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ENVIRONMENTAL BIOTECHNOLOGY

Isolation of a novel beta-cypermethrin degrading strain *Bacillus subtilis* BSF01 and its biodegradation pathway

Ying Xiao · Shaohua Chen · Yuanqi Gao · Wei Hu · Meiying Hu · Guohua Zhong

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Abstract Continuous use of the pyrethroid insecticide beta-cypermethrin (beta-cp) has resulted in serious environmental contamination problems. We report here that a novel bacterial strain BSF01, which was isolated from activated sludge and identified as *Bacillus subtilis* (collection number: CCTCC AB 2014103), showed high efficiency in degrading beta-cp. Strain BSF01 was able to utilize beta-cp as the sole carbon source for growth and degraded 89.4 % of 50 mg L^{-1} beta-cp within 7 days. The optimal conditions for beta-cp degradation were determined to be 34.5 °C, pH 6.7, and inocula amount 0.11 g dry wt L^{-1} using response surface methodology. The kinetic parameters q_{max}, K_s, and K_i were established to be 2.19 day^{-1}, 76.37 mg L^{-1}, and 54.14 mg L^{-1}, respectively. The critical inhibitor concentration was determined to be 64.30 mg L^{-1}. Seven metabolites were identified by gas chromatography–mass spectrometry. Furthermore, a novel biodegradation pathway for beta-cp was proposed on the basis of analysis of the metabolites. This strain was also capable of degrading a wide range of pyrethroid insecticides including cypermethrin, deltamethrin, cyhalothrin, and beta-cyfluthrin, which similar to beta-cp are hazardous chemicals. Taken together, our results depict the biodegradation pathway of beta-cp and highlight the promising potentials of strain BSF01 in bioremediation of pyrethroid-contaminated environments.

Keywords Bioremediation · Beta-cypermethrin · *Bacillus subtilis* BSF01 · Metabolites · Biodegradation pathway

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Y. Xiao · W. Hu · M. Hu (✉) · G. Zhong (✉)
Laboratory of Insect Toxicology, and Key Laboratory of Pesticide and Chemical Biology, Ministry of Education, South China Agricultural University, Guangzhou 510642, People's Republic of China
e-mail: humy@scau.edu.cn
e-mail: guohuazhong@scau.edu.cn

S. Chen
Guangdong Province Key Laboratory of Microbial Signals and Disease Control, South China Agricultural University, Guangzhou 510642, People's Republic of China

Y. Gao
Guangdong Tianhe Agricultural Means of Production Co., Ltd., Guangzhou, 510642, People's Republic of China

Introduction

Pyrethroid insecticides are synthetic derivatives of pyrethrins, which are natural insecticides that are produced from chrysanthemum plants (Soderlund et al. 2002). Pyrethroid insecticides act as neurotoxins (Narahashi et al. 1998), which have been used in agriculture and home formulations for over 40 years and account for about 25 % of the worldwide insecticide market (Shafer et al. 2005). Recently, with the restriction on the use of organophosphates and carbamates, pyrethroid insecticides have generally been regarded as the replacement with 160 t used for agriculture and 258 t used for nonagriculture pest control in California in 2007 (Weston and Lydy 2010). However, the accumulation and widespread use of pyrethroid insecticides have increased the public concern on potential human health risks (Mohapatra et al. 2007). Toxicological studies performed in water-only systems have found that these pesticides cause lethal and sublethal effects even at extremely low concentrations, with LC_{50} being generally less than 1 ng mL^{-1} (Coats et al. 1989). In addition, pyrethroid insecticides have been found to be unaffected by secondary treatment systems at municipal wastewater treatment facilities in California (Weston and Lydy 2010).

Beta-cypermethrin (beta-cp) [cyano-(3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-

carboxylate] is one of the most popular pyrethroid insecticides and used worldwide, which occupies more than 50 % of the total production of pyrethroids market in China (Zhang et al. 2007). Even though beta-cp was considered safe for humans, a large number of studies show that beta-cp may have reproductive toxicity (Yuan et al. 2010), endocrine disruption effects (McKinlay et al. 2008), immune toxicity (Jin et al. 2011), and genotoxic effects (Ansari et al. 2011) on nontarget organisms. Moreover, beta-cp is regarded as a possible human carcinogen by the Environmental Protection Agency (EPA) of USA (Shukla et al. 2002). The reported half-life of beta-cp in soil ranges from 94.2 to 1,103 days (Zhang et al. 2011). Hence, disposal of the residues of beta-cp from the environment is of great economic and social significance.

Biodegradation is considered to be the most significant process determining the fate of pyrethroid insecticides in nature (Fenlon et al. 2011) and has received increasing attention as an effective, safe, and cheap approach to clean up contaminated environments (Arora et al. 2012b; Cycoń et al. 2011). Up to now, several strains such as *Pseudomonas aeruginosa* (Zhang et al. 2011), *Streptomyces* sp. (Lin et al. 2011), *Stenotrophomonas* sp. (Chen et al. 2011a), and *Serratia marcescens* (Cycoń et al. 2014) have been reported to degrade pyrethroid insecticides. Several degrading enzymes involved in pyrethroids degradation have recently been purified (Guo et al. 2009; Wang et al. 2009; Zhai et al. 2012; Fan et al. 2012; Chen et al. 2013a). However, there is no information about beta-cp degradation by *Bacillus subtilis*. Moreover, the biodegradation pathway of beta-cp has rarely been characterized (Liu et al. 2014).

The present study isolated a novel *Bacillus subtilis* BSF01 that was able to efficiently degrade beta-cp and other pyrethroid insecticides. Biodegradation conditions for beta-cp were optimized. What's more, degradation products were determined, and the biodegradation pathway was proposed in this study. These results highlight the promising potentials and advantages of *Bacillus subtilis* BSF01 for bioremediation of the pyrethroid-contaminated environments.

Materials and methods

Chemicals and media

Technical grade beta-cypermethrin (95 % purity), cypermethrin (92.9 % purity), deltamethrin (98 % purity), cyhalothrin (95 % purity), and beta-cyfluthrin (95 % purity) were obtained from Jiangsu Yangnong Chemical Group Co., Ltd., China. Chromatographic grade acetonitrile was purchased from Oceanpak Alexative Chemical Co., Ltd, Sweden. All the other chemicals and solvents used in this experiment were of analytical grade. Each pyrethroid insecticide was dissolved in acetone or methanol as stock solutions (10,000 mg L^{-1}). The solutions were sterilized by filtration with 0.45 μm of filter membrane prior to use.

The mineral salt medium (MSM) containing 2.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2{\cdot}2H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$, 1.5 g $Na_2HPO_4{\cdot}12H_2O$, and 1.5 g KH_2PO_4 per liter of water and Luria–Bertani medium (LBM) containing 5-g yeast extract, 10 g tryptone, and 10 g NaCl per liter of water were used for the isolation and cultivation of degrading strains. Both media were adjusted to pH 7.0 and autoclaved to sterilize at 121 °C for 20 min (Guo et al. 2009; Chen et al. 2012; Liu et al. 2014).

Screening and isolation of beta-cp-degrading strains

Activated sludge samples were collected from pesticide-manufacturing wastewater treatment system of Guangxi Research Institute of Chemical Industry, China. One gram of the sludge was suspended with sterile water. The samples were inoculated on the LB agar plates and incubated at 30 °C for 2 days after immersing in the 80 °C water bath for 10 min. The well-isolated colonies were transferred into 250-mL Erlenmeyer flasks containing 50-mL sterilized LBM and incubated at 30 °C on a rotary shaker at 230 rpm. The final cultures were gradually diluted and inoculated on MSM agar plates containing 100 mg L^{-1} of beta-cp. The eugenic colonies were picked out as the potential strains and their degrading abilities of beta-cp were monitored by high-performance liquid chromatography (HPLC). One strain named BSF01 that showed the highest degrading efficiency of beta-cp was selected for further studies.

Identification of the beta-cp-degrading strain

Morphology of strain BSF01 was observed by electron microscope (Olympus, Japan) and scanning electron microscope (XL-30ESEM, Philips Optoelectronics Co., Ltd., Holland). Physio-biochemical tests were examined as described before (Holt et al. 1994). Genomic DNA was extracted according to protocol of E.Z.N.A.® Bacterial DNA Kit (OMEGA BioTek, USA). 16S rDNA gene was amplified using the universal primers, B1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and B4 (5′-ACGGHTACCTTGTTTACGACTT-3′). The PCR cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1.25 min, with the last cycle followed by a 10-min extension at 72 °C. The PCR products were cloned into the vector pMD20-T (TaKaRa Biotechnology Co. Ltd., China) and transformed into *Escherichia coli* DH5α cells (Chen et al. 2011b, c). Clones were sequenced by Shanghai Yingjun Technology Co. Ltd., China, after purification. The resulting

sequence (GenBank accession No. JF706263) was compared with the sequences in the GenBank database by BLAST. Multiple sequence alignments were conducted with the selection of high homology sequences using CLUSTALX 1.8.1. The phylogenetic tree was analyzed and constructed with the neighbor-joining method by MEGA 4.0 version (Tamura et al. 2007).

Relationship between the growth of strain BSF01 and the biodegradation of beta-cp

The degradation of beta-cp was performed in 250-mL Erlenmeyer flasks with 50-mL sterile MSM containing 50 mg L^{-1} beta-cp. Inocula were prepared by inoculating the individual colony into 50 mL of MSM at 32 °C with shaking at 150 rpm. The bacterial cells in the late-exponential growth phase were harvested by centrifugation at 4,000*g* for 2 min and washed with 0.9 % sterile saline and resuspended in 50 mL saline (Anwar et al. 2009). One percent of this suspension (the total biomass amount was 0.12 g dry wt L^{-1}) was used as the inocula for the subsequent studies. The experiment was conducted in triplicate at 32 °C on a rotary shaker at 150 rpm for 7 days. The noninoculated served as controls. The growth of the strain was determined by counting the colony-forming units (CFU) per milliliter of serial dilutions, and the residual beta-cp concentration was measured by HPLC.

Optimal conditions for beta-cp biodegradation

Response surface methodology (RSM) was explored to optimize the biodegradation conditions of strain BSF01. The Box–Behnken design consisting of 15 experimental runs with three replicates at the center point was used to optimize the independent variables which significantly influenced the beta-cp biodegradation. Based on preliminary study, three critical factors and their optimal ranges were selected in this experiment: temperature (22–42 °C), medium pH (5–9), and inocula (total biomass amount 0.02–0.22 g dry wt L^{-1}) (Table 1). The dependent variable was the degradation of 50 mg L^{-1} beta-cp in MSM by day 7. Empirical model equation (Eq. (1)) was created that correlated the relationship between the independent variables and the dependent variable by statistic analysis system (SAS) software version 9.0 (Chen et al. 2011b).

$$Y_i = b_0 + \Sigma b_i X_i + \Sigma b_{ij} X_i X_j + \Sigma b_{ii} X_i^2 \quad (1)$$

where Y_i is the predicted response, X_i and X_j are the variables, b_0 is the constant, b_i is the linear coefficient, b_{ij} is the interaction coefficient, and b_{ii} is the quadratic coefficient.

Table 1 Box–Behnken experimental design and the response of dependent variable for beta-cp degradation

Run	X_1	X_2	X_3	Response beta-cp degradation (%) Y_1
1	−1	−1	0	71.5±1.0f
2	−1	1	0	75.0±1.5e
3	1	−1	0	83.6±1.2cd
4	1	1	0	80.9±1.2d
5	0	−1	−1	86.6±2.3b
6	0	−1	1	85.0±0.3bc
7	0	1	−1	83.1±0.9cd
8	0	1	1	82.6±1.2cd
9	−1	0	−1	75.8±1.2e
10	1	0	−1	82.6±1.0cd
11	−1	0	1	73.7±1.1ef
12	1	0	1	83.4±1.0cd
13	0	0	0	90.2±1.5a
14	0	0	0	89.4±0.7a
15	0	0	0	91.3±1.5a

The data presented are means of three replicates with standard deviation. Different letters behind the data indicate significant differences ($P<0.05$, least significant difference (LSD) test)

X_1 temperature, −1 (22 °C), 0 (32 °C), +1 (42 °C); X_2 medium pH, −1 (5), 0 (7), +1 (9); X_3 inocula, −1 (0.02), 0 (0.12), +1(0.22)

Effect of different initial beta-cp concentrations on its biodegradation

Biodegradation experiments of different initial beta-cp concentrations were performed in 250-mL Erlenmeyer flasks with 50-mL sterile MSM containing 25–400 mg L^{-1} beta-cp. The MSM was incubated in triplicate under the optimal conditions with noninoculated as controls. Andrews equation (Eq. (2)) was used to describe the specific degradation rate (q) at different initial beta-cp concentrations (Chen et al. 2014).

$$q = \frac{q_{max} S}{S + K_s + \left(S^2/K_i\right)} \quad (2)$$

where q_{max} is the maximum specific degradation rate, K_i is the inhibition constant, K_s is the half-rate constant, and S is the beta-cp concentration. The kinetic parameters were calculated using Matrix Laboratory (MATLAB) software version 7.8 according to Chen et al. (2014).

Biodegradation kinetics of various pyrethroid insecticides

Sterile MSM was supplemented with beta-cp, cypermethrin, deltamethrin, cyhalothrin, or beta-cyfluthrin at 50 mg L^{-1} and inoculated at the optimal degradation conditions for 7 days. The experiment was performed in triplicate with noninoculated as controls. The first-order kinetic model

(Eq. (3)) was applied to explore the biodegradation kinetics of various pyrethroid insecticides (Chen et al. 2013b).

$$C_t = C_0 \times e^{-kt} \quad (3)$$

where C_0 is the initial concentration of pyrethroid insecticides at time zero, C_t is the concentration of pyrethroid insecticides at time t, and k and t are the rate constant (day^{-1}) and degradation period in days, respectively.

The half-life ($T_{1/2}$) of different pyrethroid insecticides was calculated by Eq. (4) (Chen et al. 2013b).

$$T_{1/2} = \frac{\ln 2}{k} \quad (4)$$

where k is the rate constant (day^{-1}).

Identification of beta-cp metabolites and analysis of biodegradation pathway

The cell-free filtrates of the cultures grown in MSM for 7 days containing 50 mg L^{-1} of beta-cp were collected at an interval of 1 day. The noninoculated culture containing the same amount of beta-cp was used as control. The cultures were extracted with an equal volume of dichloromethane after acidification to pH 2 with 2 M HCl. The solvent phase was concentrated using rotary evaporator (Heidolph, Germany). The extracts were dissolved in methanol and were subjected to gas chromatography–mass spectrometry (GC-MS).

Chemical analysis

Thirty milliliters of cell-free medium was extracted using 60 mL of acetone/petroleum ether (1:1, *v*/*v*) in an ultrasonic bath. After partitioning, the supernatants were passed through a 0.22 mm polytetrafluoroethylene (PTFE) membrane filter (Millipore, USA), and the filtrates were concentrated using rotary evaporator (Heidolph, Germany). The pyrethroid residues were determined by an Agilent 1260 HPLC system with an Agilent ZORBAX SB-C18 reversed phase column (4.6 nm×150 mm, 5 μm) at 235-nm wavelengths. A mixture of acetonitrile and water (85:15, *v*/*v*) was used as the mobile phase at a flow rate of 1.0 mL min^{-1} with the injection volume of 10 μL.

The metabolites of beta-cp were identified by an Agilent 6890N/5975 GC-MS system according to Chen et al. (2011b).

Results

Isolation and characterization of the beta-cp-degrading strain

In this study, one strain that was able to utilize beta-cp as the sole carbon source for growth was isolated from activated sludge and named as BSF01, which degraded 89.4 % of 50 mg L^{-1} beta-cp within 7 days. Strain BSF01 was an obligate aerobic, Gram-positive, rod-shaped bacterium with spores of 1.0 to 1.5 μm in length and 0.6 to 0.9 μm in width

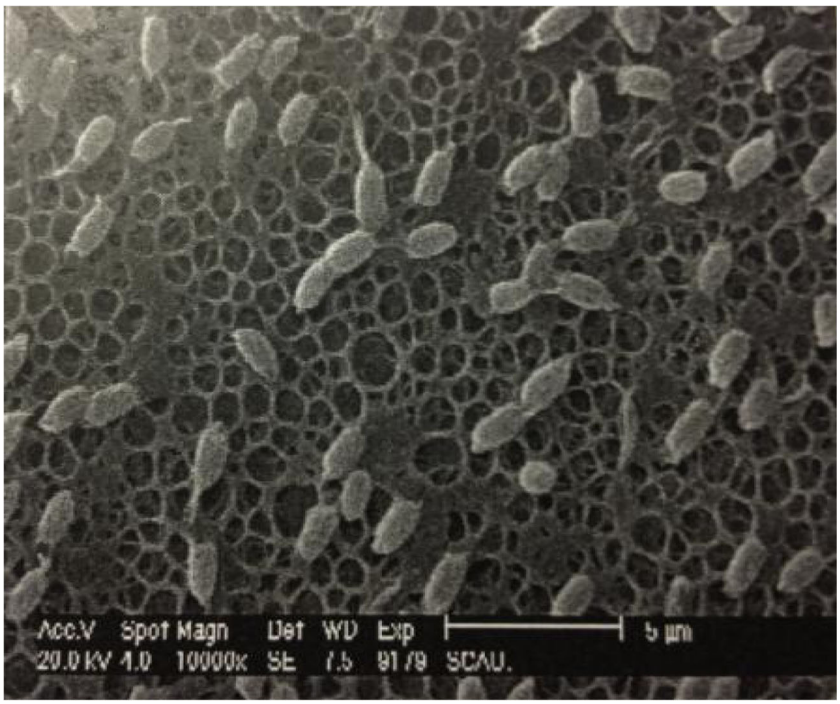

Fig. 1 Morphological characteristics of strain BSF01 under scanning electron microscope (×10,000)

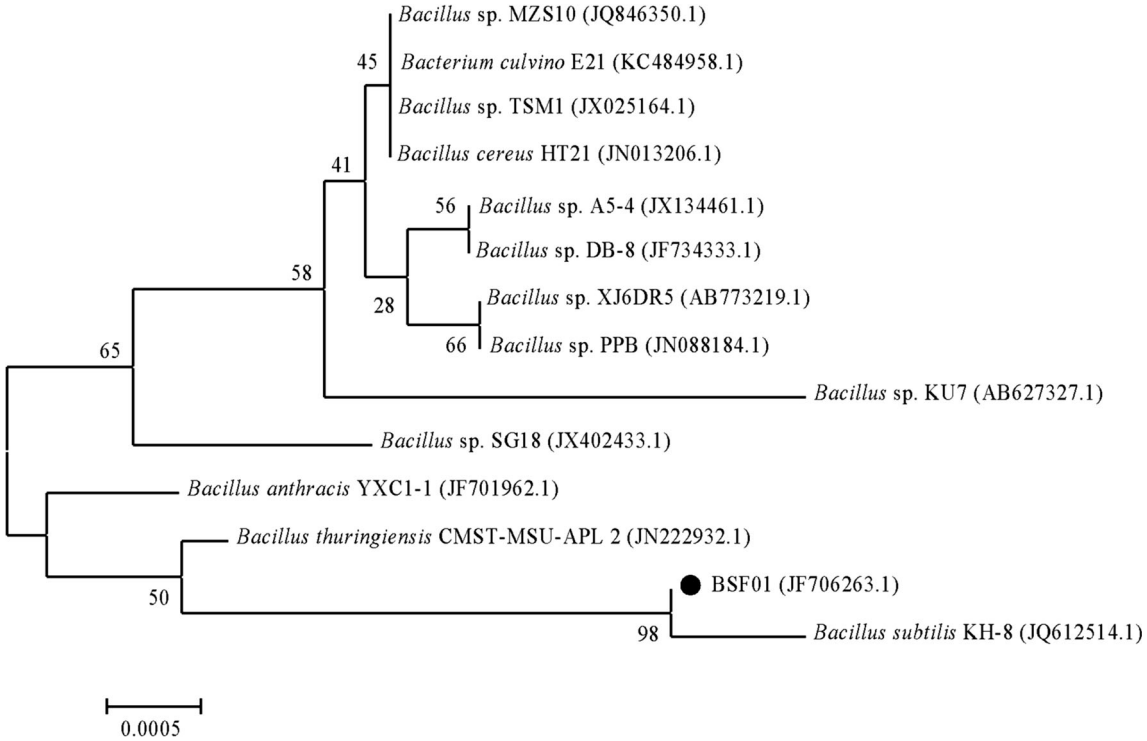

Fig. 2 Phylogenetic tree based on the 16S rDNA sequence of strain BSF01 and related strains. *Numbers in parentheses* represent the sequences accession number in GenBank. Numbers at the nodes indicate bootstrap values from the neighborhood-joining analysis of 1,000 resampled data sets. *Bar* represents sequence divergence

(Fig. 1). Colonies grown on LBM agar plates were rough, opaque, dirty white, or slightly yellow, with winkles when cultured for a long time. It was positive in tests such as amylohydrolysis, gelatin liquefaction, Voges–Proskauer (V-P), nitrate reduction, and utilization of D-glucose and D-mannitol while it was negative in utilization of L-arabinose and D-xylopyranose. PCR amplification of 16S rDNA gene from strain BSF01, a single fragment of 1,417 bp (Fig. S1), with GenBank accession number JF706263, was obtained and completely sequenced. Analysis of the 16S rDNA gene sequences demonstrated that strain BSF01 grouped among *Bacillus* species (Fig. 2). In conclusion, strain BSF01 was identified as *Bacillus subtilis* based on the morphology, physio-biochemical characteristics, and 16S rDNA gene analysis. This strain was deposited in China Center for Type Culture Collection (collection number: CCTCC AB 2014103).

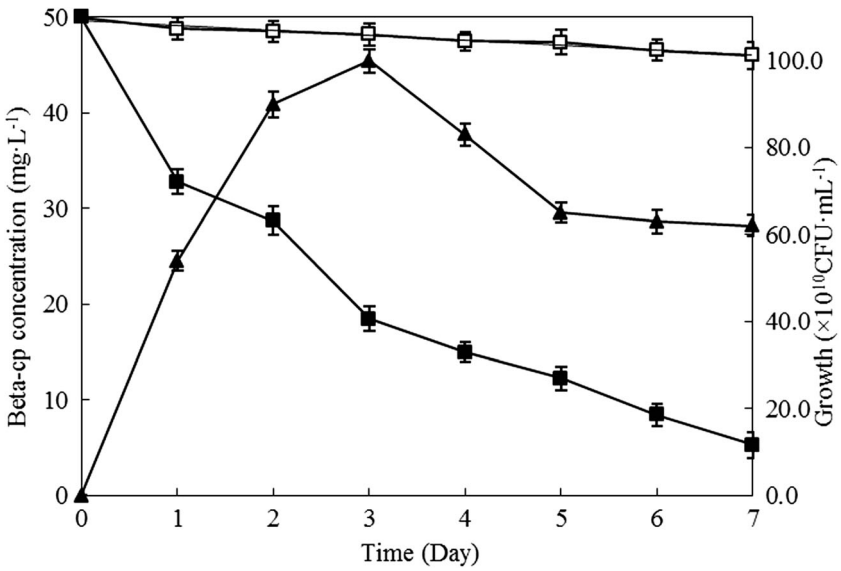

Fig. 3 Utilization of beta-cp for growth in MSM by strain BSF01. Degradation kinetics of beta-cp (*black square*), noninoculated control (*white square*), and growth of strain BSF01 (*black triangle*). Values are the means of three replicates with standard deviation

Table 2 ANOVA for the fitted quadratic polynomial model of beta-cp degradation

Source	DF	SS	MS	*F* value	*P* level*
X_1	1	148.7813	148.7813	95.4643	0.0002
X_2	1	3.2513	3.2513	2.0861	0.2083
X_3	1	1.4450	1.4450	0.9272	0.3798
X_1X_1	1	299.0769	299.0769	191.9005	0.0001
X_1X_2	1	9.6100	9.6100	6.1662	0.0556
X_1X_3	1	2.1025	2.1025	1.3491	0.2979
X_2X_2	1	46.5323	46.5323	29.8571	0.0028
X_2X_3	1	0.3025	0.3025	13.9320	0.0135
X_3X_3	1	21.7131	21.7131	13.9320	0.0135
Model	9	503.6248	55.9583	35.9052	0.0005
Error	5	7.7925	1.5585		
Total	14	511.4173			

R^2 =0.9848; CV=1.5 %

DF degrees of freedom, *SS* sum of sequences, *MS* mean square

**P* level less than 0.05 indicates that the model terms are significant

Utilization of beta-cp for growth by strain BSF01

The relationship between the growth of strain BSF01 on beta-cp and the biodegradation of beta-cp was shown in Fig. 3. The degradation rate of beta-cp increased rapidly when the strain was at logarithmic (1–2 days) and stationary (3–4 days) phase. However, the degradation rate tended to slow down when the strain arrived at the decline phase (after 5 days of incubation). An 89.4 % of 50 mg L^{-1} beta-cp was degraded by strain BSF01 within 5 days. In contrast, there was no significant change in beta-cp concentration in the noninoculated controls. Additionally, strain BSF01 grew well on MSM agar plates with beta-cp as the sole carbon source. These results drew a conclusion that strain BSF01 may be used in bioremediation of beta-cp-contaminated environments.

Optimization of beta-cp-degrading conditions by strain BSF01

The Box–Behnken design was applied to determine the effects of important variables containing temperature (X_1), pH (X_2), and inocula (X_3) according to previous single-factor experiments. The experimental design and the response of dependent variables for beta-cp degradation are presented in Table 1. Data from Table 1 were processed by response surface regression procedure of SAS software, and results were obtained by fitting with the second-order polynomial model equation (Eq. (5)):

$$Y_{BSF01} = 90.3 + 4.3125X_1 - 0.6375X_2 - 0.425X_3 - 9{X_1}^2 - 1.55X_1X_2 + 0.725X_1X_3 - 3.55{X_2}^2 + 0.275X_2X_3 - 2.425{X_3}^2 \quad (5)$$

where Y_{BSF01} is the predicted beta-cp degradation (%) by strain BSF01 and X_1, X_2, and X_3 are the coded values for the temperature, pH, and inocula, respectively.

Analysis of variance (ANOVA) for beta-cp degradation is tabulated in Table 2. The determination coefficient R^2 of

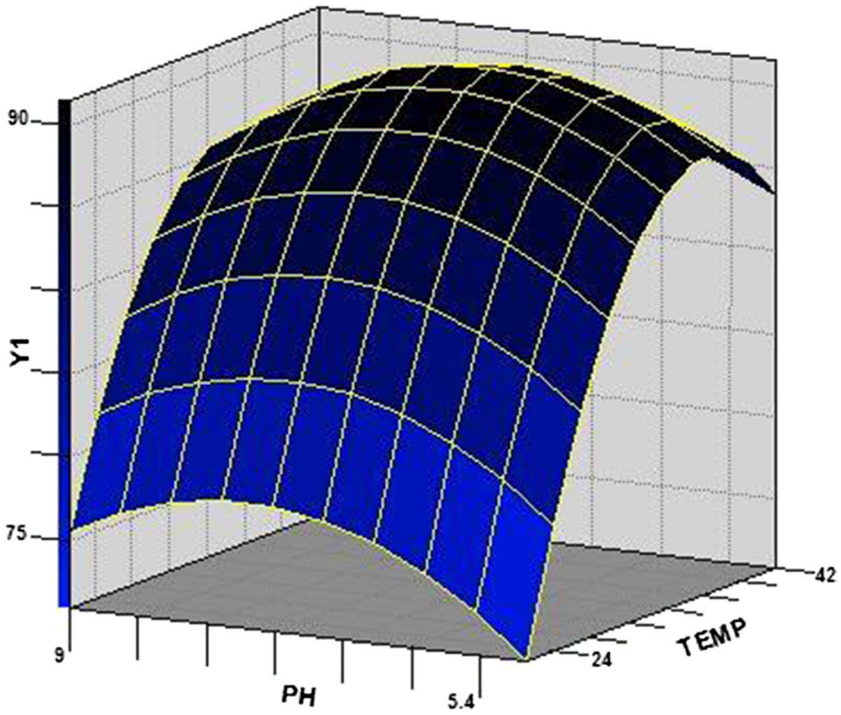

Fig. 4 Response surface plot showing the effects of temperature and medium pH on beta-cp degradation while fixing the value of inocula amount at a zero level

0.9848 indicated that approximately 98 % of responses were covered by the model, demonstrating that predicted values of the model were in good agreement with the experimental values. In general, this model for beta-cp degradation is highly significant ($p<0.01$), indicating that the established quadratic polynomial model for beta-cp degradation by strain BSF01 was adequate and reliable in representing the actual relationship between response and variables.

The results of the regression parameter estimate displayed that linear and square terms of temperature (X_1) values showed significant effects ($P<0.05$) on the beta-cp degradation by strain BSF01, while the linear and square terms of pH (X_2) and inocula (X_3) were insignificant ($P>0.05$). Figure 4 exhibited a three-dimensional response surface which was plotted to intuitively display the effects of temperature and pH with the value of inocula fixed at 0.12 g dry wt L^{-1}. There was a theoretical maximum value of 90.9 % at the stationary point. At the theoretical maximum point, the values of temperature (X_1), pH (X_2), and inocula (X_3) in terms of the uncoded units were 34.5 °C, 6.7, and 0.11 g dry wt L^{-1}, respectively. That is to say that the optimal conditions for beta-cp degradation by strain BSF01 were determined to be temperature at 34.5 °C, pH 6.7, and inocula of 0.11 g dry wt L^{-1}.

Effect of different initial beta-cp concentrations on biodegradation

Kinetic curves of beta-cp degradation at different initial concentrations (25–400 mg L^{-1}) by strain BSF01 are shown in Fig. 5a. At low concentrations of 25, 50, and 100 mg L^{-1}, the

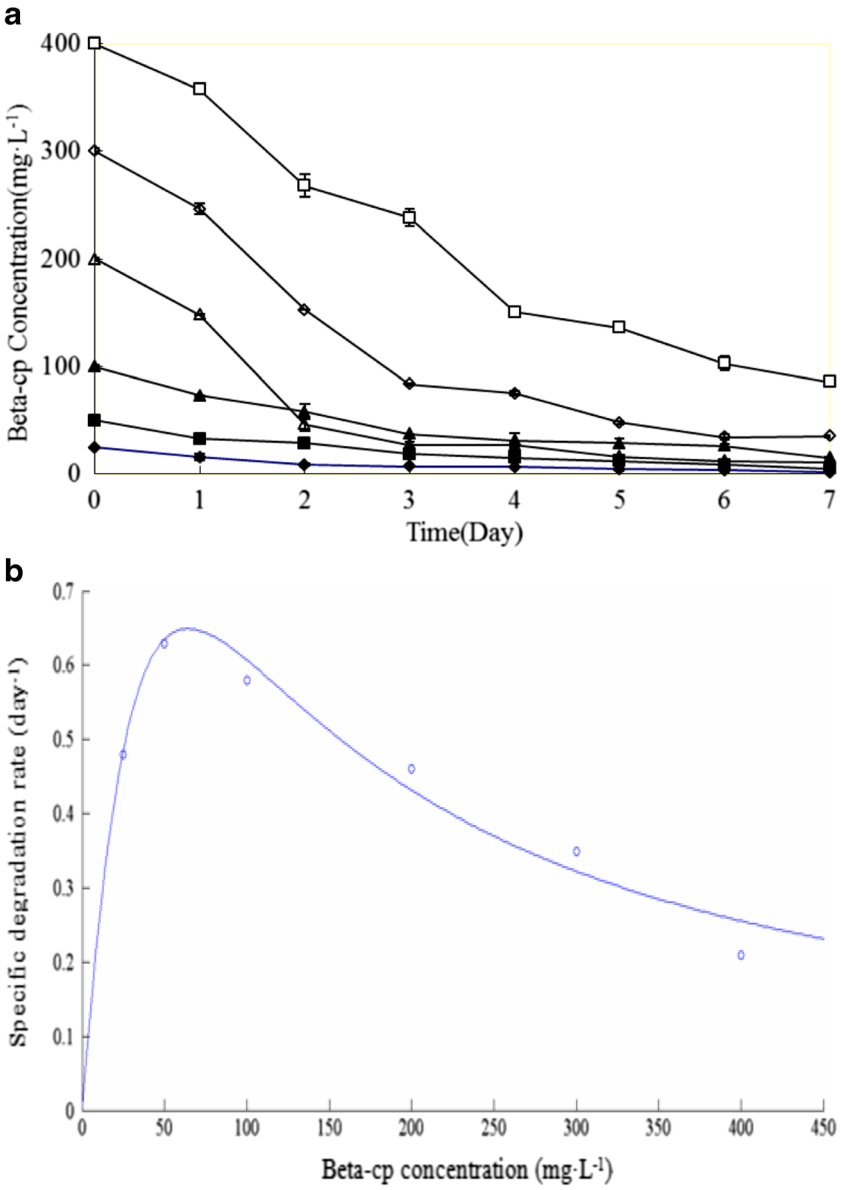

Fig. 5 Biodegradation of beta-cp in MSM with different initial concentrations by strain BSF01. **a** Degradation kinetics of beta-cp. *Black diamond*, 25 mg L^{-1}; *black square*, 50 mg L^{-1}; *black triangle*, 100 mg L^{-1}; *white triangle*, 200 mg L^{-1}; *white diamond*, 300 mg L^{-1}; *white square*, 400 mg L^{-1}. Values are means of three replicates with standard deviation. **b** Relationship between specific degradation rate and initial beta-cp concentration

degradation rates of beta-cp reached 93.9, 89.4, and 84.7 % after 7 days of incubation, respectively. Approximately 80 % of degradation rate was achieved at higher initial concentrations (>100 mg L^{-1}) with longer lag phase.

Figure 5b shows the relationship between specific degradation rate (q) and initial bata-cp concentration. The kinetic parameters q_{max}, K_s, and K_i were established to be 2.19 day^{-1}, 76.37 mg L^{-1}, and 54.14 mg L^{-1}, respectively. The value of R^2 was 0.9637 demonstrating that the experimental data were well correlated with Andrews equation. The critical inhibitor concentration was determined to be 64.30 mg L^{-1}, indicating that when the initial concentrations of beta-cp were lower than 64.30 mg L^{-1}, the specific degradation rate (q) gradually increased. The inhibition of beta-cp would be prominent at higher concentrations.

Degradation kinetics of various pyrethroid insecticides by strain BSF01

The degradation kinetics of various pyrethroid insecticides by strain BSF01 is shown in Fig. 6. The strain utilized beta-cp, cypermethrin, deltamethrin, beta-cyfluthrin, and cyhalothrin as the growth substrates with the degradation rates of 89.4, 89.2, 86.9, 86.5, and 76.8 % within 7 days, respectively.

The first-order model (Eq. (3)) was used to represent the biodegradation kinetics of various pyrethroid insecticides by strain BSF01. The degradation kinetic parameters of various pyrethroid insecticides are presented in Table 3. The degradation rate constants (k) of beta-cp, deltamethrin, beta-cyfluthrin, cyhalothrin, and cypermethrin were 0.2995, 0.3811, 0.4476, 0.2320, and 0.5164 day^{-1}, respectively, and the degradation half-lives ($T_{1/2}$) were 2.31, 1.82, 1.55, 2.99, and 1.34 days, respectively. The correlation coefficient R^2 ranged from 0.9112 to 0.9867 indicating that the degradation data were well fitted with the model.

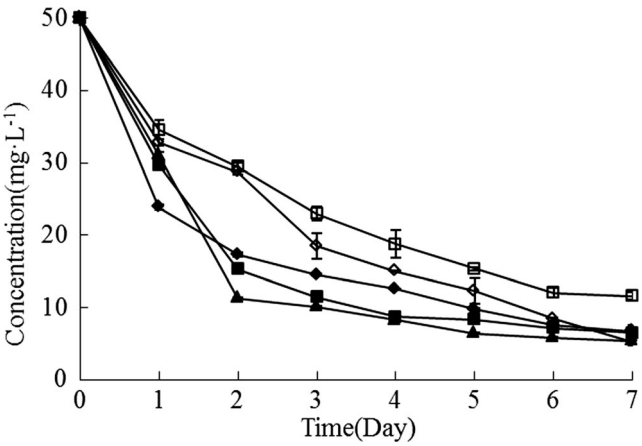

Fig. 6 Biodegradation dynamics of various pyrethroid insecticides by strain BSF01 with the initial concentration of 50 mg L^{-1}. Cyhalothrin (*white square*), beta-cp (*white diamond*), deltamethrin (*black diamond*), beta-cyfluthrin (*black square*), and cypermethrin (*black triangle*). Values are means of three replicates with standard deviation

Table 3 Degradation kinetics of various pyrethroids by strain BSF01

Pyrethroids	Regression equation	k (day^{-1})	$T_{1/2}$ (days)	R^2
Beta-cp	$y=48.5941e^{-0.2995x}$	0.2995	2.31	0.9867
Deltamethrin	$y=45.1116e^{-0.3811x}$	0.3811	1.82	0.9112
Beta-cyfluthrin	$y=48.1523e^{-0.4476x}$	0.4476	1.55	0.9565
Cyhalothrin	$y=47.5298e^{-0.2320x}$	0.2320	2.99	0.9822
Cypermethrin	$y=49.3537e^{-0.5164x}$	0.5164	1.34	0.9535

Data in the table represents the mean of three replicates

y residual concentration of pyrethroids (mg L^{-1}), x degradation period (days), R^2 correlation coefficient

Identification of beta-cp metabolites by GC-MS

The beta-cp metabolites were extracted and identified by GC-MS, and the results are summarized in Table 4. Each peak was identified on the basis of its characteristic fragment ion peaks and the National Institute of Standards and Technology (NIST, USA) library database. GC chromatogram of the culture filtrates for beta-cp degradation by strain BSF01 is shown in Fig. S2.

It was worth mentioning that two peaks, compound A (32.021 min) and compound B (32.134 min), were first observed during the beta-cp degradation by strain BSF01. These two compounds [A and B] were identified as beta-cp (*cis*) and beta-cp (*trans*), respectively, based on the similarity of their fragment retention times and molecular ions with those of corresponding authentic compounds in the NIST library database. Afterward, these two new compounds [A and B] were further metabolized to form 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate [C], α-hydroxy-3-phenoxy-

Table 4 Chromatographic properties of metabolites of beta-cp

Code	Retention time (min)	Molecular weight (MW)	Compounds
A	32.021	415	Beta-cp (*cis*)
B	32.134	415	Beta-cp (*trans*)
C	12.144	209	3-(2,2-Dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate
D	17.391	225	α-Hydroxy-3-phenoxy-benzeneacetonitrile
E	17.361	198	3-Phenoxybenzaldehyde
F	21.581	213	3-Phenoxybenzoic acid
G	20.080	154	3,5-Dimethoxyphenol

benzeneacetonitrile [D], 3-phenoxybenzaldehyde [E], 3-phenoxybenzoic acid [F], and 3,5-dimethoxyphenol [G] (Fig. S3). In the noninoculated control containing the same amount of beta-cp, only beta-cp was detected (Fig. S4). Based on analysis of the metabolites, the biodegradation pathway of beta-cp in strain BSF01 was proposed (Fig. 7). Beta-cp was firstly metabolized by hydrolysis of its ester linkage to yield 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate [C] and α-hydroxy-3-phenoxy-benzeneacetonitrile [D]. Compound D was unstable in the environment and was oxidized to form 3-phenoxybenzaldehyde [E]. Subsequent oxidization of compound E resulted in the formation of 3-phenoxybenzoic acid [F]. Compound F could be further transformed to 3,5-dimethoxyphenol [G], leading to detoxification of beta-cp.

Discussion

A number of studies had shown that it was an effective method to isolate degrading microorganisms from long-term pesticide-contaminated soil, wastewater, or activated sludge (Tallur et al. 2008; Chen et al. 2011b). *Bacillus subtilis* are widely available and have been safely used in a variety of food applications due to their properties of nontoxigenic and non-pathogenic that had been stated by the Food and Drug Administration (FDA) (Gong et al. 2014). It had been found to degrade other xenobiotic compounds, including 4-chloro-2-nitrophenol, dimethylformamide, and red M5B dye (Arora 2012a; Vidhya and Thatheyus 2013; Gunasekar et al. 2013). This study provides the first evidence that *Bacillus subtilis* participates in efficient degradation of beta-cp.

The relationship between the growth of strain BSF01 and the biodegradation of beta-cp showed that all degradation was associated with bacterial growth, and this observation is reminiscent of a previous finding by Singh et al. (2004). Strain BSF01 could utilize beta-cp as the sole carbon source and degrade it over a wide range of initial beta-cp concentration (25–400 mg L^{-1}), temperature (22–42 °C), and pH (5–9). However, longer lag phase was achieved at higher initial beta-cp concentration, and it might be because of that the greater number of bacteria was needed to initiate rapid degradation of pesticide (Karpouzas and Walker 2000; Anwar et al. 2009). Microorganisms need an acclimatization period to induce the formation of necessary degradative enzymes that may account for prolonged lag phase, which was observed at higher concentration of cypermethrin (Dubey and Fulekar

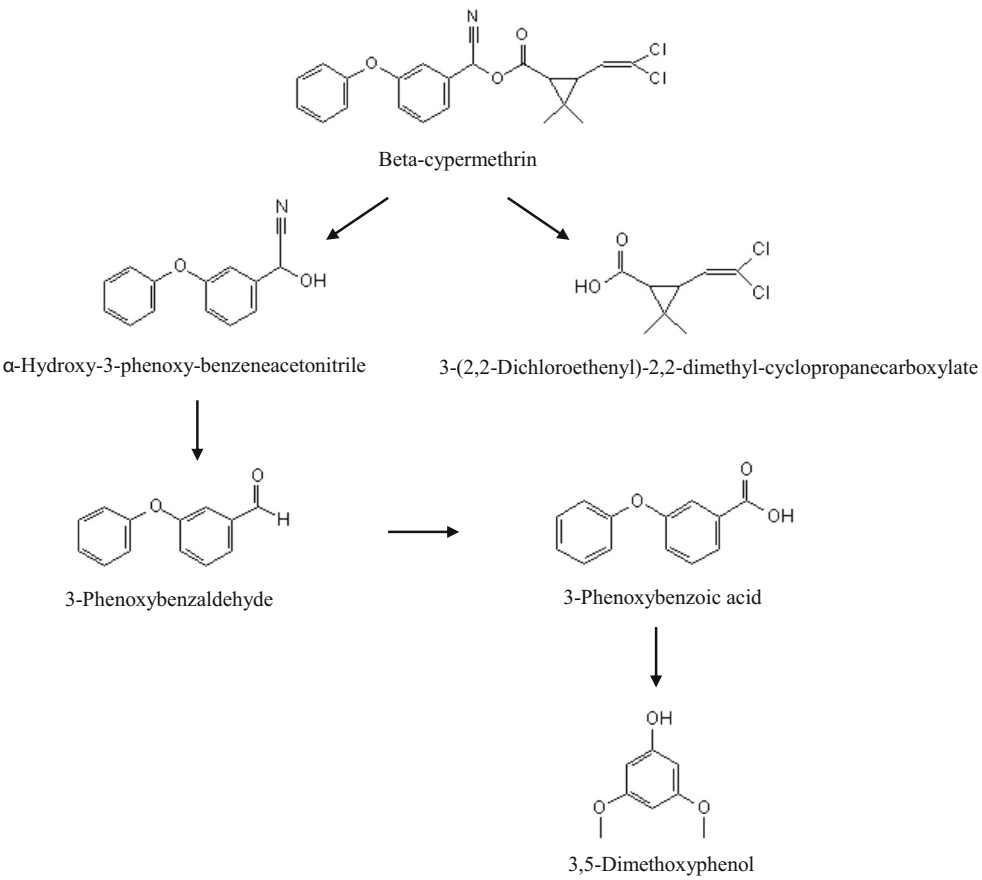

Fig. 7 Proposed pathway for the biodegradation of beta-cp by strain BSF01

2013). Moreover, strain BSF01 was capable of degrading various pyrethroid insecticides including beta-cp, cypermethrin, deltamethrin, beta-cyfluthrin, and cyhalothrin, and it performed no significant pyrethroid degradation specificity as *Brevibacterium aureum* DG-12 reported by Chen et al. (2013b). This may be because that the degrading enzyme(s) from strain BSF01 had no substrate specificity. Another possible reason could be attributed to the fact that all the tested pyrethroid insecticides share a similar structure. Similar result had been reported by Wang et al. (2011).

The basic purpose of RSM is the evaluation of the relationship between the predicted values of the dependent variable and the conditions of dependent variables (Hosseini-Parvar et al. 2009). The use of the Box–Behnken design is popular in industrial research because it is an economical design and requires only three levels for each factor (Khuri and Mukhopadhyay 2010). Zhang et al. (2010) and Chen et al. (2011b) previously used RSM based on the Box–Behnken design to optimize the pyrethroid insecticide degradation conditions at different pH, temperatures, and inocula amounts. They both demonstrated that the RSM was convenient and efficient to investigate the optimum degradation conditions for pyrethroid insecticides by various microorganisms. In the present study, a statistical model based on RSM was applied to optimize the beta-cp degradation conditions by strain BSF01, which was proved to be accurate and reliable within the limits of chosen factors.

Studies on the biodegradation pathway are very important in developing a bioremediation strategy for pyrethroid-contaminated environments because most of the pyrethroid insecticides can produce more toxic metabolites from the biodegradation process (Laffin et al. 2010). It is evident from the results that the particular strain BSF01 not only efficiently degraded beta-cp but also transformed the metabolites (compounds C, D, E, F, and G). This conclusion is in agreement with Zhang's finding (Zhang et al. 2011). It was generally accepted that ester hydrolysis by carboxylesterases was the main degradation pathway of pyrethroid insecticides which resulted in yielding an acid and an alcohol (Tallur et al. 2008; Zhang et al. 2010). In the present study, beta-cp was metabolized firstly by hydrolysis of its ester linkage to produce 3-(2,2-dichloroethenyl)-2,2-dimethyl-cyclopropanecarboxylate and α-hydroxy-3-phenoxy-benzeneacetonitrile. α-Hydroxy-3-phenoxy-benzeneacetonitrile was unstable and spontaneously transformed to yield 3-phenoxybenzaldehyde. This phenomenon is similar with the degradation of cypermethrin by different microorganisms (Lin et al. 2011; Chen et al. 2011b). 3-Phenoxybenzaldehyde can accumulate in the media or soils, and enhanced biodegradation may not occur owing to its antimicrobial activities (Chen et al. 2011b). In this study, 3-phenoxybenzaldehyde was further metabolized by strain BSF01 to form 3-phenoxybenzoic acid. The intermediates 3-phenoxybenzoic acid and 3-phenoxybenzaldehyde are both the major metabolites of pyrethroid insecticides except cyfluthrin (Laffin et al. 2010). In previous study, *Pseudomonas* strains were reported to utilize 3-phenoxybenzoic acid as a growth substrate (Halden et al. 1999); however, all these strains could not metabolize beta-cp. In this study, *Bacillus subtilis* BSF01 was found to degrade not only beta-cp but also its major metabolic intermediates 3-phenoxybenzaldehyde and 3-phenoxybenzoic acid, which are rarely seen in other pyrethroid-degrading strains. These results demonstrate that *Bacillus subtilis* BSF01 harbors the metabolic pathway for the complete detoxification of beta-cp, indicating that the isolate may be an ideal microorganism for bioremediation of the beta-cp-contaminated environments.

In conclusion, a novel bacterium *Bacillus subtilis* has been isolated and characterized in the present study, which could utilize beta-cp as the sole carbon source for growth. What's more, it was capable to degrade a wide range of pyrethroid insecticides including beta-cp, cypermethrin, deltamethrin, cyhalothrin, and beta-cyfluthrin. Furthermore, we presented evidence that the bacterium harbors the metabolic pathway for complete degradation and metabolism of beta-cp. To our knowledge, this is the first report that a pyrethroid-degrading bacterium transforms beta-cp through hydrolysis of ester linkage and cleavage of diaryl bond, which we propose is of vital importance in beta-cp biogeocycle.

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