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Short communication

Biodegradation of fenoxaprop-*p*-ethyl by bacteria isolated from sludge

Liyan Song[a,b], Rimao Hua[a,*], Youcai Zhao[b]

[a] *Center of Pesticide Residue Analysis, Anhui Agricultural University, Hefei 230026, China*
[b] *State Key Laboratory of Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Tongji University, Shanghai 200092, China*

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Abstract

A mixed bacterial population was isolated using enrichment in a basal medium containing increasing amounts of fenoxaprop-*p*-ethyl as a sole carbon source from sludge that had been exposed to fenoxaprop-*p*-ethyl production wastewater for about 2 years. Eight kinds of isolates could utilize fenoxaprop-*p*-ethyl, but only one was identified belonging to genus *Alcaligenes*, named *Alcaligenes* sp. H. In pure culture, there was 45.8, 66.0 and 69.5% loss of fenoxaprop-*p*-ethyl (initial concentration: 100, 50, 25 ppm, respectively) as the sole carbon source with biodegradation by *Alcaligenes* sp. H and fenoxaprop-*p*-ethyl degradation kinetics obeyed the first-order kinetics, the same as the fenoxaprop-*p*-ethyl biodegradation kinetics in soil. At least five degradation products of fenoxaprop-*p*-ethyl biodegradation by *Alcaligenes* sp. H and two degradation products of fenoxaprop-*p*-ethyl biodegradation by H_{uv} separated by HPTLC. It is possible that the fenoxaprop-*p*-ethyl biodegradation by *Alcaligenes* sp. H includes the same pathway as that by H_{uv} comparing with the R_f.

Keywords: Fenoxaprop-*p*-ethyl; Alcaligenes sp. H; Biodegradation products; HPTLC

1. Introduction

Fenoxaprop-*p*-ethyl {ethyl-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]propanoate} (Fig. 1) is one of the 2-(4-aryloxyphenoxy)propionic acids which is used only for control of annual and perennial grass in spring barley, winter wheat and winter rye, and for control of wild oat in fallow fields [1]. Both fenoxaprop and fenoxaprop-*p*-ethyl are members of the aryloxyphenoxypropionate herbicide family. When fenoxaprop is used as an ester or a salt, it is changed into fenoxaprop-*p*-ethyl [2]. Fenoxaprop-*p*-ethyl is widely used in China for its high performance [3–5]. By treating with the 10% fenoxaprop-*p*-ethyl EC 450–600 ml/ha, the fresh weight of wild oat was reduced over 90% [4].

Some studies showed that the control effect was over 90% with 10% fenoxaprop-*p*-ethyl EC 900 ml/ha for wild oats, safety to wheat, increasing about 12.0% of yield [5]. Fenoxaprop-*p*-ethyl is dangerous to the aquatic environment. Fish toxicity: LC50 (96 h) for rainbow trout 0.57 mg/l; Daphnia toxicity: EC50 (48 h) for Daphnia magna 0.56 mg/l; algae toxicity: LC50 (72 h) for *Scenedesmus subspicatus* 0.51 mg/l [2].

Several methods of determination fenoxaprop-*p*-ethyl have been reported, e.g. GC-ECD [3], HPLC [6], but there is no accurate information about fenoxaprop-*p*-ethyl biodegradation behavior. Many studies have showed that when the pesticide entered the environment the biodegradation usually is the main degradation style, and the high-performance pesticide degrading bacterial can be isolated from the soil and sludge which was exposed to pesticide [7–9].

In the present work, we aimed to isolate fenoxaprop-*p*-ethyl-resistant bacteria from the sludge, which had been exposed to fenoxaprop-*p*-ethyl production wastewater for about 2 years. The objective was to characterize the isolate that had the best biodegradation ability for fenoxaprop-*p*-ethyl in the mixed bacterial population and determine the fenoxaprop-*p*-ethyl biodegradation behavior of the isolate by HPLC. The

* Corresponding author. Tel.: +86 0551 2823795 3320; fax: +86 0551 2821390.
E-mail address: hrm@yeah.net (R. Hua).

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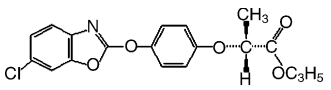

Fig. 1. Chemical structures of fenoxaprop-*p*-ethyl.

HPTLC analysis for the separation of fenoxaprop-*p*-ethyl biodegradation products was studied too.

2. Materials and methods

2.1. Reagents and chemicals

Fenoxaprop-*p*-ethyl (97%) was purchased from Huaxing Chemical Engineering Company (China); Ligroin, toluene and dichloromethane were analysis-grade; HPLC-grade methanol and ultra-pure water (Millipore ultra-pure water system, France) were used in HPLC analysis.

2.2. Isolation of microorganisms resistant to fenoxaprop-p-ethyl

The sludge used for isolation of bacteria was collected from the entrance of Huaxing Chemical Engineering Company pesticide wastewater treatment system that has been exposed to fenoxaprop-*p*-ethyl production wastewater for about 2 years.

The microorganisms were isolated in basal medium containing 100, 200, 300 and 400 ppm fenoxaprop-*p*-ethyl (pure degrade). The basal medium contained (per liter of deionized water): 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$, 0.1 g K_2HPO_4, 0.1 g $(NH_4)_2SO_4$ and 0.04 g $CaSO_4$ (pH 7.0) [10]. Fenoxaprop-*p*-ethyl was used as the sole carbon source in this medium. Fenoxaprop-*p*-ethyl was added to the flasks and the solvent methanol was allowed to evaporate for 30 min before adding the basal medium.

Five grams of sludge was added into 250 ml flasks with 100 ml solid medium [11]. These flasks were incubated at 30 °C in a rotary shaker (120 rpm) and kept from the light to avoid photo-degradation of fenoxaprop-*p*-ethyl. An aliquot of 1 ml was subcultured to fresh medium every 7 days, the concentration of fenoxaprop-*p*-ethyl increasing from 100 to 400 ppm at 100 ppm interval. Colonies that grew best on the beef extract peptone medium were isolated and stored at 4 °C in the same medium.

After isolation, each of the isolates was taken into the basic medium with 10 ppm fenoxaprop-*p*-ethyl and was incubated with the same method above for only 1 day. Biodegradation ability to fenoxaprop-*p*-ethyl of these isolates was determined by HPLC method.

2.3. Identification

The identification of bacterial isolate that has the highest fenoxaprop-*p*-ethyl biodegradation ability was made on the basis of morphological appearance and physiological characteristics [12,13]. The VITEK-AMS (bioMerieux, SA, France) was used to further characterize it with biochemical tests.

2.4. Preparation of standard solution

Stock solution of fenoxaprop-*p*-ethyl for determination by HPLC was prepared by dissolving 10 mg of its pure compound in 10 ml of HPLC-degrade methanol. Working standard solutions, from 0.5 to 10 ppm were prepared from the stock standard solution by diluting with methanol.

Stock solution of fenoxaprop-*p*-ethyl for product separation by HPTLC was prepared by dissolving 2 mg fenoxaprop-*p*-ethyl in 20 ml acetone.

All the stock solutions were stored at 4 °C in the dark.

2.5. Biodegradation

Fenoxaprop-*p*-ethyl was used as sole carbon source in basal medium, with concentration of fenoxaprop-*p*-ethyl being 25, 50 and 100 ppm, respectively. Twenty microliters of bacterial suspension (OD_{330} = 0.6) was added into 5 ml tubes with 1.5 ml basal medium. These tubes were incubated at 30 °C in a rotary shaker (120 rpm) and kept from the light to avoid photo-degradation of fenoxaprop-*p*-ethyl.

The residue was dissolved in methanol and filtered through bacteria filtration membrane (0.2 μm, Tengjiao Membrane Company, Tianjin, China). Methanol is in favor of bacterial growth as carbon source, but the bacteria will be filtrated with 0.2 μm bacteria filtration membrane after the fenoxaprop-*p*-ethyl was dissolved in methanol. As a result, the methanol effect on fenoxaprop-*p*-ethyl biodegradation can be neglected.

Twenty microliters of aliquots was used for HPLC analysis of fenoxaprop-*p*-ethyl using HP 1100 series liquid chromatography system: vacuum degasser, quaternary pump, auto-sampler and diode array detector. The column was used Nov-pak 4 mm C18 (150 × 4.6 mm). The column was operated at 25 °C. The mobile phase eventually adopted for this study was methanol/water (85:15, v/v) and the flow rate was 1.0 ml min^{-1}. Detection wavelength was 236 nm and the retention time of fenoxaprop-*p*-ethyl was 5.7 min. For the quantitation, external calibration was carried out. Standard curve of fenoxaprop-*p*-ethyl was recorded. The data were analyzed by linear regression.

2.6. Separation

1.5 ml basal medium contained 100 ppm pure fenoxaprop-*p*-ethyl was inoculated with 100 μl of *Alcaligenes* sp. H suspension (OD_{330} = 0.6) and the culture is the same as above. After 3 days, 10 flasks were collected to extract using ligroin, and then the extraction solution was enriched to 2 ml to prepare for spotting.

The samples were spotted on HPTLC glass plates with silica gel F_{254} (Qingdao Glass Company, China). At first

plates were pre-washed with a 1:1 (v:v) mixture of methanol and chloroform and activated at 110 °C for 30 min. The samples were streaked in the form of narrow bands of 5 mm length at a constant rate of 7 s μl^{-1} using a nitrogen aspirator. The volumes applied were: standard solution, 20 μl; sample 1 (*Alcaligenes* sp. H), 20 μl; sample 2 (H_{uv}: one of *Alcaligenes* sp. H ultraviolation-mutation bacteria), 20 μl. Ascending one-dimensional development was performed in a standard 24 cm × 8 cm × 14 cm twin-trough chamber (Camag).The length of the chromatographic run was 7 cm and the time required approximately 10 min. The separation was visualized by irradiation of the plates with a 236 nm ultraviolet lamp. Densitometric analysis of the fenoxaprop-*p*-ethyl biodegradation products was carried out using the Camag TLC scanner3 in the absorbance mode (λ = 236 nm). The slit dimension was 6.00 mm × 45 mm, micro, and the scan speed 20 mm s^{-1}.

3. Results and discussion

3.1. Characterization of the bacteria isolate

After incubation in basal media containing 100, 200, 300 and 400 ppm fenoxaprop-*p*-ethyl, eight isolates remained at the maximum fenoxaprop-*p*-ethyl concentration used. Each of these isolates was taken into the basic medium with 10 ppm fenoxaprop-*p*-ethyl for 1-day culture. Those isolates biodegradation ability to fenoxaprop-*p*-ethyl were determined by HPLC method (Table 1). Isolate H has the highest biodegradation ability to fenoxaprop-*p*-ethyl with 69.72% loss in 1 day. Isolate H was selected as the strain in this research.

The cells of the bacterium are coccus, 0.3–5 μm, gram-negative, colonies are round, smooth with regular edge. Optimum conditions for growth are 25–35 °C and pH 7.0–7.5. The bacterium is aerobic, catalase and oxidase-positive.

Isolate H can use some carbohydrates such as sucrose, xylose, maltose, D-glucose, arabinose, mannose, lactose, esculin for growth, but not starch and ribose.

With the VITEK-AMS system, a good similarity coefficient (0.895 in 1) was obtained for the genus *Alcaligenes*, but the isolate H could not be allocated to a particular species since the similarity coefficient values obtained (0.38 in 1) had no significance.

The isolate H, henceforth referred as *Alcaligenes* sp. H, had the "good" biodegradation ability to utilize fenoxaprop-*p*-ethyl as sole carbon source.

Table 1
Degradation rate of fenoxaprop-*p*-ethyl by different strains

Isolates	Biodegradation rate (%)
Standard sample	0
pt	
A	38.73
pt	
B	35.21
pt	
C	26.76
pt	
D	19.01
pt	
E	51.41
pt	
F	46.48
pt	
G	13.38
pt	
H	69.72
pt	

Initial concentration of fenoxaprop-*p*-ethyl: 10 ppm.

3.2. Biodegradation

The working standard solution with 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 and 10 ppm fenoxaprop-*p*-ethyl were injected into the HPLC, and peak height responses recorded. The calibration graph for fenoxaprop-*p*-ethyl was regressed seven consecutive injections and was linear at the range of 0.0005–0.01 mg/ml with a correlation coefficient (R^2) of 0.9982. The limit of detection was determined to be 11 pg. The coefficient of variation for the standard solution was 8.76, 3.13 and 3.20% with the fortified concentration of fenoxaprop-*p*-ethyl 1.0, 5.0 and 10 ppm, respectively, and the recovery of fenoxaprop-*p*-ethyl was 85.32 ± 7.47, 98.89 ± 0.25 and 95.11 ± 0.473%, respectively.

The residual fenoxaprop-*p*-ethyl content and biodegradation rate with *Alcaligenes* sp. H suspension on incubation for 5 days is given in Table 2. There was 45.76, 65.96 and 69.47% loss of fenoxaprop-*p*-ethyl (initial concentration: 100, 50 and 25 ppm, respectively) as the sole carbon source with biodegradation by *Alcaligenes* sp. H. Fenoxaprop-*p*-ethyl degradation kinetics by *Alcaligenes* sp. H obeys the first-order kinetics (Fig. 2 and Table 3). Zhu et al. reported that the fenoxaprop-*p*-ethyl biodegradation kinetics in soil obeyed the first-order kinetics also [3]. With fenoxaprop-*p*-ethyl increasing from 25 to 100 ppm, the half-time of *Alcaligenes* sp. H increases. The different half-time of *Alcaligenes* sp. H at different fenoxaprop-*p*-ethyl are 68.61, 86.63 and 135.88 h, respectively. Effect of fenoxaprop-*p*-ethyl on degradation indicated that high fenoxaprop-*p*-ethyl concentration could prevent the biodegradation rate.

Alcaligenes spp. are common in soil and it can decompose organoporous compounds [14] and dalapon [15], polychlo-

Table 2
Biodegradation rate of different concentration of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H

Initial concentration (ppm)	Bacterial strains	Residual concentration (ppm)	Biodegradation rate (%)
98.9	*Alcaligenes* sp. H	53.64	45.76
49.2	*Alcaligenes* sp. H	16.75	65.96
24.89	*Alcaligenes* sp. H	7.6	69.47

Table 3
Kinetic data of degradation of different concentration of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H

Initial concentration (ppm)	Strain	Regression equation $C_t = C_0\, e^{-kt}$ (first-order)	Coefficient of correlation r	Constant rate (h^{-1})	Half-time
100	H	$C_t = 106.96\, e^{-0.0051t}$	0.9674	0.0051	135.88
50	H	$C_t = 45.873\, e^{-0.008t}$	0.9744	0.008	86.63
25	H	$C_t = 26.851\, e^{-0.0101t}$	0.9925	0.0101	68.61

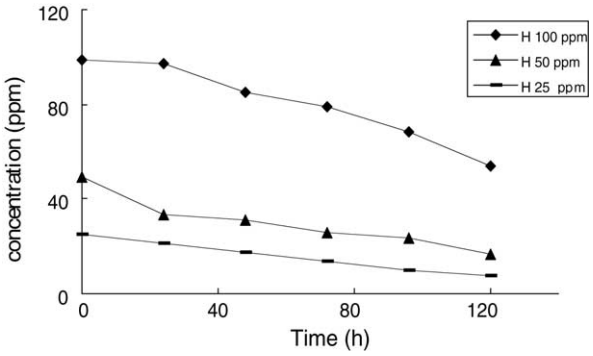

Fig. 2. Degradation of different concentration of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H.

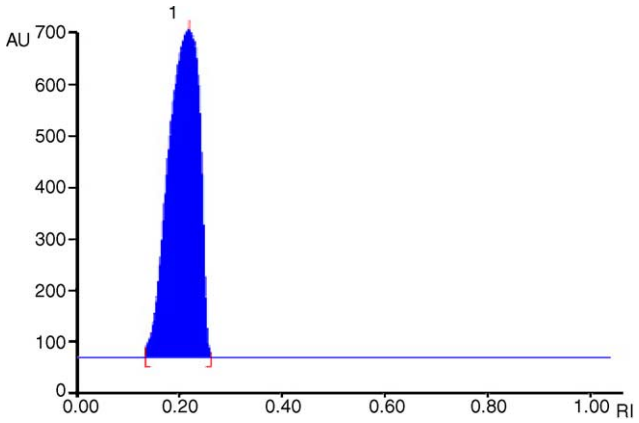

Fig. 3. HPTLC spectra of fenoxaprop-*p*-ethyl standard (100 ppm).

rinated biphenyls [16], poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [17]. The biodegradation of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. has not been recorded in the previous literature.

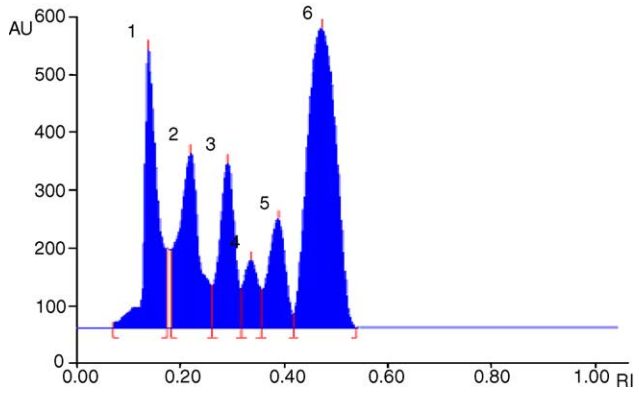

Fig. 4. HPTLC spectra of degradation sample of fenoxaprop-*p*-ethyl after 3 days by *Alcaligenes* sp. H.

3.3. Separation

HPTLC densitograms for fenoxaprop-*p*-ethyl and biodegradation products of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H (sample 1) and biodegradation products of fenoxaprop-*p*-ethyl by H_{uv} (sample 2) are shown in Figs. 3–5, respectively. R_f of fenoxaprop-*p*-ethyl is 0.22, that of sample 1 are 0.14, 0.22, 0.34, 0.39, 0.47, and that of sample 2 are 0.11, 0.22 and 0.39, respectively (Table 4).

By comparing R_f with the standard ($R_f = 0.22$), only one part of fenoxaprop-*p*-ethyl was degradated by *Alcaligenes* sp. H and H_{uv}, and H_{uv} has a better degradation ability to fenoxaprop-*p*-ethyl than *Alcaligenes* sp. H from the peaks of residual fenoxaprop-*p*-ethyl.

We can find that there are at least five degradation products of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H and two degradation products of fenoxaprop-*p*-ethyl by H_{uv} separated by HPTLC. Both samples 1 and 2 have the $0.39R_f$, and the $0.14R_f$ of sample 1 is very close to the $0.11R_f$ of sample 2. It is possible that the fenoxaprop-*p*-ethyl biodegradation by *Alcali-*

Table 4
Different characteristics of fenoxaprop-*p*-ethyl and biodegradation products of fenoxaprop-*p*-ethyl on thin-layer plate

	Peak	R_f	Height (AU)	Area (AU)
Fenoxaprop-*p*-ethyl standard	1	0.22	633.6	33954.3
Fenoxaprop-*p*-ethyl biodegradation sample 1 (*Alcaligenes* sp. H)	1	0.14	481.9	10463.5
	2	0.22	302.2	10294.5
	3	0.29	285.8	7182.0
	4	0.34	116.6	2859.2
	5	0.39	188.6	5303.8
	6	0.47	518.7	24503.5
Fenoxaprop-*p*-ethyl biodegradation sample 2 (H_{uv})	1	0.11	455.6	20521.7
	2	0.22	284.4	9378.4
	3	0.39	263.0	8742.1

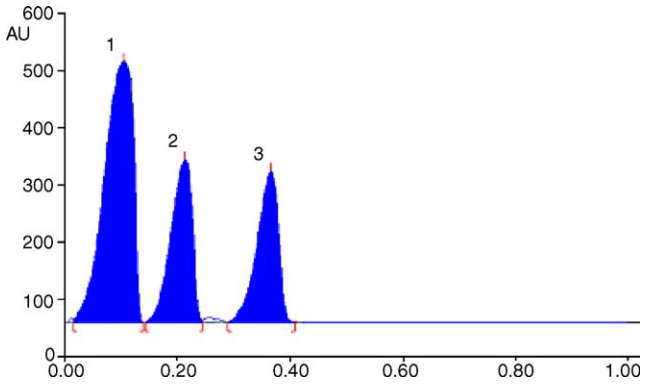

Fig. 5. HPTLC spectra of degradation sample of fenoxaprop-*p*-ethyl after 3 days by H_{uv}.

genes sp. H includes the same pathway as that by H_{uv} though they have different biodegradation ability to fenoxaprop-*p*-ethyl.

4. Conclusions

Determined by HPLC method isolate H was selected as the strain in this research with highest biodegradation ability to fenoxaprop-*p*-ethyl, and after identification isolate H was referred as *Alcaligenes* sp. H. Fenoxaprop-*p*-ethyl degradation kinetics by *Alcaligenes* sp. H obeys the first-order kinetics, the same as the fenoxaprop-*p*-ethyl biodegradation kinetics in soil. At least five degradation products of fenoxaprop-*p*-ethyl by *Alcaligenes* sp. H and two degradation products of fenoxaprop-*p*-ethyl by H_{uv} separated by HPTLC. It is possible that the fenoxaprop-*p*-ethyl biodegradation by *Alcaligenes* sp. H includes the same pathway as that by H_{uv} though they have different biodegradation ability to fenoxaprop-*p*-ethyl comparing with the R_f, and further research is needed.

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