergillus niger* could metabolize MCPA to a variety of hydroxylated products (Evangelista et al., 2010). However, catechol was undetermined in this study, which indicated the degradation pathway of MCPA by strain E41 was different with these microbes. The hypothetical biodegradation pathway of MCPA by strain E41 in MSM was illustrated in *Fig. 6*.
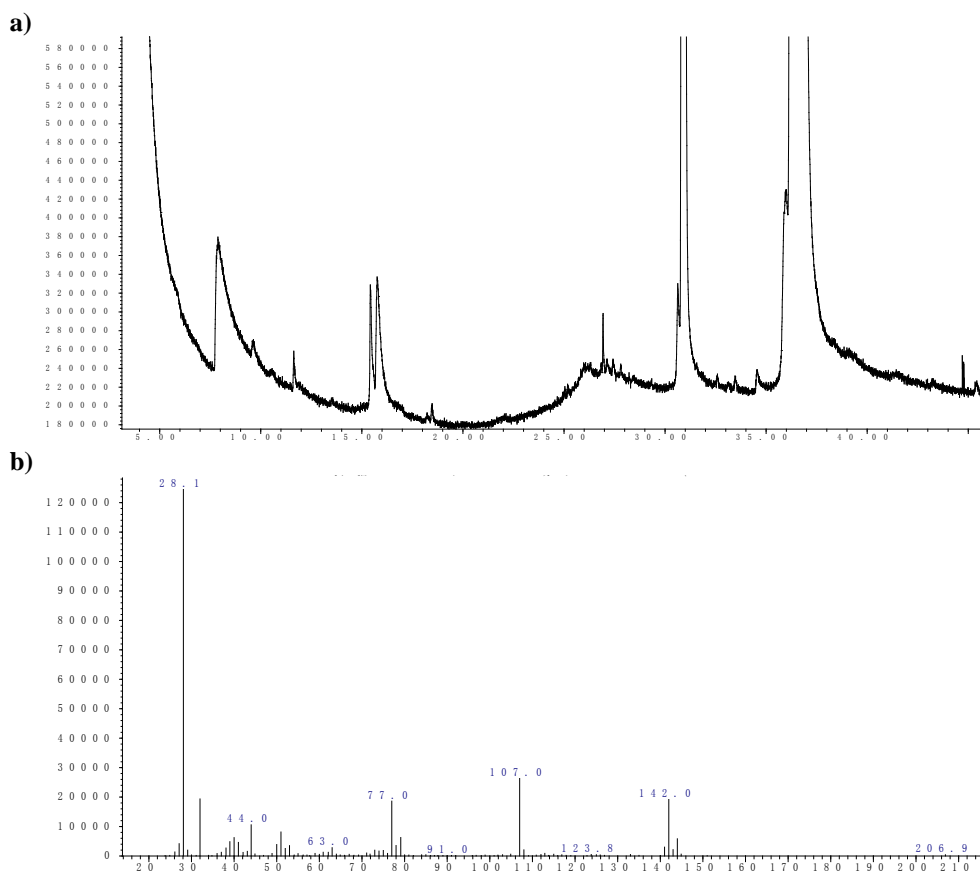
Figure 5. *The total ion chromatogram (a) and mass spectrogram (b) of the metabolite by GC-MS*
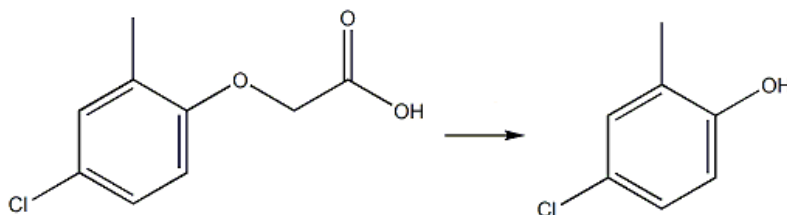

Figure 6. *Hypothetical biodegradation pathway of MCPA by strain E41 in MSM*

The amount of this metabolite was also detected during the biodegradation process. The amount of 4-chloro-2-methylphenol increased to 12.18 mg/L until 5 days of incubation and then decreased to 6.73 mg/L at 14 days, probably due to the hydroxylation and its own degradation (Bojanowska-Czajka et al., 2006). Finally, the metabolite was not detected in the culture medium after 21 days of incubation. These results indicated that the added MCPA in MSM was completely degraded by strain E41 without any accumulative metabolites at the end of incubation.

Biodegradation of MCPA in soil

To investigate the bioremediation of MCPA in contaminated soils by strain E41, the biodegradation experiment was performed in soils with initial MCPA concentration of

2.5 mg/kg, and the analytic results were shown in *Table 1*. The results showed that the half-lives of MCAP were 12.84, 2.07, 27.73, and 2.73 d in non-sterile soil, non-sterile soil+strain E41, sterile soil, and sterile soil+strain E41, respectively. The MCPA in non-sterile soil degraded faster than that in sterile soil, which showed that the indigenous microbes in soil probably played a role in MCPA degradation. After inoculated with strain E41, the degradation of MCPA was 6.2 and 10.2 times faster than that in non-sterile soil and sterile soil. It was obvious that the biodegradation of MCPA was significantly enhanced in soils with the presence of strain E41. Attaining highly efficient degradation strain was critical to the bioremediation of environmental pollutants and the usefulness of bioaugmentation with pesticide-degrading microorganisms in the clean-up of polluted soil was proven some years ago. Endophytic fungi have the capability to degrade organic compounds, decompose environmental contaminants, and improve the soil microenvironment (Ortega et al., 2020). Previous studies have demonstrated that *Phomopsis* sp. could accelerate leaf litter decomposition, soil phenol transformation, and biodegrade soil resvertrol (Chen et al., 2013b; Wang et al., 2019). The result in our study indicated that endophyte *Phomopsis* sp. E41 was a valuable microbial resource for potential applications in agricultural soil. However, it was noticeable that this result was mere an academic observation and further experiments should be conducted as to its large-scale applicability.

Table 1. Degradation dynamics of MCPA by strain E41 in different soils

Soil treatment	Equation	Correlation coefficient (R^2)	Half-life (day)
non-sterile soil	$y = 2.2196e^{-0.054x}$	$R^2 = 0.8080$	12.84
non-sterile soil+E41	$y = 4.2441e^{-0.335x}$	$R^2 = 0.8609$	2.07
sterile soil	$y = 2.4031e^{-0.025x}$	$R^2 = 0.9521$	27.73
sterile soil+E41	$y = 3.5932e^{-0.254x}$	$R^2 = 0.8331$	2.73

Conclusion

An entophytic fungus strain E41 could degrade MCPA in liquid medium and soil. The optimal degradation conditions for MCPA by strain E41 were at 30 ºC and pH 6–7. The main metabolite was 4-chloro-2-methylphenol. The strain *Phomopsis* sp. used to remove and detoxify the MCPA contamination in practice needs to be investigated in detail in the future, for instance, degradation mechanism and the effect factors on the bioremediation of MCPA in soil.

Declaration of interest statement. No potential conflict of interests was reported by the authors.

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