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Analysis of Bifenthrin Degrading Bacteria from Rhizosphere of Plants Growing at Tannery Solid Waste

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Abstract

Bifenthrin is an insecticide which is used to control insects, mites, and ticks. It poses a solemn environmental threat and health risk to living organisms. It may be bioaccumulated or biomagnified at different trophic levels in the food chain by biota. Microbes are hidden creature of earth's biodiversity. For isolation of bifenthrin degrading bacteria, rhizospheric soil samples of plants like *Pisum sativum*, *Triticun aestvum*, *Chenopodium album* were taken from tannery solid waste, Kasur, Pakistan. Enrichment culture techniques were used for the isolation of bacterial strains that showed luxurious growth on minimal growth media with bifenthrin dose was selected for biodegradation study. Bacteria were further screened out based on their morphological, biochemical parameters and degradation efficiency. Furthermore the effect of different growth factors like temperature, pH, inoculum concencentration, minimal inhibitory concentration of heavy metals and antibiotics were also studied. Bacterial strains of *Xanthomonas* and *Bacillus* sp. were identified as efficient degrading microbes. Maximum bifenthrin utilization were observed at 25˚C (pH 7), with 500 µL inoculum of *Bacillus* sp., while *Xanthomonas* sp. gave optimm utilization at 30˚C (pH 7) at the same inoculum volume of bacteria. The Rf values of *Bacillus* sp. and *Xanthomonas* sp. were 0.91 and 0.90 respectively, which indicated their potential to metabolize bifenthrin into non-toxic forms. These strains can be used to clean up the sites polluted with pesticides and tannery wastes when present in rhizosphere of plants.

Keywords

Bifenthrin, Biodegradation, Tannery Solid Waste, Rhizosphere

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1. Introduction

Pesticide applications have become an essential component of modern age agriculture. Their use for the protection of crops from the pests, insects, mites and ticks is increasing steadily [1] [2]. Pyrethroids have also been used in agriculture to control broad spectrum insects and in sheep dip [3] [4]. Bifenthrin (2-methyl-1, 1-biphenyl-3-y1)-methyl-3-(2-chloro-3, 3, 3-trifluoro-1-propenyl)-2, 2-dimethyl cyclopropane carboxylate) is the natural pyrethrin [5], which is sold in Pakistan under the trade name of Talstar, Resham, Jatara and Biflex. It is used on cereals, cotton, corn, alfalfa, ornamentals, and vegetables and on some fruits against insects and mites. However, the uncontrolled and excessive use of pesticides creates environmental pollution by contaminating soil and groundwater [6] [7]. Microbes such as bacteria are the hidden creature of earth's biodiversity and microbial community of soil is physiologically versatile in metabolizing and mineralizing a wide variety of organic pollutants [8]. Rhizosphere is a region in vicinity of plant roots, influenced by root exudates and soil micro-flora. Microbial action in zone of plant roots offers a conducive environment for metabolism of recalcitrant chemicals, as the root derived substances may enhance the growth of soil micro-flora as compared to non-vege- tated soils [9] [10].

Biodegradation is the potential of microbes to metabolize organic pollutants into nontoxic and environment friendly products that can enter into trophic levels of food chain without posing any threat to life. The rate of degradation may be affected by availability of nutrients, oxygen supply, pH values, concentration of compounds and agronomic characteristics of soil. Biodegradation of chlorinated pesticides involved dehalogenation, oxidation-reduction, hydrolysis and cleavage of aromatic rings, by the enzymatic action of different microbes [11]. The application of microbes to clean up environment polluted with xenobiotics may be a solution for this problem [12].

The potential use of bacteria for bioremediation of sites contaminated with bifenthrin has not yet achieved the significance that it deserves. Therefore, the present work deals with the isolation, morphological, physiological and biochemical characterization of bifenthrin degrading bacteria. After degradation study by thin layer chromatography (TLC) [13] and bacterial growth curve analysis through optical density (O.D), it is concluded that these strains have potential to use as a biological tool for bioremediation of polluted environment.

2. Materials and Methods

2.1. Samples Collection

Four different types of soil samples (A, B, C and D) were collected at depth of 10 - 12 cm from rhizoplane of different plants growing around the tannery solid waste of district Kasur Punjab, Pakistan, where pesticides have been used for many years. Collection and transportation of soil samples were done aseptically in labeled plastic bags for further processing in the laboratory.

2.2. Isolation of Bifenthrin Degrading Bacteria

Enrichment culture techniques were used for the isolation of bifenthrin degrading bacteria from different soil samples using Bushnell-Haas Broth (BHB) [14] [15]. The LB media containing trypton, 10; Yeast extract, 5; NaCl, 5; and agar, 15 ($g \cdot L^{-1}$) with pH adjusted to 7.0 was used for the primary cultivation and to obtain discrete colonies. A 20 g of soil sample was added in 100 ml of BHB medium with 24 hours shaking at 25˚C aerobically. After 24 hours of shaking at 200 rpm, solid particles were allowed to settle down for 1 hour. Supernatant (1 ml) was taken from the source flask and mix with 9 ml of BHB media in 50 ml conical flask. Each flask was spiked with 100 $\mu g \cdot ml^{-1}$ bifenthrin and was incubated at 30˚C aerobically for two weeks for the completion of first round of enrichment techniques. After two weeks of incubation 0.1 ml of culture was transferred to 10 ml of fresh BHB media containing 150 $\mu g \cdot ml^{-1}$ of bifenthrin and further incubated for two weeks for the second round of enrichment techniques [16] [17].

2.3. Bifenthrin Degrading Monoculture

Pure culture of single strain was obtained from second round of enrichment; bacterial culture was centrifuged for 20 minutes at 3500 rpm. The supernatant was discarded and pellet was resuspended in 500 µl BHB media. 50 µl suspensions was taken and spread on plates of BHB media with bifenthrin by adding 2% agar. The plates

were incubated at 30˚C till the discrete colonies were appeared. Twelve different strains of bacteria were isolated from four soil samples. The colonies of these strains were streaked on BHB with bifenthrin 100 $\mu g \cdot ml^{-1}$ and the strains that showed growth at this concentration were re-streaked on higher concentration of bifenthrin 200 $\mu g \cdot ml^{-1}$. Maximum growth showing strains (B-B_1, B-B_2) was further streaked on BHB at concentration of 250 $\mu g \cdot ml^{-1}$.

2.4. Characterization of Bacteria

Morphological and biochemical characterization was performed as mentioned by [18]. Microbact Gram-negative 24E system kit (Oxoid, Wade Road, UK) was used for the identification of Gram-negative bacteria. Bacterial suspensions for the Microbact 24E tests were prepared using isolates pre-grown on agar plates. Colonies were suspended in 0.85% sterile normal saline. The suspension was added to wells and change in colour of each well was taken immediately after 24 and 48 hours incubation at 37˚C. Physiological characterization as effects of different antibiotics discs (Chloramphenicol, Ampicillin, Fusidic acid, Carbenicillin, Linomycin and Clarithromycin) (Oxide, Wade Road, UK) as per Clinical Laboratory Standard Institute (CLSI) guidelines and heavy metals solutions ($CuSO_4$, $MnSO_4$, $ZnSO_4$, $NiCl_2$, $CoSO_4$, Na_2SO_4, K_2Cr2O_7) with concentration 100 $\mu g \cdot ml^{-1}$ and 300 $\mu g \cdot ml^{-1}$ were checked by incubating plates of L-agar supplemented with antibiotic discs and salt solution at 30˚C for 24 - 48 hours.

2.5 Growth Curve and Influence of Physiochemical Conditions

Bacterial growth curve was obtained with LB-broth and BHB media along with bifenthrin inoculated with bacteria, incubated at 30˚C on rotatory shaker at 150 rpm. The optical density (O.D) was taken at 600 nm with intervals of 0, 2, 4, 6, 8, 10, 12, 14 and 15 hours using spectrophotometer. Physical growth factors as temperature, pH, Inoculums volume, minimum inhibitory concentration (MIC) were studied by growing strains on different temperatures (25˚C, 30˚C, 37˚C, 42˚C, 56˚C and 57˚C), pH (5, 6, 7, 8, 9, 10), Inoculums volume (125 µl, 250 µl, 500 µl), MIC (50 $\mu g \cdot ml^{-1}$, 100 $\mu g \cdot ml^{-1}$, 150 $\mu g \cdot ml^{-1}$, 200 $\mu g \cdot ml^{-1}$, 250 $\mu g \cdot ml^{-1}$) respectively.

2.6. Biodegradation Study

Growth experiments on bacteria were carried out by bifenthrin as sole carbon source in 50 ml BHB media with 100 $\mu g \cdot ml^{-1}$ bifenthrin in 100 ml Erlenmeyer flasks. The BHB media was aseptically inoculated with seed suspension and incubated for one week at 30˚C with shaking at 160 rpm on orbital shaker OS-752 (Optima Japan). Each treatment was set with control samples in which no bifenthrin was added. Samples were withdrawn periodically from cultures to examine the growth by recording the O. D values at 600 nm using spectrophotometer.

2.7. Analytical Procedure

Bifenthrin residues were extracted by mixing ethyl acetate in sample culture (1:1 by volume) in 50 ml conical flask and flask was kept on shaker at 160 rpm for 1hour. The organic phase was carefully separated and passed through anhydrous sodium sulphate column (6 cm) to remove water contents. The organic phase was allowed to elute drop wise by gravity. The column was made in pasture pipette stopped with glass wool. The organic solvent (ethyl acetate) was evaporated on rotary evaporator. The dried sample was dissolved in methanol and then applied to TLC plate.

2.8. Thin Layer Chromatography (TLC)

Pre-coated silica gel plates (silica gel 60 F254 0.25 mm thicknesses, 20 × 20 cm, Merck Ltd., Germany) were used for TLC of bifenthrin [13]. TLC plates were spotted with 5 µl sample volume at 1cm apart with micropipette with same volume of standard bifenthrin in lane 1 for comparison of RF values. The plates were dried and chromatogram was developed in pre-saturated tank with Benzene: Ethyl acetate (6:1 by volume) as solvent system. After developing the plates, the solvent front was immediately marked and extra solvent was evaporated in fume hood. The plates were kept under U.V at 245 nm for 20 minutes. Three spots were clearly visible upon exposure to UV. The spots were marked and RF values were calculated.

3. Results

3.1. Isolation and Identification of Bifenthrin Degrading Bacteria

Two isolates B-B_1 *Bacillus* sp. and B-B_2 *Xanthomonas* sp. that were able to grow with bifenthrin as sole source of carbon were identified (**Figure 1** and **Figure 2**) (**Table 1** and **Table 2**).

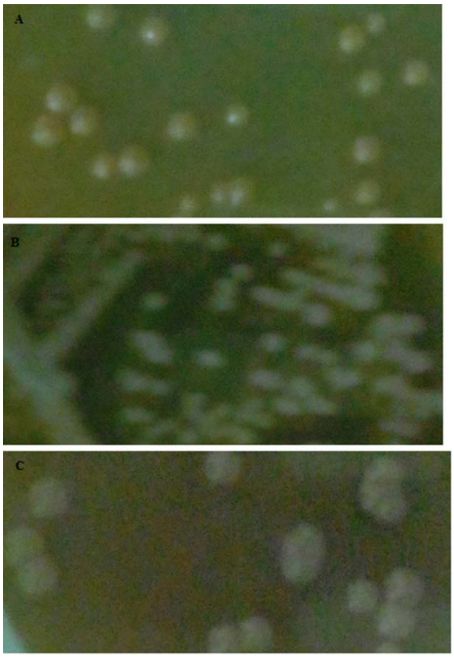


Figure 1. Colony morphology of bacterial strains.

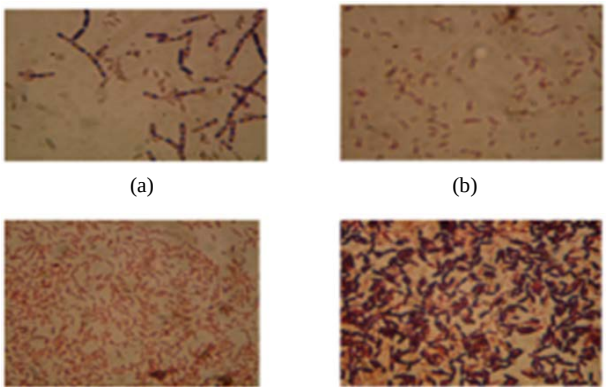


Figure 2. Cell morphological characteristics of bacterial strains (a) Gram Positive spore formers (b), (c) Gram Negative rods (d) Gram Positive rods.

Table 1. Morphological characterization of bacteria (colony characteristics).

Strains	Growth on Condition		Color	Size	Elevation	Shape	Margin	Texture
	L-Broth	**MM-Bifenthren**						
B-B_1	Pellicle	+ +	Off white	Medium	Convex	Irregular	Undulate	Entire
B-B_2	Uniform turbidity	+ + +			Raised	Circular	Entire	Rough

No growth (−), Less (+), Good (++), Excellent (+++).

Table 2. Cell morphology and biochemical characterization of bacteria.

Strains	Gram Staining	Cell Shape	Spore Formation	Motility	Catalase Production	Oxidase Production	Nitrate Reduction	Mac Conkey Agar	EMB	
									Color	Growth
B-B_1	+	Rod	+	+	+	+	-	–	Pink	+
B-B_2	–	Rod	–	+	–		+		–	–

Biochemical tests using Microbact 24E system for B-B_2

Lycine	Ornithine	H_2S	Glucose	Manitol	Xylose	ONPG	Indole	Urease	VP	Citrate	TDA	Gelatin	Malonate	Inositol	Sorbitol	Rhaminose	Suoscr	Lactose	Arabinose	Adonitise	Raffinose	Salicin	Arginine
+	–	–	–	–	–	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+

3.2. Effect of Different Antibiotic and Heavy Metal Salts

Bacillus sp. showed resistance to almost all the antibiotics and heavy metals whereas *Xanthomonas* sp. were sensitive to FD and CLR and $ZnSO_4$ and K_2CrO_7 salts at 300 μg·ml^{-1} (**Table 3** and **Table 4**).

3.3. Optimization of Conditions for Bifenthrin Degradation with Bacteria

The optimum conditions for bifenthrin utilization by *Bacillus* sp. B-B_1 were at 25˚C, pH 7, with 500 μL inoculum and MIC 150 μg·ml^{-1}, whereas for *Xanthomonas* strain B-B_2 were at 30˚C, pH 7 with inoculum 500 μL and MIC 50 μg·ml^{-1}. These conditions were determined by taking mean O.D at 600 nm in triplicates (**Figure 3**).

3.4. Growth Curve

It was observed that there was increase in cell biomass in both media up to 12 hours ($O.D_{600nm}$ = 2) and after 12 hours, cells entered into decline phase (**Figure 4**).

3.5. Biodegradation Study

Comparative growth response of two strains *Xanthomonas* sp. B-B_2 and *Bacillus* sp. B-B_1 in presence of two different concentrations of bifenthrin (100 μg·ml^{-1}, 250 μg·ml^{-1}) is shown (**Figure 5(a)** and **Figure 5(b)**) along with control (without bifenthrin). The O.D were 1.115 to 1.50 in experimental and no growth appeared in control flask. The increase in O.D might be due to utilization of bifenthrin by bacteria as carbon and energy source. Degradation of pesticide was also determined by TLC. The RF value obtained for standard was 0.87 while the RF value of B-B_1 spot was 0.91 and for B-B2, it was 0.90 in lane No. 2 and 3 (**Table 5**). The intensity of color of B-B_2 was lighter as compared to B-B1 that represented greater rate of degradation in B-B_2 as compared to B-B1. The RF values of all the tested samples are in close agreement with that of standard one (**Figure 6**).

4. Discussion

Bacteria have the potential to eliminate the hazardous compounds such as bifenthrin that is discharged by the human activities by breaking them into less persistent metabolites in soil [19]. In present study two active isolates of B-B_1 *Bacillus* sp. and B-B_2 *Xanthomonas* sp. which were able to metabolize bifenthrin were identified. Similarly more than five cypermethrin utilizing bacterial isolates including *Bacillus* sp. from soil cultivated *Solanum melagena* were identified by [20], while isolates related to *Bacillus pumilus*, *Bacillus subtillus*, *Pseudomonas fluorescence*, *Streptomyces* spp. *Xanthomonas maltophilia* and *Saprobic coryneform* were studied by [10] from rhizosphere of wheat and barley. Previous work has revealed that potential bifenthrin degrading microorganisms were mostly *Bacillus*, *Pseudomonas*, *Serratia*, yeast and *Fusarium* [16] [21]-[25]. Present study revealed that both strains exhibited resistance to heavy metals were also resistant to antibiotics. These results are

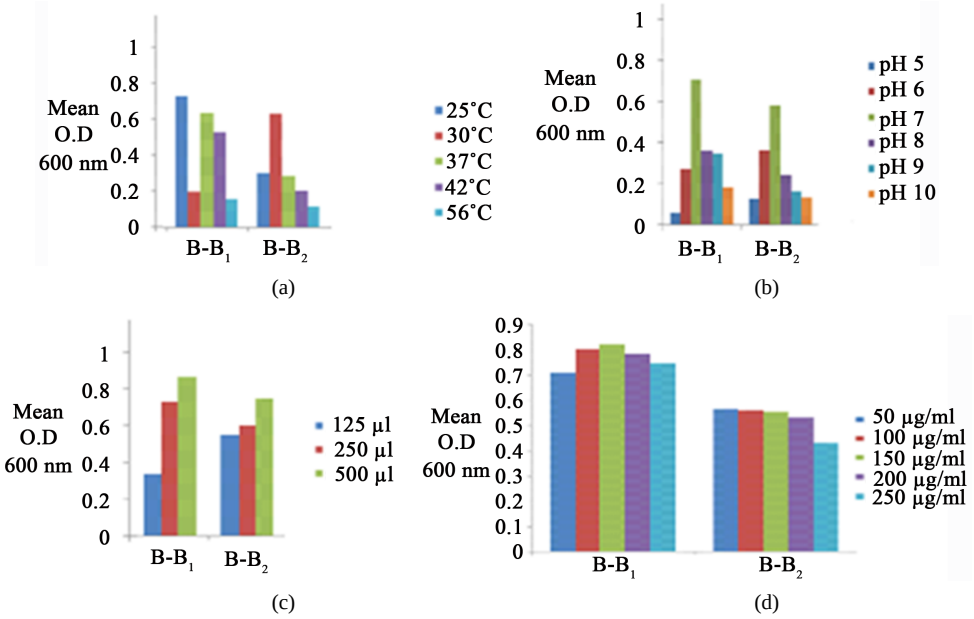


Figure 3. (a) Effect of different temperatures on Bifenthrin degrading bacteria; (b) Effect of different pH on Bifenthrin degrading bacteria; (c) Effect of different inoculums volume Bifenthrin degrading bacteria; (d) Effect of MIC of Bifenthrin on Bifenthrin degrading bacteria.

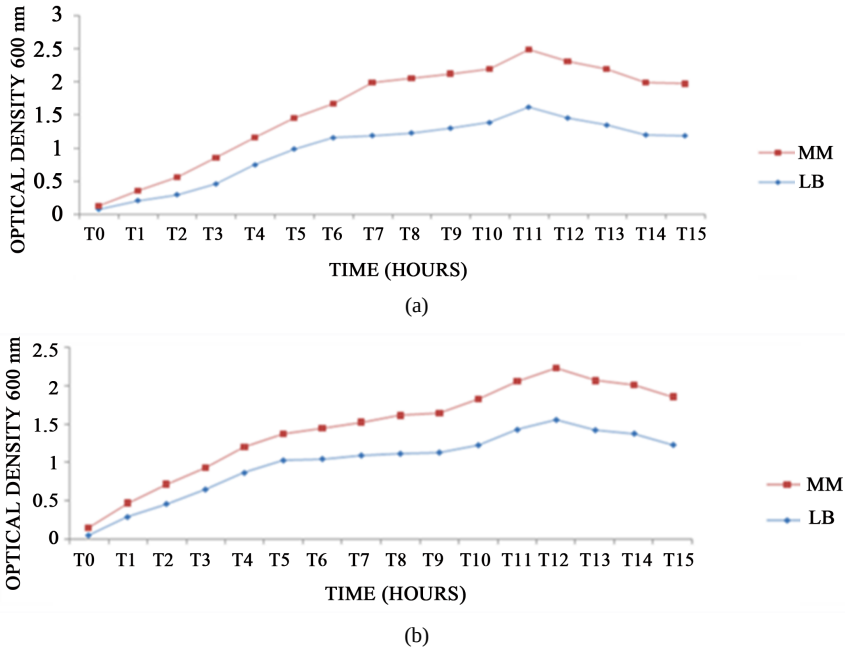
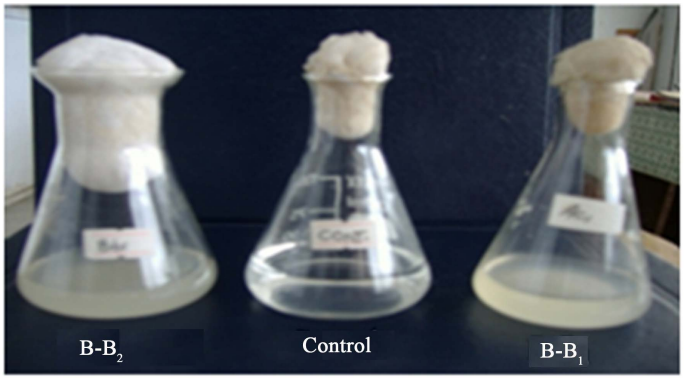


Figure 4. Growth curve of bacterial strains (a) *Xanthomonas*B-B_2 and (b) *Bacillus sp.* B-B1.

Table 3. Growth of Bacteria on media with different antibiotics.

Strains	C	CAR	MY	AMP	FD	CLR
Bacillus Sp. B-B_1	−	+	+	+	+	+
Xanthomonas sp. B-B_2	−	+	+	+	−	−

C = Chloramphenicol 30 µg; CAR = Carbenicillin 100 µg; MY = Linomycin 15 µg; AMP = Ampicillin 10 µg; FD = Fusidic acid; CLR = Clarithromycin 15 µg.



(a)

(b)

Figure 5. (a) Growth of *Xanthomonassp.*B-$B_2$and *Bacillus sp.* B-B_1 in BHB with 100 µg·ml^{-1} bifenthrinalong with negative control; (b) Growth of *Xanthomonassp.* B-B_2 and *Bacillus sp.* B-B_1 in BHBwith 250 µg·ml^{-1} bifenthrin along with negative control.

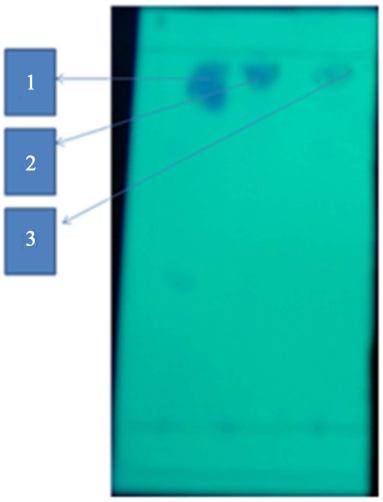


Figure 6. The chromatograms showing intensity of spotson TLC plate.

Table 4. Growth of bacteria on media with different metallic salts.

Sr. No	Strain	$NiCl_2$		$ZnSO_4$		$COSO_4$		Na_2SO_4		$PbNO_3$		K_2CrO_7		$CuSo_4$	
	Conc. (µl·ml^{-1})	100	300	100	300	100	300	100	300	100	300	100	300	100	300
1	*Bacillus Sp.* B-B_1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	*Xanthomonas sp.* B-B_2	+	+	+	−	+	+	+	+	+	+	−	+	+	+

Table 5. Rf Values of spots in Chromatogram.

Lane #	Strain	Rf value
1	*Bifenthrin* (*Standard*)	Rf = 0.87
2	*Bacillus Sp.*	Rf = 0.91
3	*Xanthomonas maltophilia*	Rf = 0.09
	Each Rf Value is the mean of triplicate observation	

in close agreement with findings of [25]. These metals are present in industrial effluents as in case of tannery solid waste. The presence of these metals is detrimental and may be dangerous to health.

Previous findings have shown that environmental conditions like temperature and pH have significant effect on degradation process of microorganisms having ability to break xenobiotic compounds [26]-[28]. Our results showed that these strains were capable of degrading bifenthrin over wide range of temperature 25˚C - 56˚C and pH 5 - 10. The increases in bacterial mass and substantial disappearance of bifenthrin represented greater rate of degradation in *Xanthomonas* sp. as compared to *Bacillus*. The results correlated with work of [29] [30] who investigated the enrichment of endosulfan biodegrading bacterial cultures. Rf value for bifenthrin was 0.87 which was very close to the Rf value of experimental samples (0.90) and the findings by [24] (0.71). The difference in Rf values from experiment described [24] might be due to different development systems that employed in our study. However, the exact identification of compounds produced by breakdown of bifenthrin by these bacteria required the use of HPLC, GCMS or other more precise techniques and after identification of metabolites we could propose the degradation of bifenthrin by these strains.

The use of pesticides like cypermethrin, bifenthrin, cyfluthrin, deltamethrin, fenvalerate, fenpropathrin and heavy metals at industrial level have led to pollution of environment. The removal of these pollutants is a major issue for environmental management. To our knowledge there has not been any report of bacterial strains resisting such high doses of metals coupled with wide range of antibiotics and bifenthrin degradation. Therefore the dual expression of antibiotics and heavy metal resistance makes valuable applications of these isolates for decontaminating sites polluted with bifenthrin and rich in heavy metals, as these bacteria are able to withstand heavy metals and break bifenthrin into metabolites that are not persistent in environment and do not cause potential threat to life.

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