

ISOLATION AND CHARACTERIZATION OF BIFENTHRIN CATABOLIZING BACTERIAL STRAIN *BACILLUS CIBI* FROM SOIL FOR PYRETHROIDS BIODEGRADATION

Preeti Pandey, Geetika Pant and G. Sibi

Department of Biotechnology,
Indian Academy Degree College, Centre for Research and Post Graduate Studies, Bangalore, India

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ABSTRACT

Pyrethroids are commonly used in most parts of the world and are reported to have potential health risks. Bifenthrin, a third generation pyrethroid used as insecticide has caused potential effect on aquatic life and human health. Bioremediation is a practical approach to reduce pesticide in the environment and reports of microbial degradation of bifenthrin are meagre. This study was aimed at isolating and characterizing bacterial isolates for the efficient removal of bifenthrin residues in the environment. A bacterial strain PGS-4 isolated from sewage of pesticide industry was tested for growth at higher concentration of bifenthrin (800 mg L⁻¹) and the optimum pH and temperature were determined. The strain utilized bifenthrin as sole carbon source for growth over a wide range of pH (4.0-9.0) and temperatures (16-37°C). On the basis of growth kinetics studies, the optimal conditions were determined to be pH 7.0-8.0 and 30°C. 16S rRNA gene sequence analysis showed that strain PGS-4 forms a distinct phylogenetic lineage within the evolutionary radiation encompassed by the genus *Bacillus* and showed 99% similarity to that of *Bacillus cibi*. This study depicts the ability of *B. cibi* to utilize bifenthrin at higher concentration under in vitro thereby can be used in eliminating bifenthrin from contaminated soils as a practical approach to reduce pyrethroid toxicity in the environment.

Keywords: Bifenthrin, Pyrethroids, Biodegradation, *Bacillus cibi*, Insecticides

1. INTRODUCTION

Pyrethroids are replacing organophosphates and carbamates due to their restriction (Hintzen *et al.*, 2009; Smith and Stratton, 1986) and are one of the dominant insecticides (Weston *et al.*, 2009). Pyrethroids have potential health risks (Tewary *et al.*, 2005; Mohapatra *et al.*, 2007; Chen *et al.*, 1991; Bradberry *et al.*, 2005; Miyamoto, 1991) and the use is expected to increase further (Wang *et al.*, 2007). They are reported to have neurotoxicity (Shafer *et al.*, 2005; Wolansky and Harrill, 2008), immunotoxicity (Dutta and Das, 2011; Jin *et al.*, 2011), reproductive toxicity (Yousef, 2010; Perry *et al.*, 2007) and cytotoxicity (Zhang *et al.*, 2010a; Wang *et al.*,

2009a; Cavas and Ergene-Gozukara, 2003) on non-target organisms. Some of pyrethroids have been classified as a possible human carcinogen by the Environmental Protection Agency (EPA) of US (Tallur *et al.*, 2008; Zhang *et al.*, 2010b).

The disposal methods for pesticide residues include photolysis, hydrolysis and biodegradation (Demoute, 1989; Fan *et al.*, 2012). Degradation of pyrethroids in the soil is mostly by chemical and microbial action and the rate of degradation depends on the type of pyrethroids, soil type, climate and the species of microbes. Microbes play a significant role in the degradation of bifenthrin and other pyrethroids in nature (Fenlon *et al.*, 2011). Microbial degradation of pesticides is an effective, cheap

Corresponding Author: G. Sibi, Department of Biotechnology, Indian Academy Degree College, Centre for Research and Post Graduate Studies, Bangalore, India

and safe approach to clean up contaminated environment (Singh and Walker, 2006). Many microbes have been isolated and reported to degrade pyrethroids (Maule *et al.*, 1993; Grant *et al.*, 2002; Tallur *et al.*, 2008; Guo *et al.*, 2009; Zhang *et al.*, 2010b; Wang *et al.*, 2011).

Bifenthrin (2-Methyl-3-Phenyl phenyl methyl (1S, 3S)-3-(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl 2,2-dimethylcyclopropane-1-carboxylate) is a third generation pyrethroid used as insecticide (Gupta *et al.*, 2009; Reddy and Rao, 2002; Chinniah and Ali, 2000; Ali and Karim, 1994). It is also extensively used for the control of residential pests such as termites in urban areas (Baskaran *et al.*, 1999). Bifenthrin has been accounted as greatest toxic unit among pyrethroids (Hintzen *et al.*, 2009) and is classified as Toxicity Class II moderately hazardous (WHO, 2009). Due to the potential toxic effects to humans, ecosystem and persistence of bifenthrin in the environment, there is an urgent need to develop efficient strategies to remove the residues. The objective of this study was to identify and characterize a bifenthrin degrading bacteria and to optimize its degradation conditions.

2. MATERIALS AND METHODS

The Mineral Salt Medium (MSM) containing (g L⁻¹) (NH₄)₂SO₄, 2.0; MgSO₄.7H₂O, 0.2; CaCl₂.H₂O, 0.01; FeSO₄.7H₂O, 0.001; Na₂HPO₄.12H₂O, 1.5; KH₂PO₄, 1.5 and pH 7.2 was used for the isolation of bifenthrin degrading bacteria.

2.1. Isolation and Screening of Bifenthrin Degrading Bacteria

Primary wash and sewage from pesticide industry, Peenya (13°03'N, 77°52'E), Bangalore were collected as inoculum and directly inoculated into 250 mL Erlenmeyer flasks containing 100 mL of Mineral Salt Medium (MSM) added with bifenthrin dissolved in acetone at a final concentration of 1, 5 and 10 mg L⁻¹. The flasks were incubated at 37°C for 7 days in orbital shaker at 150 rpm. From 10 mg L⁻¹ bifenthrin flask culture, 2% of the inoculum was transferred into 50 mL of fresh medium containing 50 mg L⁻¹ of bifenthrin for enrichment and incubated for 7 days. Five additional successive transfers made into media containing 100, 200, 400, 600 and 800 mg L⁻¹ of bifenthrin. The final culture from the highest concentration of bifenthrin (800 mg L⁻¹) were serially diluted and spread on Luria Bertani (LB) agar plates. The plates were incubated at 37°C for 5 days and the colonies were purified by streak plate method.

2.2. Phenotypic Characterization of the Isolates

A total of six strains (PGS-1, 2, 3, 4, 5, 6) were isolated by enrichment technique and were subjected to Gram's staining, motility test, endospore staining, IMViC, catalase, oxidase, urease tests, starch and gelatin hydrolysis.

2.3. Growth Kinetics Studies for Bifenthrin Degrading Bacteria

The growth curve of the isolates (PGS-1, 2, 3, 4, 5, 6) grown on LB agar were inoculated into mineral salt media with 800 mg L⁻¹ bifenthrin and incubated at 37°C. Growth of the isolates was measured at 600 nm at different intervals for a period of 3 days. MSM without bifenthrin was served as negative control and based on the good log phase, the best strain was selected.

2.4. pH Optimization

Different aliquots of pH (4.0, 5.0, 7.0, 8.0 and 9.0) were set up in 100 mL of mineral salt medium containing bifenthrin (800 mg L⁻¹) and inoculated with the selected strain. The flasks were incubated at 37°C and the growth was measured on 3, 19, 27, 47, 51 and 75 h of incubation period at 600 nm. Control flask without the inoculum was used as blank and a graph was plotted using the values of absorbance and pH. From the graph, optimum pH for the strain was determined.

2.5. Temperature Optimization

The optimum temperature for the selected strain was determined by inoculating 100 mL of mineral salt medium containing bifenthrin (800 mg L⁻¹) and incubating at 16, 24, 30, 37 and 45°C temperature. The growth was measured spectrophotometrically on 3, 19, 27, 47, 51 and 75 h intervals at 600 nm using blank.

2.6. Isolation of Genomic DNA

The genomic DNA of the selected strain PGS-4 was extracted using guanidium thiocyanate method. 1.5 mL of bacterial culture was pelleted at 8000 rpm for 5 min. To the pellet, 600 µL of cell lysis buffer (2 M guanidine thiocyanate, 10 mM Na₃EDTA, 50 mM HEPES, pH ~5.3) was added and the content was mixed by inverting for 5 min. To this, 700 µL of isopropanol was added and gently mixed till the solution becomes homogeneous. The DNA strands were spooled out and transferred to a fresh tube followed by the addition of 700 µL of 70% ethanol and vortexing. The contents were centrifuged at 10,000 rpm for 10 min and the DNA pellet was air dried, dissolved in 300 µL of TE buffer and incubated at 55°C

for 10 min. The isolated genomic DNA was run on agarose gel for purity and quantity.

2.7. 16S rRNA Sequencing

The 16s rRNA gene was amplified with 5'-AGAGTTTGATCMTGGCTCAG-3' (FW) and 5'-ACCACAGTCCATGCCATCAC-3' (RV) to amplify ~1.4 Kb gene. Amplification was carried out in 50 μ L reaction mixture containing 5 μ L of 1x Taq AB buffer, 2 μ L of 10 mM dNTPs, 1 μ L of 100 ng each primer, 1 μ L of genomic DNA, 1 μ L of Taq DNA polymerase and 39 μ L of ultrapure water. Reaction conditions consisted of initial denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 2 min with the last cycle followed by a 5 min extension at 72°C. Polymerase chain reaction product containing the amplified 16S rRNA gene fragment was gel purified and sequenced with 16S rRNA forward primer. The resulting sequence was compared with the genes available in the GenBank nucleotide library by a BLAST search through the National Centre for Biotechnology Information (NCBI) genbank database. Multiple alignments of 16S rRNA were carried out using CLUSTAL W software and phylogenesis was analyzed using MEGA 4.0 software.

3. RESULTS

Bifenthrin was used a sole carbon source in mineral salt medium for the isolation of pyrethroid degrading strains by enrichment technique. In the

isolation procedure, 6 strains were able to grow well on MSM agar plates containing 800 mg L⁻¹ of bifenthrin. Pesticide tolerance abilities of the stains were determined by growth kinetics studies and the highest growth rate strain PGS-4 at maximum concentration of bifenthrin was selected for further identification and studies. The colonial morphology of strain PGS-4 was large, irregular, convex, wrinkled, yellowish cream with lobate margin. Morphological characteristics of the strain revealed Gram variable, motile and endospore forming *Bacillus*. Phenotypic characteristics of the other strains are depicted in **Table 1**.

Growth kinetics studies at highest bifenthrin concentration (800 mg L⁻¹) were performed to determine the efficient strain among the isolates. Absorbance values were taken at regular intervals of 12, 24, 36, 48, 60 and 72 h. Among the isolates, strain PGS-4 has recorded maximum growth rate at the end of 3 days incubation period followed by strain PGS-6 (**Fig. 1**). From the growth kinetics studies, strain PGS-4 was selected and determined for its optimum pH and temperature. Various pH in the range of 4.0-9.0 were prepared in MSM containing 800 mg L⁻¹ bifenthrin and inoculated with the selected strain. It was found that maximum growth rate was observed at pH 7.0 and 8.0, whereas at low pH levels (pH 4.0 and 5.0) the growth rate was not stable after 27 h of incubation (**Fig. 2**). Poor growth of achieved at pH 9.0 throughout the incubation period. Optimum temperature for strain PGS-4 was determined as 30°C during the 51 h incubation period followed by 37°C (**Fig. 3**).

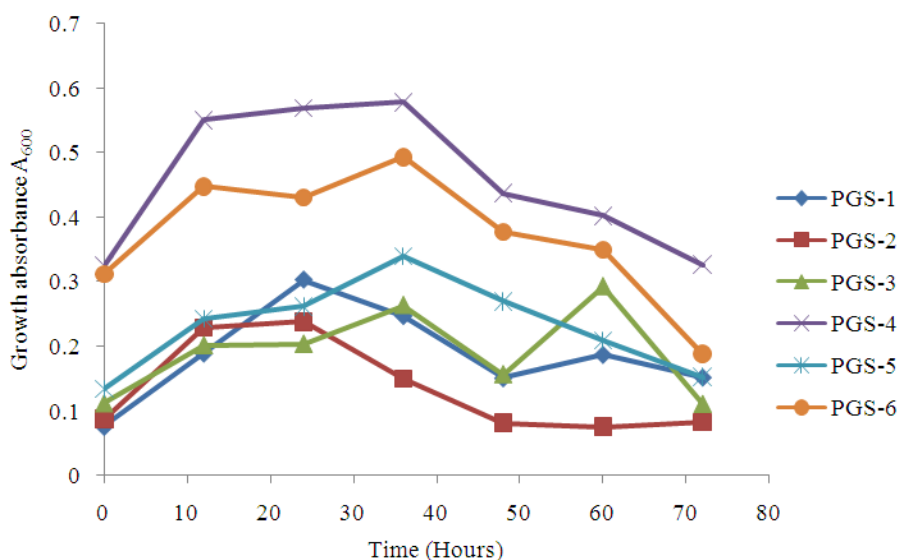


Fig. 1. Growth kinetics studies of the isolates for bifenthrin tolerance (800 mg L⁻¹)

Table 1. Phenotypic characteristics of the isolates

Characteristics	PGS-1	PGS-2	PGS-3	PGS-4	PGS-5	PGS-6
Gram's reaction	-ve	-ve	-ve	Variable	-ve	-ve
Appearance	<i>bacilli</i>	<i>bacilli</i>	<i>bacilli</i>	<i>bacilli</i>	<i>bacilli</i>	<i>cocci</i>
Endospore	-	+	-	+	-	-
Motility	-	-	+	+	-	-
Catalase	-	+	-	-	+	+
Oxidase	+	+	-	+	-	-
Indole	-	+	-	-	-	-
Methyl red	-	-	+	-	-	-
Voges-Proskauer	-	-	-	-	-	-
Citrate	+	+	+	+	+	+
Urease	-	+	+	-	+	+
Starch hydrolysis	-	-	-	+	-	-
Gelatin hydrolysis	-	+	-	-	+	-

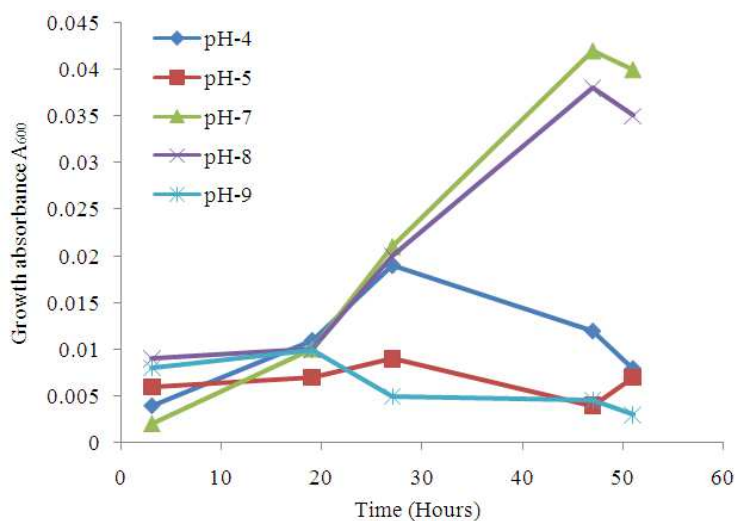


Fig. 2. pH optimization of strain PGS-4 for bifenthrin utilization (800 mg L⁻¹)

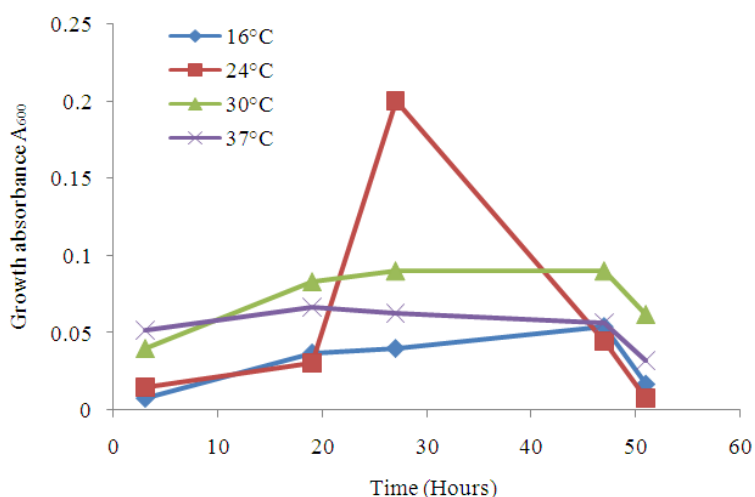


Fig. 3. Temperature optimization of strain PGS-4 for bifenthrin utilization (800 mg L⁻¹)

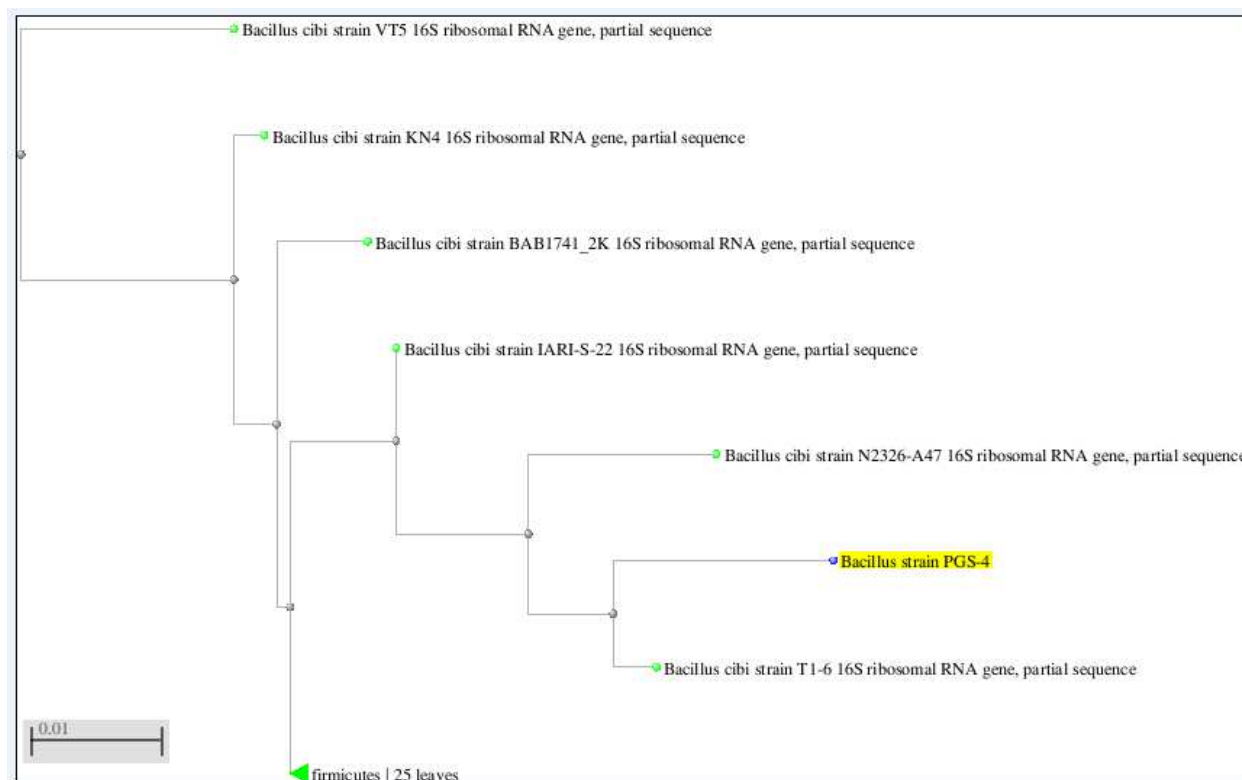


Fig. 4. Neighbour joining tree, based on 16S rRNA gene sequences showing the phylogenetic positions of *Bacillus* strain PGS-4

However, maximum growth rate was observed with 27°C but was declined drastically after 27 h of incubation. It was noteworthy that strain PGS-4 was tolerant to higher concentration of bifenthrin (800 mg L⁻¹) irrespective of incubation period, pH and temperature. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain PGS-4 fell within the radiation of the cluster comprising *Bacillus* species. The results obtained from the phenotypic and phylogenetic analyses justify a taxonomic position for strain PGS-4 as a member of the genus *Bacillus* and showed 99% similarity to that of *Bacillus cibi* (**Fig. 4**).

4. DISCUSSION

Environmental conditions are important for the isolation and screening of degrading strains (Xu *et al.*, 2008). In this study, primary wash and sewage from pesticide industry were collected as inoculum. Biodegradation is an important method mainly involves the use of living organisms to breakdown organic pollutants. It is generally considered as safe and efficient

way of removing environmental contaminants (Alexander, 1981). Bifenthrin has been used throughout the world for pest control resulting in widespread contamination. Although several microorganisms were reported to degrade pyrethroids, few reports are available on bifenthrin degrading strains (Chen *et al.*, 2012). The possible reason could be that bifenthrin is persistent and refractory to microbial degradation (Wang *et al.*, 2009b). It was observed that constant growth rate was achieved in strain PGS-4 whereas other strains exhibited poor growth rate in the presence of higher bifenthrin concentration. In order to identify the strain, DNA from PGS-4 was isolated and the PCR amplified product was subjected to 16 S rRNA sequencing and identified as *Bacillus cibi*. Temperature and pH are the two important factors influencing the microbial degradation and in this study the optimum conditions for the isolate *Bacillus cibi* strain was determined. It was noteworthy that the isolated strain was not only tolerable to the higher concentration of bifenthrin (800 mg L⁻¹) but was able to utilize it as sole carbon source

during its growth. Bacteria of the genus *Bacillus* are active group to degrade pyrethroids and organophosphate pesticides (Maloney *et al.*, 1992; Anwar *et al.*, 2009). In a study by (Lee *et al.*, 2004), 56 strains of synthetic pyrethroid degrading bacteria have been isolated from contaminated sediments. Reports of hydrocarbon degradation by *Bacillus cibi* was found in literature (Cerqueira *et al.*, 2011) and studies on pyrethroid degradation by *B. cibi* was not found. This study depicts the ability of *B. cibi* to utilize bifenthrin at higher concentration under optimum pH and temperature which can be used for degradation of bifenthrin.

5. CONCLUSION

Bacillus cibi isolated in this study appeared to be highly efficient in degrading bifenthrin at higher concentration. The bacteria utilized bifenthrin as sole carbon source under the optimum temperature of 30°C and pH 7.0-8.0. Further studies are needed to evaluate the degrading potential of the isolated strain and the pathways involved in the degradation of bifenthrin. The scope of this study extends by using this strain in eliminating bifenthrin from contaminated soils for bioremediation purposes at field conditions as a practical approach to reduce pyrethroid toxicity in the environment.

6. ACKNOWLEDGMENT

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