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Degradation of Cypermethrin Using Potent Nitrogen Fixing Bacteria Isolated from Wheat Soil System Nearby Ambala Region

CHAHAT SHARMA, POOJA SHARMA* AND RAJ SINGH

Department of Bio-Sciences and Technology, MMEC, Maharishi Markandeshwar (Deemed to be) University, Mullana, Ambala-133 207 (Haryana), India
**(e-mail: pooja0029@gmail.com; Mobile: 92505 86619)*

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ABSTRACT

Despite the benefits, over use of pesticide has a negative impact on human health as residues transfer in food chain and ground water through leaching, percolation and bioaccumulation. To investigate the possibility of microbial degradation of hazardous chemicals in the natural environment, nitrogen-fixing bacteria isolated from wheat soil were employed. Nitrogen fixing bacterial isolates were identified using molecular technique 16SDNA sequencing. The isolates belonged to *Bacillus subtilis* species. These bacteria were initially grown in nitrogen free media (Burks media plates) having different concentration of cypermethrin (10, 25, 50, 75 and 100 ppm) after the primary screening of pesticide tolerance efficiency. Six chosen bacterial isolates, A10, KU40, KA23, Y18, Y11 and Y13 were used for further biodegradation studies using carbon nitrogen deficient media. Secondary screening was performed by growing them in different concentration (50 and 100 ppm) of cypermethrin in minimal salt media under the standard conditions (30ºC, 7 pH; 120 rpm). Bacterial growth increased with gradual days and OD for maximum tolerant strain was recorded as 0.406 for KA23 at 600 nm on 10^{th} day of degradation analysis. The percentage of degradation of cypermethrin was calculated by analyzing its residue in MSM at 280 nm using UV spectrophotometer. At 50 ppm of CP, three isolates (Y13, Y11 and KU40) showed complete degradation (99.9%) and other isolates (Y18, A10 and KA23) showed more than 90% of degradation after 10 days. At 100 ppm of CP, half of the conc. was reduced on 6^{th} day (50%) and on 10^{th} day, more than 80% of CP was degraded by all the nitrogen fixing bacterial isolates under shaking conditions. The active role of diazotrophs in cypermethrin degradation under various conditions was investigated which could be beneficial in large-scale pollutant treatment.

Key words: Pesticide, cypermethrin, nitrogen, bacteria, minimal salt media, diazotrophs

INTRODUCTION

According to the United Nations Food and Agriculture Organization, invasion from pests, weeds and diseases kills approximately 40% of crops and 6-7% of pesticides may persist in the field even after harvesting of crop (Devi *et al.,* 2017). Application of pesticides in increasing agricultural productivity has been well recognized, and their critical input in agricultural production is known (Atreya *et al.,* 2020). It has been seen that around 2/3rd million tonnes of pesticides is developed globally out of which 0.3 kg/ha is being utilized by India (Umadevi *et al.,* 2017). Worldwide, maximum pesticide consuming countries are Italy, Turkey, Colombia, India and Japan (Sharma *et al.,* 2019). In order to kill these harmful pests, basic pesticide production began in 1952 with benzene hexachloride (BHC), which was soon followed by DDT. After that the use of pesticide has grown dramatically. In 1958, India developed approximately five thousand metric tonnes of chemicals, primarily insecticides (Chahal *et al.,* 2016). Cypermethrin is a synthetic pyrethroid insecticide that is similar to naturally occurring pyrethrins derived from plants. It is commonly used in farming, forestry, gardening, healthcare and at home to protect materials and prevent invasions of insects (Gangola *et al.,* 2018). In 2023, cypermethrin was classed as restricted pesticide by Ministry of Agriculture & Farmers Welfare, India. Central Insecticide Board and Registration Committee (CIBRC) have approved cypermethrin for application in eight crops, including cabbage, wheat, cotton, rice, sugarcane, brinjal, sunflower and okra (Pandey, 2023). In India, cypermethrin output was estimated to be 6.5 MT in 2005-06 and 2473 MT in 2009-10 (Kaur and Singh, 2021). Cypermethrin is a carcinogenic pesticide that interacts with sodium channels in the central

nervous system, causing hyperexcitability. It not only affects voltage-dependent sodium channels and the ATPase system in neuronal membranes that bind to nuclear DNA, but it also causes DNA instability and protein unfolding (Indratin *et al.,* 2019). Cypermethrin, a full-spectrum pesticide, kills both good and harmful insects. Continuous usage of cypermethrin may result in resistance to insects, deterioration of the environment and negative consequences on human health. It is extremely poisonous to fish and aquatic invertebrates, resulting in low quantities of erythrocytes and protein levels in the blood (Farag *et al.,* 2021). Chemical and physical strategies have been developed to minimize the degree of toxicity in the environment. However, these procedures are more expensive and insufficient to minimize contamination and restore natural environmental conditions (Sharma *et al.,* 2023). As a result, using the microbial system for decomposition of harmful organic compounds is seen as a more cost-effective and environmentally-benign method to pollution remediation (Wirsching *et al.,* 2020). Since 1940s, researchers are investigating biological methods of degradation process of pesticides/toxins and their mechanism (Pankaj *et al.,* 2016). Rhizospheric isolated bacterial strains with the ability of metabolizing the pyrethroid family is an ecofriendly and *in situ* detoxification approach for environmental sustainability. In the presented manuscript, the cypermethrin degradation at different concentrations was evaluated using16 nitrogen fixing bacterial isolates isolated from wheat soil which belonged to *B. subtilis*.

MATERIALS AND METHODS

In the present study, analytical grade 97% cypermethrin (CP) used was procured from Pesticide Distributor, Ambala. The stock solution (1000 ppm) of cypermethrin was prepared in acetone (Hi media) by dissolving 0.1 g of CP in 100 ml of acetone. The soluble solution was sterilized by syringe filtration through 0.22 µm pore size membrane filters and stored at 4°C (Attya, 2020).

Burk's medium (Hi media) containing (per L) 0.800 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.130 g $CaCl_2$, 0.000253 g Na_2MoO_4, 0.00145 g $FeCl_3$ and 20.00 g sucrose was used for isolated bacterial growth and primary screening of pesticide tolerant efficacy. The pH of Burk's media was adjusted to 7.0 prior to autoclaving. Mineral salts medium (MSM) broth containing 1.6 g K_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2$, and 1 ml salt stock solution (1.8 g $MnSO_4.H_2O$, 0.2 g $ZnSO_4$, 0.1 g $CuSO_4$, 0.25 g Na_2MoO_4, in 1000 ml distilled water) was used for secondary screening of pesticide degradation assay. The initial pH of MSM medium was adjusted to 7.0 prior to sterilization by autoclaving at 121°C for 20 min. Furthermore, all chemicals and solvents used in present study were of analytical reagent-grade.

Sixteen nitrogen-fixing bacterial isolates were procured from the Department of Bio-Sciences and Technology, MMEC, MMDU, Mullana, Ambala, Haryana. Each culture was revived in nitrogen deficient medium (Burk's) and further pure colonies from each isolate were isolated using Burk's agar plates. Glycerol stock of 16 isolates was stored at -80^0 C for future use.

The growth range of isolates on Burk-agar plates with CP (cypermethrin) at various doses was examined to identify pesticide tolerant isolates. Gradient plates were made by adding a base layer of 15 ml Burk agar media, cooling it, and then adding CP (initially 10 ppm). Isolates were streaked down the pesticide gradient, and bacterial growth was measured after 18 to 72 h of incubation at 30°C. The colonies that survived at a lower concentration (10 ppm) were then exposed to escalating concentrations of cypermethrin on Burk-agar plates (25, 50, 75 and 100 ppm). Bacterial colonies that were surviving at the maximum concentration (100 ppm) were marked as a pesticide tolerant isolate. During this study, two controls (one without inoculum and one without pesticide) were kept to check the contamination and growth comparison.

In vitro breakdown of cypermethrin was carried out in 200 ml flasks containing 100 ml of minimum salt medium supplemented with various cypermethrin concentrations (25, 50 and 100 ppm). Each flask was infected with 10 µl of each bacterial culture, which was then incubated in an incubator shaker at 120 rpm and 30°C. The blank media without culture served as a control. Bacterial growth was examined after every two days using a UV-Visible spectrophotometer at 600 nm. Then, 10 ml of supernatant from each culture was

centrifuged (8000 rpm for 10 min) and used in biodegradation studies of cypermethrin. After 10 times dilution of the supernatant, the cypermethrin content was measured at 280 nm. A standard curve was generated using a standard solution of cypermethrin, and from the above readings of 280 nm, the value of the unknown conc. was obtained, and the percentage of degradation was calculated as:

% of degradation of CP = [(Initial conc. - Final conc)/(Initial conc.0] x 100

RESULTS AND DISCUSSION

A cypermethrin-degrading bacterial strain was collected from pesticide-contaminated soil in an agricultural area using an enrichment approach and characterized based on biochemical, morphological and molecular characteristics. According to 16s RNA technology, the bacteria shared almost complete homology with *Bacillus subtilis*. In the current investigation, the dual activity of bacteria was analyzed which included both nitrogen fixation and pesticide degradation.

Total 16 bacterial isolates were isolated from wheat soil system revived in Burk's media. Each of the bacterial isolates was examined under salt conditions and it was observed that only six A10, KU 40, KA23, Y18, Y11 and Y13 showed improved wheat seedling growth at 50 mM salt concentration in comparison to wild type. These six isolates were selected for further biodegradation of cypermethrin.

Revived bacterial isolates were cultivated in a Burk-agar plate till 100 ppm concentration to determine maximum tolerance level of cypermethrin. Colonies were off-white and round in form. Some were slimy, some were opaque. Colonies developed after 18 h of incubation at 30°C with CP concentration of 10 ppm (Table 1). The selected six isolates were further subjected to degradation. These isolates grew successfully on the Burk's plate in the presence of pesticides up to 100 ppm, confirming their pesticide tolerance (Fig.1). The increasing concentration of cypermethrin in medium caused stress in the developing bacteria, and as a result, these isolates took longer to grow on pesticide gradient plates. At 100 ppm of CP, bacterial isolates grew in 72 to 96 h (Table 1). No bacterial isolates survived over 100 ppm of CP in Burk's media, indicating that 100 ppm was the saturation limit for isolates to flourish in a cypermethrin containing environment. Pesticides of the functional class must be cleaved using specialized genes and enzymes. Microbes require optimal environmental conditions to function properly, which aid in the successful biodegradation of pesticides (Gangola *et al.*, 2018; Asim *et al.*, 2021).

OD for bacteria was observed to be increasing along with cypermethrin concentration throughout a 10-day trial and then turned constant as the incubation proceeded until the end of the experiment. The study's findings showed that an isolated potential degrader bacterium degraded cypermethrin more efficiently. The rise in OD was caused by bacteria growing under shaking with cypermethrin as the sole carbon source in MSM at concentrations of 25, 50 and 100 ppm. Growth parameters were recorded using spectrophotometer and growth curve of bacterial isolates was plotted for 25 ppm (Fig. 2), 50 ppm (Fig. 3) and 100 ppm (Fig. 4), respectively. Microorganisms require an acclimatization period to produce the essential degradative enzymes. This might be the reason behind the longer lag phase observed at higher cypermethrin concentrations, such as 100 ppm CP (Fig. 4). Several soil bacteria are known that eliminated 80.5% (50 ppm) after 10 days (Malla *et al.*, 2022). In the current work, three strains of isolated bacterium *B. subtilis* (Y13,

Table 1. Time taken for colonies appearance by different bacteria under different concentrations of cypermethrin

Name of bacterial strain	At 10 ppm conc. of CP	At 25 ppm conc. of CP	At 75 ppm conc. of CP	At 100 ppm conc. of CP
	After 18 h	After 18 h	After 36 h	After 36 h
KA 23	+	+	+	+
Y 18	+	+	+	+
KU 40	+	+	+	+
A10	+	+	+	+
Y 13	+	+	+	+
Y 11	+	+	+	+

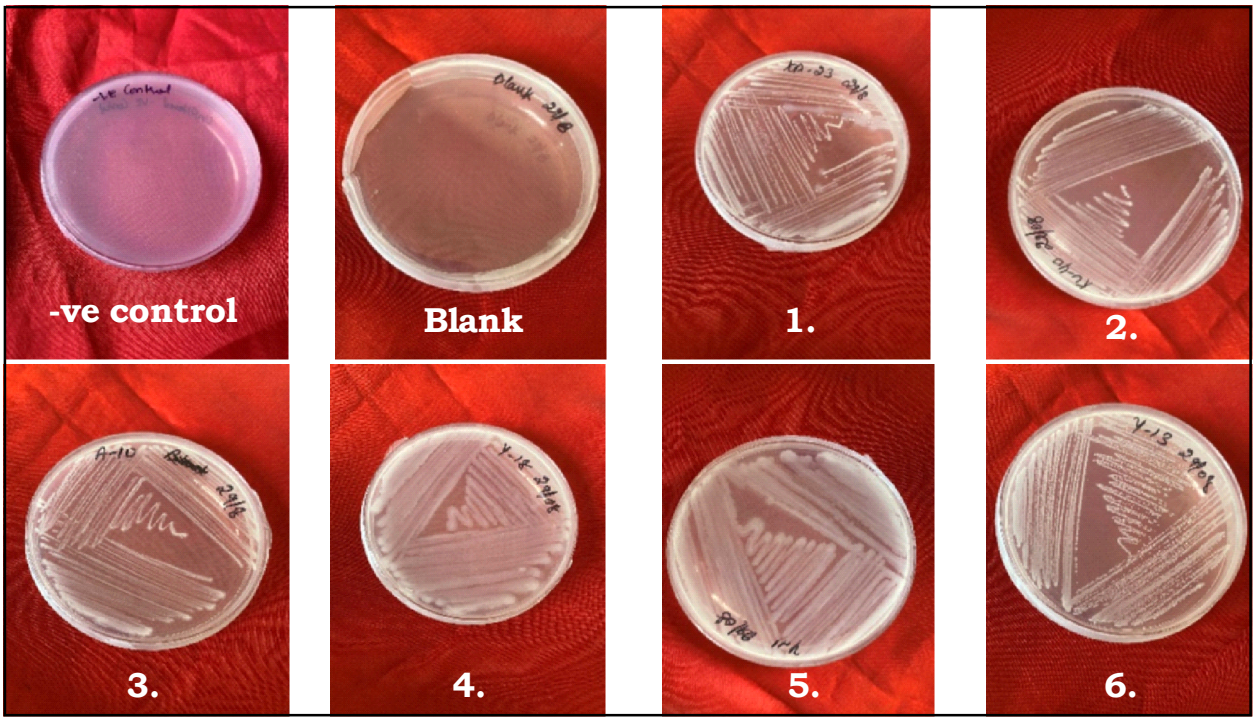

Fig. 1. Growth of six bacterial isolates at 100 ppm conc. of cypermethrin on burk agar plate with two controls.

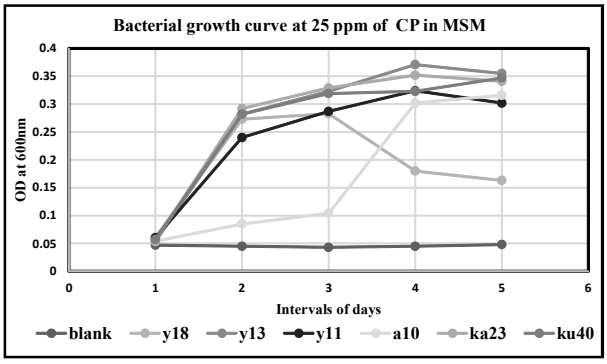

Fig. 2. Growth curve of six bacterial isolates at 25 ppm of CP in MSM.

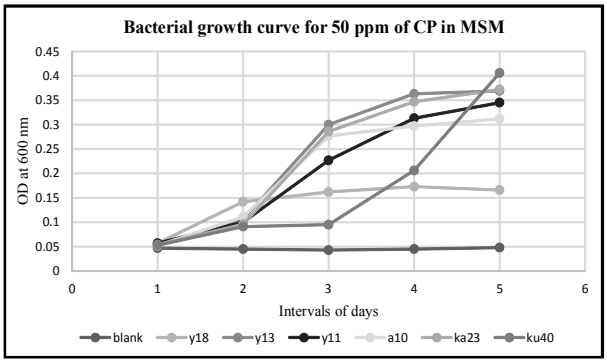

Fig. 3. Growth curve of six bacterial isolates at 50 ppm of CP in MSM.

Y11 and KU40) totally degraded cypermethrin at 50 ppm in 10 days under laboratory conditions (Table 2). It was discovered that

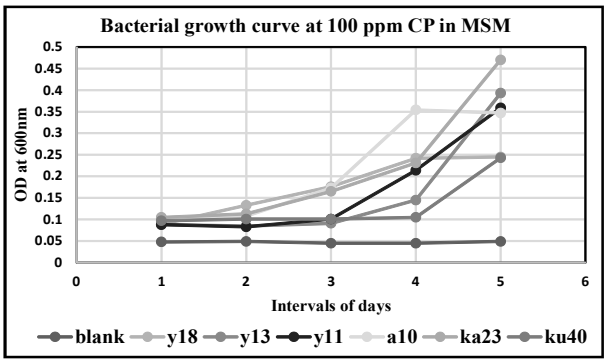

Fig. 4. Growth curve of six bacterial isolates at 100 ppm of CP in MSM.

these isolates used cypermethrin as their principal carbon and nitrogen source. Further all the isolates destroyed more than 80% of cypermethrin after 10 days of incubation when grown with maximum conc. of cypermethrin at 25 and 100 ppm (Tables 3 and 4). All the

Table 2. Per cent degradation shown by different bacteria at 50 ppm of cypermethrin under the incubation period of 10 days

Bacterial isolates	Day 2 (%)	Day 4 (%)	Day 6 (%)	Day 8 (%)	Day 10 (%)
Y18	48.72	62.26	83.76	85.80	84
Y13	51.44	68.68	96.78	98.20	99.99
Y11	42.68	56.22	92.64	96.34	99.32
A10	23.24	35.46	88.68	92.12	97.10
KA23	35.68	41	88.16	91.74	94.62
KU40	18.77	50.92	94.71	98.34	99.99

isolates reduced 50% of cypermethrin within four days of incubation which meant fastening of half-life of cypermethrin by isolated bacteria (Table 4). This was confirmed by the reduction in OD of cypermethrin at 280 nm, which increased the percentage of degradation over time (Fig. 5).

Table 3. Degradation (%) shown by different bacteria at 25 ppm of cypermethrin under the incubation period of 10 days

Bacterial isolates	Day 2 (%)	Day 4 (%)	Day 6 (%)	Day 8 (%)	Day 10 (%)
Y18	14.56	24.84	30.80	44.82	79.56
Y13	19.2	36.86	39.64	56.87	59.04
Y11	14	18.84	18.84	25.68	73.60
A10	24.84	59.88	62.48	71.84	84.68
KA23	14.81	23.12	31.68	59.76	71
KU40	24.84	59.88	64.43	70.12	78.34

Table 4. Per cent of degradation shown by different bacteria at 100 ppm of cypermethrin under the incubation period of 10 days

Bacterial isolates	Day 2 (%)	Day 4 (%)	Day 6 (%)	Day 8 (%)	Day 10 (%)
Y18	7.8	21.85	46.97	79.35	90.15
Y13	15.34	30.04	49.36	87.91	88.47
Y11	14.97	26.51	59.44	81.22	89.40
A10	3.43	23.98	67.82	79.17	90
KA23	6.22	22.77	54.79	83.26	86.8
KU40	6.22	18.53	54.42	86.61	91.18

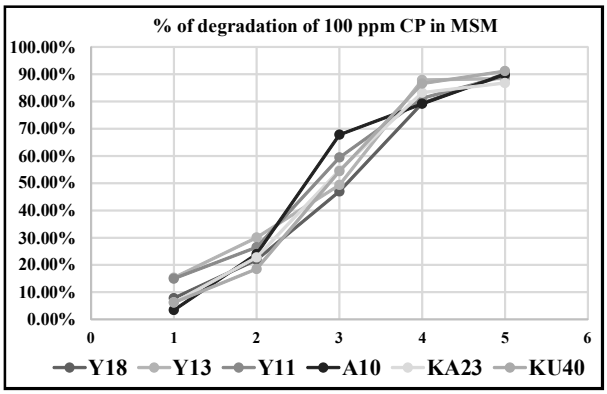

Fig. 5. Increase in per cent of degradation of CP within 10 days (at 100 ppm).

The optimal degradation temperature and pH were 30°C and 7, respectively. All of the bacterial isolates showed high tolerance (100 ppm) and digested cypermethrin up to 100 ppm in MSM media. This demonstrated bacteria's ability to thrive in very hazardous circumstances and degrade in different conditions. He *et al.* (2022) had already documented many *Bacillus* species with the ability to degrade several pesticides (carbendazim, fipronil, profenofos and cypermethrin) as well as other xenobiotic chemicals (azo dyes). Based on the results of the studies, it was anticipated that catabolic suppression had no effect on bacterial metabolic activity at higher pesticide concentrations, which might account for the highest level of resistance to pesticide stress. Furthermore, researchers discovered that microorganisms required an acclimatization period to produce the requisite degradative enzymes. This may explain the extended lag phase observed at higher cypermethrin concentrations. As a result, the bacterial isolate *Bacillus* species could provide more efficient elimination of harmful chemicals from the environment (Asim *et al.*, 2021). Researchers proved that the rise in population density in medium was a reflection of the degradation process. As a result, as degradation progressed, the isolates' population density rose in the experimental conditions compared to the control, indicating metabolic activity as seen by the higher cell count (Jabeen *et al.*, 2017). Present findings suggested that increase in turbidity of the MSM with gradual days indicated bacterial growth which was measured by using spectrophotometer and this proliferation of bacteria was clear indication of cypermethrin degradation because bacteria utilized CP as an energy source. The present study demonstrated the rhizosphere bioremediation, which used *B. subtilis* species, particularly appealing, possibly economical, and successful method of removing cypermethrin residues from soil. The association between growth of bacteria and CP biodegradation demonstrated that all degradation was connected with bacterial growth. *B. subtilis* was discovered to breakdown not just CP but also its key metabolic intermediates, such as 3-phenoxybenzoic acid, which were uncommon in other pyrethroid-degrading cultures (Zhao *et al.*, 2021). These findings showed that *B. subtilis* contained the metabolic route for full CP detoxification, suggesting that the isolate might be a suitable bacterium for bioremediation of CP-contaminated settings. Finally, in this investigation, a novel bacterium, *B. subtilis* was isolated and characterized, which had the potential to utilize cypermethrin as sole energy source. Furthermore, it is capable of nitrogen fixation, salt tolerance and seed germination under stress circumstances. Probably, this is

the first report of a pyrethroid-degrading bacterium which shows dual activity of nitrogen fixation and pesticide degradation. Furthermore, these isolates are salt-tolerant, and exhibit PGPR properties thus emerging as most prominent bioremediation species to sustain the environment.

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