

Cometabolic biotransformation of fenpropathrin by *Clostridium* species strain ZP3

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Abstract A novel bacterial strain capable of degrading the pyrethroid pesticide fenpropathrin was isolated from mixed wastewater and sludge samples. Phylogenetic analysis of the 16S rDNA sequence revealed that the organism belongs to the genus *Clostridium*. The organism can co-metabolically transform fenpropathrin at 100 mg l⁻¹ at 35°C and pH 7.5 in 12 days. Metabolic products of fenpropathrin from strain ZP3 were examined by gas chromatography/mass spectrometry, and the results showed that the organism degraded fenpropathrin with an oxidization process to yield benzyl alcohol, benzenemethanol, 3,5-dimethylamphetamine. Analyses of cell-free extracts from this strain showed that the optimal degrading conditions for degrading fenpropathrin were 35°C and pH 7.5, and degradation efficiency was 20.0 mg l⁻¹ day⁻¹, and it might be potential using for rapid treating fenpropathrin, for example, on the surface of fruits and vegetables.

Keywords Biodegradation · Fenpropathrin · Metabolic products · Benzyl alcohol · Benzenemethanol · 3,5-dimethylamphetamine

Introduction

Pyrethroid pesticides, which are based on the chemical pyrethrin, are insecticidal esters derived from the flower heads of certain *Chrysanthemum* species (George and Kalyanasundaram 1994). Pyrethroid pesticides have been widely used in crops, including vegetables and fruit trees, because of their high toxicities to insects and low toxicities to mammals (Ross et al. 2006). However, pyrethroid pesticides are extremely toxic to the aquatic environment, and a concentration of as little as 10.0 ng l⁻¹ is enough to eradicate all of the invertebrate life in entire rivers and lakes (Antwi and Peterson 2009). Furthermore, some of the pyrethroid pesticides have been classified as potential human carcinogens by the US Environmental Protection Agency (EPA 1996). Currently, organophosphorous pesticides are increasingly being replaced by pyrethroid pesticides, and the impact of the pyrethroid pesticides vestige on the environment is significantly increased (Liu et al. 2005).

Recently, due to growing concern about the pollution caused by pyrethroid pesticides release, intensive efforts have been made to minimize this

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adverse effect. Several studies indicate that biodegradation is a practical solution for detoxifying pyrethroid pesticides (e.g., cypermethrin and deltamethrin) over a wide range of conditions, including synthetic media, dip troughs or rivers (Maloney et al. 1993; Lee et al. 2004; Tallur et al. 2008).

Traditionally, pyrethroid pesticides can be grouped into two subclasses (type I and II) based on their chemical structure and toxicological actions. Type I pyrethroids (e.g. Bifenthrin) do not have cyano-bases in their chemical structure, while type II pyrethroids (e.g. Fenpropathrin) have chemical structures that contain an α -cyano base (Ray and Fry 2006). The biodegradation pathway of type II pyrethroids for the most part is well understood (Kaufman et al. 1981; Maloney et al. 1993; Lee et al. 2004; Zhang et al. 2009, 2010), especially for cypermethrin, which can be completely mineralized to CO_2 and H_2O by strains of the genus *Micrococcus* (Tallur et al. 2008). However, studies on the metabolism and identification of intermediate products for some of the newer type II pyrethroids such as fenpropathrin thus far have been limited (Wang et al. 2007). This is despite the fact that type II pyrethroids are the primary cause of toxicity to aquatic invertebrates due to residential and agricultural use (Weston et al. 2005).

Here we aimed (i) to isolate and identify a novel bacterial strain capable of degrading fenpropathrin; (ii) to extract and identify the metabolic products to elucidate the degradation pathways; and (iii) to examine the factors influencing the process of fenpropathrin degradation using cell-free extracts from this organism.

Materials and methods

Media and chemicals

Fenpropathrin (95.0% purity) was purchased from Hainan Zhengye Pesticide Chemical Company, China. Fenpropathrin was dissolved in acetone as a stock solution ($10,000 \text{ mg l}^{-1}$). Authentic standard fenpropathrin (99.0% purity) was purchased from Tianjin Orient Green Technology and Development Company, China. The enrichment medium was mineral salt medium (MSM), which is composed of (per liter deionized water): $(\text{NH}_4)_2\text{SO}_4$, 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 g; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 1.5 g;

KH_2PO_4 , 1.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g; Glucose, 5.0 g; adjusted to pH 7.5 with 10 M NaOH. In order to investigate whether isolated degrader can use fenpropathrin as the sole source of carbon, an amended MSM (AMSM) was prepared, which contained 100 mg l^{-1} fenpropathrin substituted for glucose as the sole carbon source.

All other chemicals were of analytical grade and were obtained from Shanghai Sango Biological Engineering Technology & Services Co., Ltd (Shanghai, China).

Enrichment and isolation of fenpropathrin-degrading strain ZP3

The mixed wastewater and sludge samples were taken from effluent in a pesticide factory in Hunan, China. Samples (10 g) were added to 90 ml of MSM containing 25 mg l^{-1} of fenpropathrin in 250 ml flasks. The suspension was incubated at room temperature on a rotary shaker. 10 ml of the culture broth was transferred weekly to 90 ml of fresh medium, while its fenpropathrin concentration was doubled using a stock solution. After the fourth enrichment transfer, aliquots of each culture were spread on respective plates of solid MSM (containing 1.5% agar and 100 mg l^{-1} of fenpropathrin) and incubated at room temperature until visible growth appeared. Strain ZP3 was selected and pre-cultured in MSM and monitored by spectrophotometry at 600 nm (Tu-1901, China). The starting number of cells (OD 600 value) for subsequent tests was adjusted to approximately 0.5.

Identification of the isolated strain ZP3

Primary PCR was performed for bacterial 16S rDNA, using the universal primers 27f (5'-AGAGTTTGAT CMTGGCTCAG-3') and 1492r (5'-TACGGYTACC TTTGTTAC-3') (Xu et al. 2008). The DNA sequences amplified by PCR were cloned into *Escherichia coli* using pGEM-T Easy Vector System I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cloned DNA fragments were sequenced using an ABI Prism 377 automated sequencer (PE Applied Bio-systems, Foster City, CA, USA). The phylogenetic tree was generated using MEGA 3.1 software (Kumar et al. 2004) with the Kimura 2-parameter model and the Neighbor-Joining method.

Extraction and determination of fenpropathrin levels

The extraction and quantification of fenpropathrin residue was modified slightly from the method described in Zhang et al. (2009). Briefly, the broth of one total flask was sacrificed as a sample. The sample was extracted three times with 100 ml of petroleum ether. The extracts were passed through anhydrous sodium sulfate, collected in a 250 ml flat-bottom flask, concentrated approximately to dryness on a rotary evaporator, and then dissolved in 5 ml of petroleum ether for determination by gas chromatography (GC).

Quantitative analysis was performed using a GC-6890 (Agilent, USA) equipped with an electron capture detector. A fused silica capillary column HP-5 (30 m $\times$ 0.32 mm $\times$ 0.25 μ m) (Agilent, USA) was employed. The temperature of the injection port was 250°C. The column was held at 160°C for 5 min, ramped at 10°C min⁻¹ to 200°C (first ramp), held at 200°C for 1 min, ramped at 10°C min⁻¹ to 280°C (second ramp), and then held at 280°C for 8 min. The temperature of the electron capture detector was 320°C. The detector makeup gas was nitrogen with high purity at 1 ml min⁻¹. The injection volume was 1 μ l. Concentrations were determined by analyzing peak area with an authentic fenpropathrin standard.

Biodegradation of fenpropathrin by strain ZP3

The degradation ability and growth of strain ZP3 were assessed in 90 ml MSM supplemented with 100 mg l⁻¹ of fenpropathrin and 10 ml of strain ZP3 in 250 ml flasks incubated at 35°C. At periodical intervals, individual flasks were sacrificed and their contents were used to monitor the growth and fenpropathrin residue. The growth was monitored by spectrophotometry at 600 nm (Tu-1901, China), and the residue was assayed by GC. Assessment of the degradation ability of strain ZP3 in AMSM was carried out as for MSM.

Isolation and identification of metabolites

Metabolites were isolated from the culture filtrates of the organism grown in fenpropathrin (100 mg l⁻¹) by extraction with ethyl acetate before and after acidification to pH 2 with 2 N HCl, and the residue

obtained was dissolved in methanol. The residues were analyzed for metabolites by GC/MS. All GC/MS analyses were conducted using an Agilent 6890N/5975 series GC-MSD (Agilent, USA) equipped for electron ionization (EI). Electron Ionization (70 eV) was performed with a trap current of 100 mA and a source temperature of 200°C. Full-scan spectra were acquired over the ranges of m/z of 45–500 at 2 s per scan. Data collection and processing were performed using Agilent MSD chemstation software containing the Agilent chemical library.

Preparation of cell-free extracts

The strain ZP3 was grown in MSM containing fenpropathrin (100 mg l⁻¹). Growth was monitored, and cells were pelleted by centrifugation at 6,000 $\times$ g for 10 min. Pellets were washed three times and re-suspended in 3 ml phosphate buffer (0.05 M, pH 7.0). After the disruption of cells by ultrasonication on ice and centrifugation at 4°C, the supernatants were recovered as the cell-free extract. The protein concentration was adjusted to 1 μ g μ l⁻¹ prior to use.

Enzymatic degradation of fenpropathrin by cell-free extracts

The reaction mixture (10 ml) contained phosphate buffer (0.05 M, pH 7.0), fenpropathrin (100 mg l⁻¹) as the substrate, and cell-free extracts (protein concentration 1 μ g μ l⁻¹). Reactions were performed at a range of pH (5.5–9.5) and temperature (25–45°C), and the fenpropathrin residues were periodically quantified as described above. All experiments were performed in triplicate, and controls lacking cell-free extracts were employed.

Results

Isolation and characterization of strain ZP3

The 16S rDNA sequence of the fenpropathrin-degrading strain ZP3 showed the greatest similarity to the reference sequence of *Clostridium celerecrescens* (AJ 295659) within the GenBank database. A phylogenetic tree (Fig. 1) depicts the position of strain ZP3 within the genus *Clostridium*. Based on these observations, the isolate was tentatively

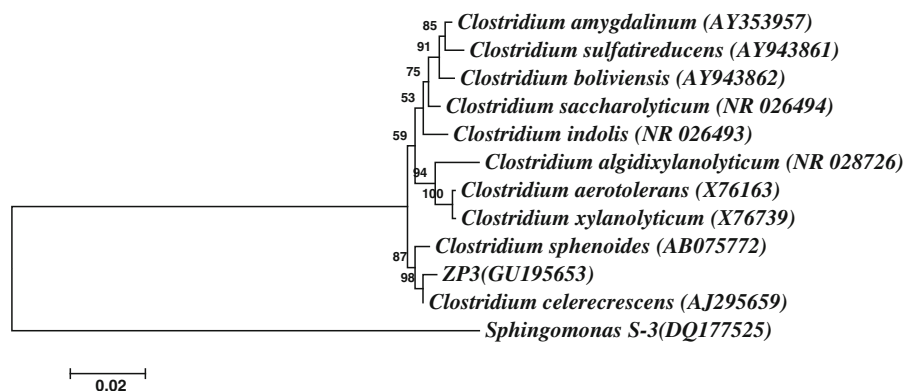


Fig. 1 Phylogenetic tree based on the 16S rDNA sequences of strain ZP3 and related species. GenBank accession numbers are given in brackets. The bootstrap values obtained for 1,000

repetitions are indicated as percentages at all branches. *Sphingomonas* S-3 (DQ177525) was selected as an outlier to root the tree

designated as a member of the genus *Clostridium* (Holt et al. 1994).

Bacterial growth and degradation ability

With fenpropathrin as the sole carbon source for 12 days, strain ZP3 degraded fenpropathrin slightly in AMSM, but completely in MSM (Table 1). This contrasting result suggested that strain ZP3 degraded fenpropathrin co-metabolically, which required an extra carbon source (glucose) (Liang et al. 2009; Zhang et al. 2009).

In a biodegradation assay, complete degradation of 100 mg l⁻¹ fenpropathrin was observed within 12 days (Fig. 2). Degradation of fenpropathrin followed a pattern of transient tardiness, but the fenpropathrin was subsequently degraded rapidly and eventually disappeared. In controls lacking incubation, abiotic degradation was negligible throughout all studies. The highest bacterial population density was observed on Day 7, and the population density was rapidly increasing from 3 to 7 days. After that, the bacterial population seemed to reach the equilibrium.

Table 1 Degradation capacity of strain ZP3 in different medium following 12 days

Media	Degradation ratio (%)
MSM	100
AMSM	12.58 ± 2.37

Analysis of fenpropathrin biodegradation metabolites

Analysis of the culture extracts from strain ZP3 grown on fenpropathrin by GC/MS revealed the presence of several compounds. The mass spectra of metabolic compounds showed that compound I corresponded well with benzyl alcohol (Fig. 3a), compound II corresponded well with benzenemethanol (Fig. 3b), compound III corresponded well with 3,5-Dimethylamphetamine (Fig. 3c). The retention times of compounds were 6.800, 7.657, and 7.942 min, respectively.

Enzymatic degradation of fenpropathrin using cell-free extracts

Cell-free extracts were able to degrade fenpropathrin efficiently at temperature in the range of 30–40°C and the optimal temperature was at 35°C (Fig. 4a). The most rapid degradation pH occurred at pH 7.5, while in the pH range of 6.5–8.5, cell-free extracts were capable of degrading fenpropathrin efficiently (Fig. 4b).

The degradation capacity of fenpropathrin by cell-free extracts from strain ZP3 under optimal conditions is shown in Fig. 4c. Fenpropathrin was degraded rapidly in the first 3 days by cell-free extracts that were pre-cultured with fenpropathrin. The enzymatic degradation rate for fenpropathrin was 20.0 mg l⁻¹ day⁻¹. Only slight degradation was observed in the absence of cell-free extracts.

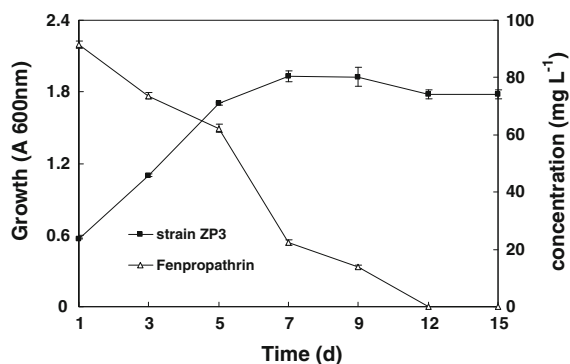


Fig. 2 Degradation capacity of strain ZP3 and biomass as monitored by measuring OD at 600 nm

Discussion

This study reports the isolation and characterization of strain ZP3, an organism capable of degrading fenpropathrin co-metabolically. The biodegradation characteristics of fenpropathrin by strain ZP3 were

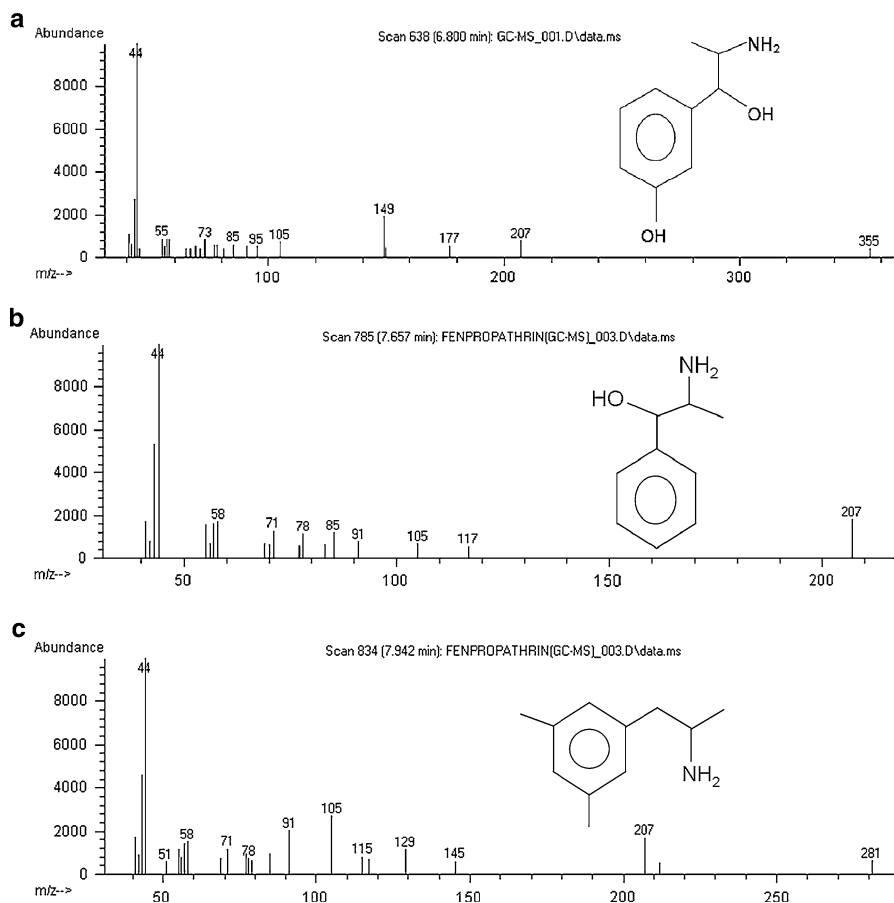
analyzed by GC and GC/MS. The biodegradation of fenpropathrin by cell-free extracts from strain ZP3 was also evaluated.

Several studies have verified that the insecticidal activity of some of the type I and II pyrethroid pesticides is destroyed leading to its detoxification by inception hydrolysis of ester linkage (Abernathy and Casida 1973; Lee et al. 2004; Tallur et al. 2008; Hong et al. 2010). Much additional empirical evidence has testified that some of the type I and II pyrethroid pesticides can be biodegraded by oxidation, such as the expressed products of P450 genes in *Anopheles funestus*. Otherwise, the metabolic pathway for pyrethroid degradation is as yet uncovered (Charles et al. 2009; Cuamba et al. 2010). It is clear evident from our results that strain ZP3 degraded fenpropathrin with an oxidation process to yield benzyl alcohol, benzene-methanol and 3,5-dimethylamphetamine.

The results in present study suggest that strain ZP3 might provide a promising method to degrade

Fig. 3 GC/MS spectrum of metabolic products.

- a** Benzyl alcohol.
- b** Benzenemethanol.
- c** 3,5-dimethylamphetamine



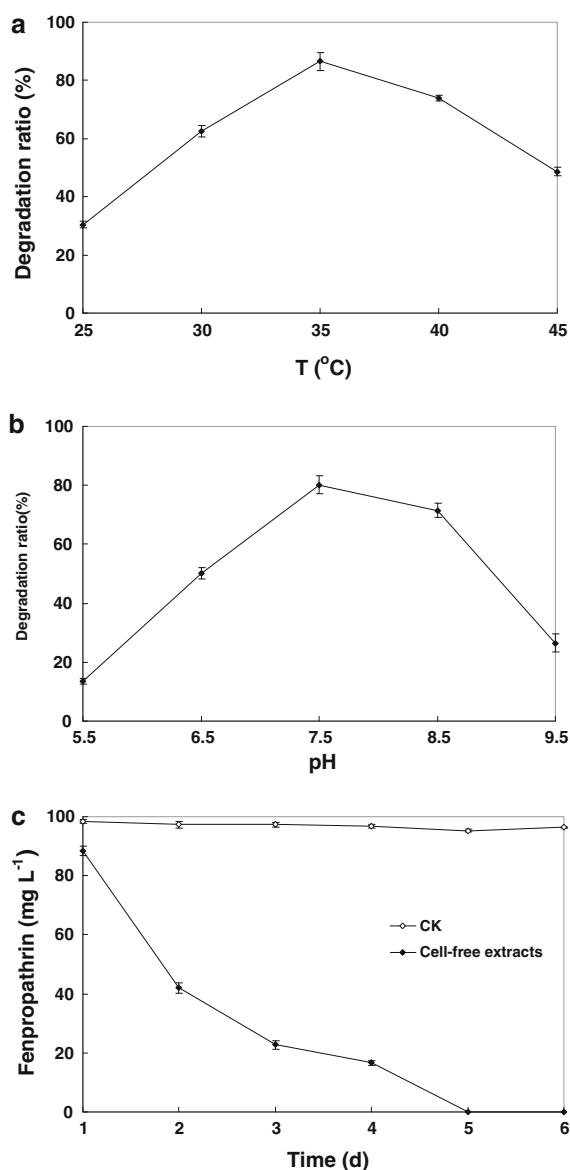


Fig. 4 Enzymatic degradation of fenpropathrin by cell-free extracts from strain ZP3. **a** Enzymatic degradation capacity of cell-free extracts under different temperature on 4 days. **b** Enzymatic degradation capacity of cell-free extracts under different pH on 4 days. **c** Enzymatic degradation capacity of cell-free extracts under optimal conditions. Values are means $\pm$ SD of three replicates

fenpropathrin, and compared to cell of the organism, cell-free extracts have higher fenpropathrin-degrading efficiency probably because of direct contacting to zymolyte (fenpropathrin) without separating by cell membrane, so cell-free extracts might provide degradation potential for rapid treating fenpropathrin

on the surface of fruits and vegetables (Xu et al. 2008).

Conclusions

An efficient fenpropathrin degrader, strain ZP3, was isolated from the mixed wastewater and sludge samples and identified as a member of the genus *Clostridium*. Strain ZP3 was able to degrade fenpropathrin at high concentration with co-metabolic way, which would be suitable for wastewater treatment, especially for eutrophic wastewater with fenpropathrin contaminants. Three compounds (benzyl alcohol, benzenemethanol and 3,5-dimethylamphetamine) were detected, and this result also hinted there are complex redox reactions in fenpropathrin degrading process by strain ZP3. The experiments of cell-free extracts displayed its potential using for rapid treating fenpropathrin, for example, on the surface of fruits and vegetables.

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