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Original Research

Prospects of Plant Growth Promoting Bacterium, *Bacillus megaterium* for the Biodegradation of Selected Novel Pesticides

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ABSTRACT

Bacillus megaterium, a phosphorus-solubilizing bacterium, is exploited as a biofertilizer for increasing crop yield. Thiamethoxam and chlorantraniliprole, the novel insecticides, are applied as granular, and foliar formulations for insect pest control. The present study evaluated the potential of *B. megaterium* for bioremediation of these novel pesticides in natural and amended soils. The survivability of *B. megaterium* was studied in liquid half-strength nutrient broth supplemented with thiamethoxam or chlorantraniliprole (5-100 mg L^{-1}). In addition, soil microcosm studies were conducted (21 days) to explore the bio-stimulating effect on the degradability of *B. megaterium* in pesticide-treated soils (@ 10 mg kg^{-1}) using organic amendments *viz.*, vermicompost and Vesicular Arbuscular Mycorrhiza (VAM). The impact of pesticides was evaluated by calculating the enzymatic activity of dehydrogenase, phosphatase, and β-D glucosidase. The experimental results revealed that *B. megaterium* could survive in pesticide-supplemented conditions with maximum optical density observed as 0.734 AU and 0.965 AU at 100 mg kg^{-1} for thiamethoxam and chlorantraniliprole, respectively. Further, these cultures of *B. megaterium* also exhibited colony-forming units when plated on the nutrient agar supplemented with thiamethoxam (21 x 10^5) and chlorantraniliprole (43 x 10^5) at the end of 21 days, indicating its adaptability. Soil application of *B. megaterium* combined with vermicompost or VAM has exhibited higher degradation efficiency for thiamethoxam (2.61 and 2.16 mg kg^{-1}) and chlorantraniliprole (2.58 and 3.92 mg kg^{-1}), resulting in quick degradation. The observed half-life values in these combined treatments were 11-12 days (thiamethoxam), 11 and 15 days (chlorantraniliprole) that are on par with each other and significantly differed (ANOVA two factor, $p<0.05$) when compared to

natural attenuation (29 - 35 days). The enzymatic activity was negatively impacted for all the enzymes under study. However, vermicompost amendments could recoup the enzymatic activity over time. Thus, *B. megaterium* has the potential to bioremediate thiamethoxam and chlorantraniliprole, and the application of soil amendments can reduce the sublethal effects of these pesticides.

INTRODUCTION

Bioremediation is a prospective method for revival of contaminated soils. The soil microbiota helps to degrade many xenobiotic compounds and maintain soil fertility and health. Natural attenuation is a natural way of degradation of toxic pesticides by the native soil microorganisms belonging to bacteria, fungi, and actinomycetes. They produce various extracellular enzymes that aid in the metabolic pathway of degradation. The chemical nature of the pesticides varies widely, and so does the requirement of various enzymes and microbes to degrade pesticides at various phases. Native microbial communities of actinobacterial species including *Azotobacter*, *Pseudomonas*, *Bacillus*, *Klebsiella*, etc. have been employed to degrade a wide range of pesticides to recover the soil (Bokade et al. 2021). *Ochrobactrum thiophenivorans* and *Sphingomonas melonis* could degrade 86% of methomyl, a carbamate pesticide, in 8 days (Tatar et al. 2020). Similarly, Raimondo et al. (2020) demonstrated that natural attenuation in lindane-contaminated microcosms could degrade lindane by 40 per cent, which is attributed to the participation of the soil native microbiota.

In recent decades, novel insecticides have attracted farmers and are replacing conventional pesticides for plant protection due to their lower doses and site-specific action. Thiamethoxam, the neonicotinoid, is a neurotoxicant used for seed treatment and foliar application and has a share of 60% of global neonicotinoid usage (Jeschke et al. 2011). Chlorantraniliprole, the diamide pesticide, acts on the ryanodine receptors and controls the lepidopteran borers by interrupting the muscle contractions. These chemicals belonging to the novel group are assumed to degrade faster than conventional pesticides. However, the reported half-lives for these pesticides are in the range of 23.89 - 170 days (Sahu et al. 2019, Li et al. 2018) in soil. There are also reports that thiamethoxam and chlorantraniliprole reduce soil microbial biomass and enzymatic activity indicating their ill effect on soil microorganisms (Wu et al. 2021).

Pesticides cause diverse effects on the established soil microbial community structure, which participates in various biological processes in soil (Prashar & Shah 2016). There are reports that organochlorine-contaminated soils were predominated by certain types of proteobacteria and mortiriella fungi (Egbe et al. 2021). The numerous enzyme exudates from microorganisms play a key role in various bio-geochemical processing of chemical elements and nitrogen, phosphorus, sulfur, and carbon cycles (Mahdi et al. 2017). Pesticide-affected soils due to altered microbial community structure exhibit lesser enzymatic activity for these enzymes due to reduced microbial abundance, thus affecting soil health.

Farmers use different beneficial microorganisms in the form of biofertilizers, biostimulants, and biopesticides to promote plant growth, and improve soil fertility. These organisms, referred to as Plant Growth Promoting

Rhizobacteria (PGPR), have profound beneficial effects that include biological nitrogen fixation, phosphorus & potassium solubilization, and hydrolytic enzymes (Khatoon et al. 2020). Apart from growth-promoting effects, these PGPRs are reported to have the ability to degrade pesticides. There are reports on the degradation of imidacloprid and thiamethoxam by *Pseudomonas* sp. (Zamule et al. 2021) and chlorantraniliprole by *Bacillus subtilis* (Fahmy et al. 2022).

Bacillus megaterium, a gram-positive, phosphorus solubilizer exploited as a biofertilizer, was also reported for biodegradation of insecticide, *viz.*, fipronil (Prado et al. 2022) and allethrin (Huang et al. 2022). *B. megaterium* secretes several organic acids such as oxalic, malic, fumaric acid, etc., to enable the mineralization of phosphorus with subsequent release of OH^- ions (Castagno et al. 2021). This leads to the chelation of ions, affecting the adsorption and desorption of pesticides. In addition, *B. megaterium*, is reported to absorb toxic contaminants in soil through various mechanisms that include biosorption, exopolysaccharide and siderophore production (Saeed et al. 2021). The nitroreductase genes from *B. megaterium* were reported to aid in the enzymatic degradation of mesotroine (Carles et al.2021).

The previous studies on bioremediation were majorly focused on the degradation of conventional pesticides by isolated organisms from contaminated sites. There is a gap in utilizing the established beneficial plant growth-promoting microorganisms for the degradation of a new class of pesticides. Similarly, the sublethal effects of novel pesticides on the beneficial microbes are not understood. Studies on the prospects of a proven PGPR such as *B. megaterium* for pesticide degradation would give an added advantage of the removal of pesticide contaminants besides fulfilling the nutrient requirements of plants. In this study, the degradation potential and survivability of *B. megaterium* was evaluated through laboratory studies for 21 days for the removal supplemented concentration of thiamethoxam and chlorantraniliprole. In addition, soil microcosm studies were conducted (21 days) to explore the bio-stimulating effect on degradability in pesticide-treated soils (@ 10 mg/kg) when *B. megaterium* is used along with organic amendments *viz.*, vermicompost and Vesicular Arbuscular Mycorrhiza (VAM). The Degradation half-lives were estimated based on their residual concentration over 21 days in soil samples using an HPLC-PDA. Further, the effect of the pesticides on the soil dehydrogenase, β-D- glucosidase, acid, and alkaline phosphatase activity was determined to have an analysis of the microbial activity in natural attenuated, bioaugmented, and biostimulated treatments.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents:

Nutrient Agar and Nutrient Broth for maintaining bacterial cultures were procured from Hi-media. Chemicals for enzymatic analysis viz., p-Nitrophenyl - β - D glucopyranoside (purity ≥ 99.0 %), p-Nitrophenyl-phosphate disodium salt (≥ 98.00), Standard p-nitrophenol (≥ 99.0%), TRIS (hydroxy methyl) amino methane (≥ 99.0%), 2,3,5-triphenyl tetrazolium chloride (≥ 99.0%), 1,3,5-Triphenyl formazan (TPF) (≥ 90.0%) were procured from Hi-

media. Sodium Hydroxide (M/S. Fisher Scientific, AR, ≥99.0%), Maleic acid (M/S. Himedia, AR, ≥ 99.0%), Boric acid (M/S. Fisher Scientific, AR, 99.5%), Citric acid (M/S. Fisher Scientific, AR, 99.5%), Calcium Chloride (M/S. Fisher Scientific, AR, 99.5%) were used for preparation of various reagents. Primary Secondary Amine (PSA) residue grade (M/S. Agilent Technologies Ltd.), Magnesium Sulphate (M/S. Fisher Scientific, AR, 99.5%), Sodium Chloride (M/S. Fisher Scientific, AR, 99.5%) were used in the estimation of residual concentration in soil samples.

2.2. Standard solutions:

The individual stock solutions of thiamethoxam and chlorantraniliprole were prepared in methanol to contain 10000 mg L^{-1} concentration of pesticide. The individual standard solution was serially diluted further in the range of 2.5 to 100 mg L^{-1} in 25 mL volumetric flasks.

2.3. Preparation of pure cultures:

B. megaterium cultures from soil were isolated on Pikovskayas agar media using the dilution plate technique and observing the phosphate solubilizing ability of *B. megaterium*. A clear zone around the microbial colony on the medium indicated the presence of phosphate solubilizer. The bacterial cultures were reinoculated and incubated at 28 ± 2 ° C for 24 hours to get pure culture on the nutrient agar medium.

2.4. Acclimatization of *B. megaterium*:

The cultures of *B. megaterium* were revived on nutrient broth for 24 hours. To acclimatize to the presence of pesticides, in the initial phase, the bacteria were cultured on a Minimal salt Medium under aerobic conditions at 28 ± 2° C. The minimal medium provided the required amounts of minerals, carbon, and nitrogen sources to support the growth of *B. megaterium.* The minimal medium is composed in g/L of $(NH_4)_2 SO_4$ (4.99 g), KH_2PO_4 (1.36 g), Na_2HPO_4 2.13 g, $MgSO_4.7 H_2O$ (0.2 g) and L-tryptophan (1.0 g) (Bouknight & Sadoff 1975). *B. megaterium* was inoculated (1 mL, OD ~ 0.987-1.313) aseptically into 100 mL of the minimal medium and incubated for 48 hours. Subsequently, *B. megaterium* was inoculated in the minimal medium supplemented with thiamethoxam or chlorantraniliprole (5 mg kg^{-1}), replacing the carbon source (L-tryptophan) to establish that the bacterium could utilize the pesticides as a carbon source. The cultures were incubated for 48 hours under non-shaking aerobic conditions at 28 ± 2° C and subcultured for 3-4 generations. In the later phases of the experiment, *B. megaterium* was inoculated on half-strength nutrient broth supplied with thiamethoxam or chlorantraniliprole in the range of 5-100 mg L^{-1} and cultured under aerobic conditions at 28 ± 2° C for 21 days in three replications along with control to understand the inhibitory concentrations and degradation efficiency in nutrient limited conditions.

Bacterial cultures were sampled on 0 (18 hours), 3, 7, 14, and 21 days after inoculation, and bacterial growth was measured at 600 nm in a UV-VIS spectrophotometer to understand their survivability at various concentrations of pesticides under the study. It was also verified by plating the cultures from half-strength nutrient broth at the end of 21 days on nutrient agar and the bacterial colony count was taken. Subcultures of *B. megaterium* were prepared

for several generations on pesticide-supplemented (10 mg L^{-1}) half-strength nutrient broth from which bacterial cultures were prepared for bioremediation studies.

2.5. Preparation of cultures of *B. megaterium* for bioremediation experiments:

B. megaterium cultures for bioremediation experiments were prepared from 72-hour incubated, pesticide-spiked, half-strength nutrient broth cultures of *B. megaterium*. The cells were harvested by centrifuging at 10,000 rpm at 4º C for 10 min. The biomass was washed two times with sterile water and resuspended in 10 mL of saline (0.85% NaCl), and the optical density of the suspension was measured in the range of 0.8-1.00 AU, equivalent to 10^8 CFU/mL. The soil treatments under microcosm experiments are mixed with this solution at 2 mL kg^{-1} of soil (Raimondo et al. 2019)

2.6. Experimental design for evaluation of bioremediation potential of *B. megaterium* in soil microcosms:

Bioremediation experiments were conducted in rectangular trays with 30 × 15 × 5 cm (L × W × H) filled with soil collected at a depth of 5 to 15 cm from pesticide contamination-free areas and analyzed for physicochemical properties. The collected soils were shade-dried and sieved through a 2 mm sieve. The bio-stimulants used for the experiment were vermicompost and VAM and were collected from the bio-fertilizer production unit of the National Institute of Plant Health Management, Hyderabad, India.

The trays were filled with 1 kg of sieved soil and were fortified with thiamethoxam or chlorantraniliprole @ 10 mg kg^{-1}. This spiked soil was combined with vermicompost (BS1) or VAM (BS2) at the rate of 2% and /or *B. megaterium* (BA) suspension at 2 mL kg^{-1} of soil as per the treatment. The experiment was set up with six treatments and three replications in a block design along with a positive control without the addition of amendments or *B. megaterium* inoculum but supplemented with pesticides (10 mg kg^{-1}) to understand the degradation of pesticides under natural attenuated conditions. Another treatment without the addition of pesticides or amendments or inoculum served as a negative control. The experiment was set up at room conditions and observations were taken for 21 days. The soil moisture content was at 20 -30 % of water holding capacity, and the room temperature varied between 30-34º C. The soil was mixed daily to ensure uniform distribution and aeration in microcosms. Soil was sampled on 0 (18 hours), 3, 7, 14, and 21 days to determine the pesticide content. The estimated residual concentrations were analyzed to interpret the half-life of pesticides under bioaugmented, bio-stimulated and natural attenuated conditions and statistically evaluated by ANOVA two factor ($p<0.05$) to understand the significant differences. The effect of pesticides on soil enzymatic activity was observed for the soil samples drawn on 3, 7, 14, and 21 days from the treatments along with ANOVA single factor ($p<0.05$) to evaluate the significant values

2.7. Quantitative estimation of residual content in culture media and soil samples:

High-performance liquid Chromatography (Shimadzu model: Nexara) with binary pump and photodiode array (PDA) detector was used for the quantification of pesticide residues of thiamethoxam and chlorantraniliprole along

with a 250 x 4.6 mm, and 5 µm particle reverse phase (C_{18}) column. The pesticide residues in liquid bacterial cultures were determined on 0 (18 hours), 3, 7, 14, and 21 days by taking 5 mL of culture into a centrifuge tube. The extract was centrifuged at 10,000 rpm for 10 minutes at -10º C to separate the biomass, and filtered (0.22 µm) to quantify the residues on HPLC-PDA. Quantification of residues was by linear calibration standards (2.5 – 100 mg L^{-1}).

Residual thiamethoxam content in soil microcosms was estimated on 0 (18 hours), 3, 7, 14, and 21 days using a Quick, Easy, Cheap, Effective, and Rugged method of Analysis (QuEChERS) (Anastassiades et al. 2003). Soil samples (5 g) from each treatment were weighed in a 50 mL centrifuge tube and were mixed with 5 mL of water followed by 10 mL of extraction solvent i.e. acetonitrile with 1 % acetic acid. The samples were hand shaken vigorously for 5 min, and vortexed for about 3 min., followed by the addition of extraction salts i.e., 4 g $MgSO_4$ and 1 g NaCl. The contents were mixed properly for 1 minute by handshaking and centrifuged at 3000 rpm for 3 min. After centrifugation, the supernatant (6 mL) was taken into a 15 mL centrifuge tube containing 150 mg of Primary Secondary Amine (PSA) and 900 mg of $MgSO_4$ for clean–up to remove the co-extracts. The extract was filtered through a 0.22 µm membrane filter into an injection vial for quantifying the residues as per the Analytical Conditions (Table 1).

Table 1: Analytical Conditions for quantification of residues in culture media and soil samples

Name of the pesticide	Mobile phase	Identification of Analytes	Flow rate mL min^{-1}	Retention time (minutes)
Thiamethoxam	ACN: Water 75:25	HPLC-PDA at 256 nm	1.0	3.70 ± 0.1
Chlorantraniliprole	ACN: Water 80:20	HPLC-PDA at 260 nm	1.0	4.26 ± 0.1

2.8. Method Validation:

The method for quantifying the residues was validated for specificity, sensitivity/linearity, limit of detection (LOD), limit of quantification (LOQ), and accuracy (recovery). Specificity of the method, the ability to measure quantitatively the analyte in the presence of components that may be expected to be present in the sample matrix, was evaluated by ensuring that there is no interference at the retention time of the analyte from standard, solvent and/or blank extract with each other. The linearity of the method was evaluated by plotting a matrix-matched calibration curve in the range of 2.5 – 100 mg L^{-1} (n=3) against their responses on the detector. The LOD and LOQ of the method were evaluated from this linear calibration curve and determined the standard deviation of y-intercept and slope using regression analysis at 95% confidence. LOD is calculated as 3 x (standard deviation of intercept/slope), and LOQ is calculated as 10 x (standard deviation of intercept/slope). The method's accuracy was determined by fortification experiments by spiking the control or blank samples with known concentrations of

pesticides (n=5). Precision was evaluated by calculating the relative standard deviation (RSD %) between different measurements (n=5) for residue estimation.

2.9. Degradation Kinetics:

Half-life ($t_{1/2}$) of the pesticides, the time required to reduce the initial concentration by 50 %, was calculated from the residue data subjected to statistical analysis (Hoskins 1961). A first-order kinetic equation was fitted ($C_t = C_0 \times e^{-kt}$), where C_0 is the initial concentration of thiamethoxam, k is the rate constant, t is the days of pesticide application, and C_t is the recovered concentrations of pesticides over 21 days in different treatments. The half-life of pesticides in different treatments was determined as $t_{1/2} = \ln(2)/k$.

2.10. Determination of soil enzymatic activity:

A UV-Vis spectrophotometer (Shimadzu) was used to measure the colour intensity of the substrate-enzyme complex formed at different wavelengths during the evaluation of soil enzymatic activity. Dehydrogenase activity was determined as per the method outlined in ISO 23753-1:2005 by the estimation of reduced triphenyl formazan (TPF) was given in terms of µg of TPF g^{-1} h^{-1}. The activity of β- D- glucosidase, Acid and alkaline phosphatase was evaluated by measuring the absorbance of the yellow-coloured extract at 400 nm using a p-nitrophenol standard and the results were expressed as µg of pNP g^{-1} h^{-1} (Deng & Popova 2015, Eivazi & Tabatabai 1988, Acosta-Martínez & Ali Tabatabai 2015, Eivazi & Tabatabai 1977).

2.11. Statistical analysis

Experiments were carried out in triplicate, and the results were expressed as average values. The recovered log concentration of thiamethoxam and chlorantraniliprole were plotted against time (days after application) and fitted with a first-order kinetic equation to calculate the half-life values. The significant difference among the treatments in the observed half-lives of thiamethoxam and chlorantraniliprole was analyzed by ANOVA two factor ($p < 0.05$). The results of enzymatic activity over 21 days were analyzed by One-way ANOVA with a two-sample t-test ($p < 0.05$) to compute significant differences among the treatments.

3. RESULTS AND DISCUSSIONS

3.1. Survivability and biodegradability of *B. megaterium* in pesticide-supplemented liquid media:

The results of the evaluation of the growth of *B. megaterium* on half-strength nutrient broth supplemented with thiamethoxam or chlorantraniliprole indicated that acclimatized cultures could tolerate pesticides (5-100 mg L^{-1}). The maximum absorbance values were observed as 0.734 AU and 0.965 AU at 100 mg/kg of thiamethoxam and chlorantraniliprole, respectively, demonstrating the bacterium's adaptability to both pesticides (Table 2). The

growth was more prominent in the treatments supplemented with chlorantraniliprole than thiamethoxam. This could be attributed to the variation of molecular structures and degradation products from these pesticides, which could act as energy sources. Carbon sources taken by the bacterial cells enter the metabolic processes of energy generation at different points. Depending on the sources available, bacterial cells may utilize different or preferred sources over the other. The differential growth rates of *B. megaterium* in the presence of thiamethoxam and chlorantraniliprole as carbon sources could be due to these variations in metabolized products, which are utilized as carbon sources (Wang et al. 2019). At the end of 21 days, these liquid cultures of *B. megaterium* were plated on nutrient broth, and the colony-forming unit count was taken (Fig. 1d) *B. megaterium* exhibited colony-forming units 21 x 10^5 and 43 x 10^5 in cultures with thiamethoxam and chlorantraniliprole, respectively, correlating with the intensity of optical density in these cultures and confirming its survivability.

Table 2: Measured Optical Density of *B. megaterium* in liquid half-strength nutrient broth cultures with thiamethoxam and chlorantraniliprole (5-100 mg L^{-1})

Concentration of supplemented Pesticides (mg L^{-1})	Measured optical density (AU)* of *B. megaterium*	
	Thiamethoxam	Chlorantraniliprole
5	0.418 ± 0.09	0.847 ± 0.18
10	0.453 ± 0.08	0.878 ± 0.12
25	0.456 ± 0.11	0.877 ± 0.10
50	0.503 ± 0.13	0.905 ± 0.09
100	0.734 ± 0.21	0.964 ± 0.17

Data is the mean of three replicat ions ± SD

Further, experiments to confirm the adaptability aimed at the relative spread of *B. megaterium* plated as 10 µL bacterial culture spots on filter paper discs on agar plates spiked with thiamethoxam and chlorantraniliprole revealed retarding effect of the pesticides compared to the control with no pesticide (Fig.1 a, b & c).

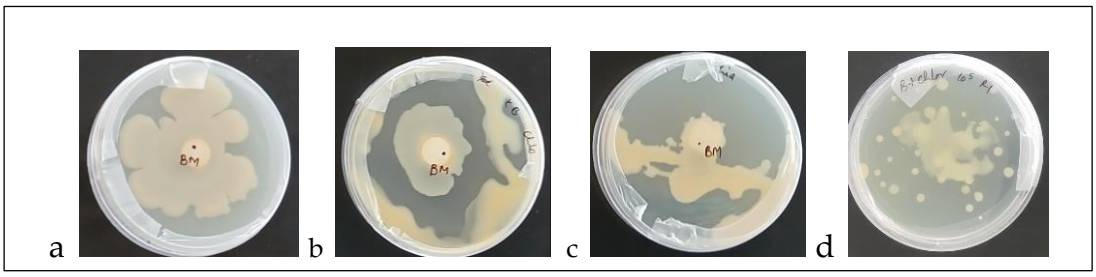


Fig. 1: Relative spread of *B. megaterium* on agar plates with a) no pesticide addition b) chlorantraniliprole c) thiamethoxam d) CFU of *B. megaterium* on NA

3.2. Method Validation :

Method validation results evaluating the suitability of the method to estimate the concentration of pesticides indicated that there was no interference from coextraactives of the sample components, at the retention times of the

principal peaks of thiamethoxam and chlorantraniliprole, establishing the specificity of the method and allowing unambiguous identification of analytes based on the retention time with a tolerance of ±0.1 min. The linearity of the method was evaluated in the range of 2.5 to 100 mg kg^{-1} by a matrix-matched calibration curve with the coefficient of regression value $R^2 \geq 0.99$ exhibiting a good linearity. The method's LOD was determined as 1.0 mg kg^{-1} and LOQ as 3.0 mg kg^{-1} for thiamethoxam and chlorantraniliprole in nutrient broth extractions and soil samples. Recovery tests were conducted to verify the accuracy of the method by fortifying the control or blank sample at 5.0 and 10.0 mg kg^{-1}. The mean recovery was in the range of 87.73 - 96.87 % for thiamethoxam and 81.82 - 97.81 % for chlorantraniliprole. The RSD values between individual measurements (n=5) to evaluate the repeatability were in the range of 1.03 to 6.04 %. The results were found to be satisfactory and are as per the SANTE guidelines for specified limits of mean recovery in the range of 70-120% and less than ±30% RSD between measurements.

3.3. Degradation of thiamethoxam and chlorantraniliprole in liquid cultures:

The residual concentrations of thiamethoxam and chlorantraniliprole were estimated in the extracted liquid cultures over 21 days. Thiamethoxam was degraded to less than the detection limits in treatments supplemented with 5 and 10 mg L^{-1} thiamethoxam compared to 39 and 43 per cent reduction, respectively, in chlorantraniliprole-supplemented treatments at the same concentration (Table 3). Thiamethoxam, belonging to the neonicotinoid group, undergoes degradation *via* nitro reduction to form Urea, which through the Urea cycle transforms to fumarate, and enters Kreb's cycle to yield energy (Pang et al. 2020). Similarly, amide hydrolysis by the amidases is reported to cause degradation of chlorantraniliprole (Gao et al.2019). Thus, it could be inferred that the amidases produced from *B. megaterium* might have aided in the chlorantraniliprole degradation in its supplemented cultures. The higher growth rate (0.847-0.964 AU) and CFU (43 x 10^5) observed in chlorantraniliprole-supplemented treatments might be due to the co-utilization of chlorantraniliprole by *B. megaterium* along with other carbon sources in the nutrient broth. Though the observed OD and CFU are less in the thiamethoxam-supplemented cultures (0.418-0.734 AU, 21 x 10^5), the preferential utilization of thiamethoxam as a carbon source over the other resources might have led to higher degradation efficiencies with its complete degradation. However, there is no significant difference between chlorantraniliprole and thiamethoxam treatments at higher concentrations (50-100 mg L^{-1}) exhibiting per cent reduction that is on par with each other, implying the potential of *B. megaterium* to degrade both pesticides. *B. megaterium* has been previously reported to grow in fipronil-supplemented media (0.6 g L^{-1}), degrading up to 94 % of its initial concentration and utilizing it as the main carbon source, indicating its adaptability to pesticide exposure (Prado et al. 2021)

Table 3: Degradation of thiamethoxam and chlorantraniliprole in supplemented half NB cultures

Treatment	Supplemented Conc. (mg L^{-1})	Thiamethoxam		Chlorantraniliprole	
		Residual Conc. at 21 days[1]	Per cent reduction in conc.	Residual Conc. at 21 days[1]	Per cent reduction in conc.

		(mg L^{-1})		(mg L^{-1})	
T1	5	ND ± 0.53	~ 100	3.05 ± 0.13	39.00
T2	10	ND ± 0.65	~ 100	5.70 ± 0.46	43.00
T3	25	9.62 ± 0.54	61.52	14.55 ± 0.83	41.80
T4	50	20.48 ± 0.10	59.04	13.59 ± 1.08	72.82
T5	100	32.98 ± 3.50	67.02	19.75 ± 3.64	80.25
T6	10*	6.93 ± 0.03	30.7	7.52 ± 0.18	24.80

[1] Data is the mean of three replications each ± SE of regression.* uninoculated control supplemented with pesticide at 10 mg/L

3.4. Degradation and Half-Life estimation for thiamethoxam and chlorantraniliprole in soil microcosms:

The degradation rate of thiamethoxam and chlorantraniliprole was studied under natural attenuated, bioaugmented, and biostimulated conditions through soil microcosm experiments under laboratory conditions. The experimental trays were filled with silt clay loam textured soil with a pH of 7.75. The kinetics of degradation for thiamethoxam and chlorantraniliprole were evaluated by quantifying their residual concentration in soil samples on 0 (18 hours), 3, 7, 14, and 21 days on HPLC-PDA (Fig. 2). The residues were quantified using a standard solution prepared with the extracted solution of blank treatments (matrix-matched standard) in the range of 2.5 to 100 mg L^{-1}. The dissipation pattern of thiamethoxam and chlorantraniliprole was fitted to a first-order kinetic equation (Table 4), and half-lives were determined by $t_{1/2}= \ln(2)/k$, where the half-life is independent of the initial concentration of pesticides. The treatments amended with vermicompost combined with *B. megaterium* have exhibited shorter half-lives for both pesticides (11-18 days) compared to natural attenuation (29-35 days). Pesticide degradation rate is dependent on various factors such as soil pH, organic matter content, clay content, pesticide characteristics, temperature and moisture content in soil, etc. The faster degradation rates of pesticides under amended conditions than under natural attenuated conditions may be attributed to the high nutrient availability and carbon content, which could have aided in the establishment of augmented microorganisms. Further, the enriched nutrient conditions and carbon abundance might have stimulated the natural degraders to degrade the pesticides. The microbial abundance in the vermicompost might also have increased the microbial biomass, contributing to the quick degradation of pesticides in amended soils compared to natural attenuated soils

The secretion of organic acids by *B. megaterium* during mineralization of phosphorus with subsequent release of OH- ions, might have contributed to the adsorption-desorption of pesticides, leading to their increased bioavailability for degradation. The adsorption-desorption of chlorantraniliprole has been reported to be strongly affected by soil pH. Release of the organic acid by *B. megaterium* leading to the acidification of rhizosphere might have affected the desorption of chlorantraniliprole, causing quick degradation in augmented and biostimulated soils. Further, the release of hydroxyl groups from the organic acids might have caused metabolic transformation of thiamethoxam to its hydroxyl and urea derivatives (Xiang et al. 2024). The production of enzymes such as nitroreductases by *B. megaterium* might also have contributed to the quick degradation of the thiamethoxam. Vermicompost with high nutrient content and enriched microbial diversity (Raza et al. 2022) might have

contributed to the enhanced degradation rate. Vermicompost has been previously reported to improve the removal rates of diuron (Romero et al. 2024), and phosmet (Dias et al. 2021) in the bio-bed system and could be an organic source for reducing the soil persistence of pesticides besides improving the quality

3.5. Evaluation of soil enzymatic activity in microcosms

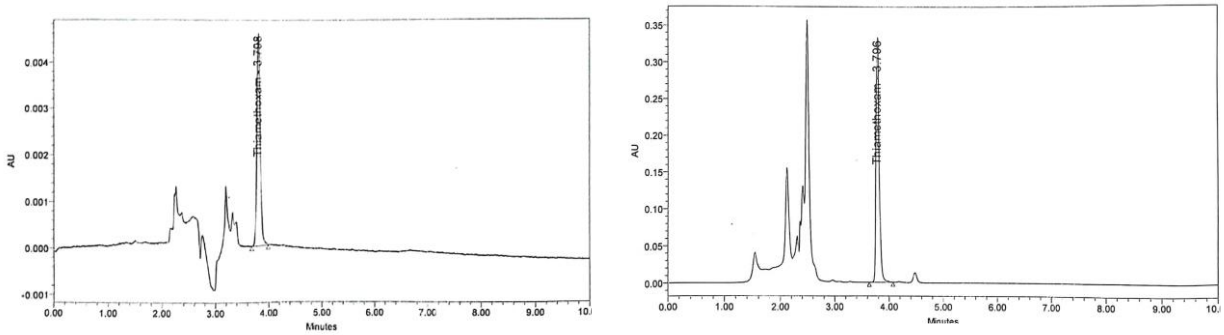
The activity of soil dehydrogenase (DH), β-D glucosidase (BDG), acid phosphatase (ACP), and alkaline phosphatase (AKP) was evaluated on 3, 7, 14, and 21 days after application of thiamethoxam and chlorantraniliprole in all the treatments, including control. The enzymatic activity was significantly low for all the enzymes under study in natural attenuated treatments compared to the control.

Table 4: Degradation of thiamethoxam and chlorantraniliprole in Soil microcosms

	Thiamethoxam			Chlorantraniliprole		
Treatment	**Residual Conc. at 21 days (mg L^{-1})[1]**	**Regression equation**	**Half-life ($t_{1/2}$) in days**	**Residual Conc. at 21 days (mg L^{-1})[1]**	**Regression equation**	**Half-life ($t_{1/2}$) in days**
B. megaterium	4.85 ± 0.18	y = -0.0292x + 2.1781	23	3.67± 0.75	y = -0.0447x + 2.171	16
Vermicompost	3.33 ± 0.57	y = -0.0508x + 2.2204	14	2.84± 0.67	y = -0.0557x + 2.249	12
B. megaterium + Vermicompost	2.61 ± 1.03	y = -0.0593x + 2.1264	12	2.58± 0.50	y = -0.0626x + 2.2343	11
VAM	2.57 ± 0.46	y = -0.0594x + 2.3616	12	4.33± 0.76	y = -0.0383x + 2.1747	18
B. megaterium + VAM	2.16 ± 0.35	y = -0.0654x + 2.3247	11	3.92 ± 0.77	y = -0.0456x + 2.2537	15
Natural attenuation	6.59 ± 0.28	y = -0.0127x + 2.3072	35	6.04± 0.13	y = -0.024x + 2.3265	29

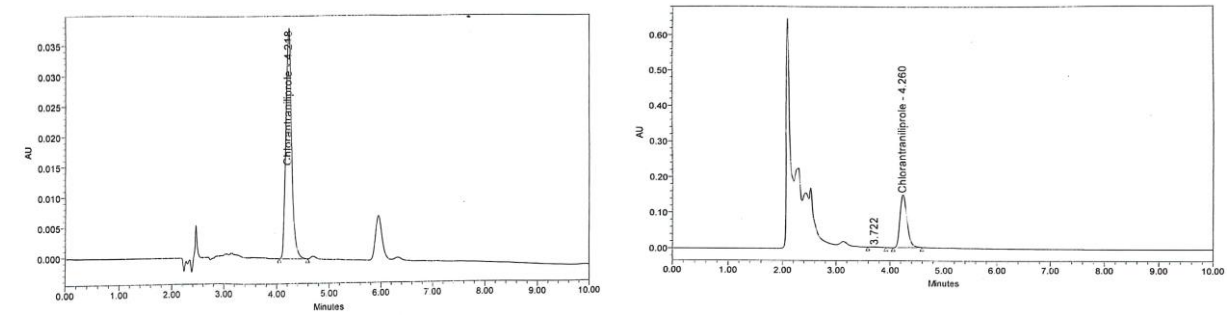
[1] Data is the mean of three replications each ± SE of regression.

1. Thiamethoxam



a) b)

2. Chlorantraniliprole



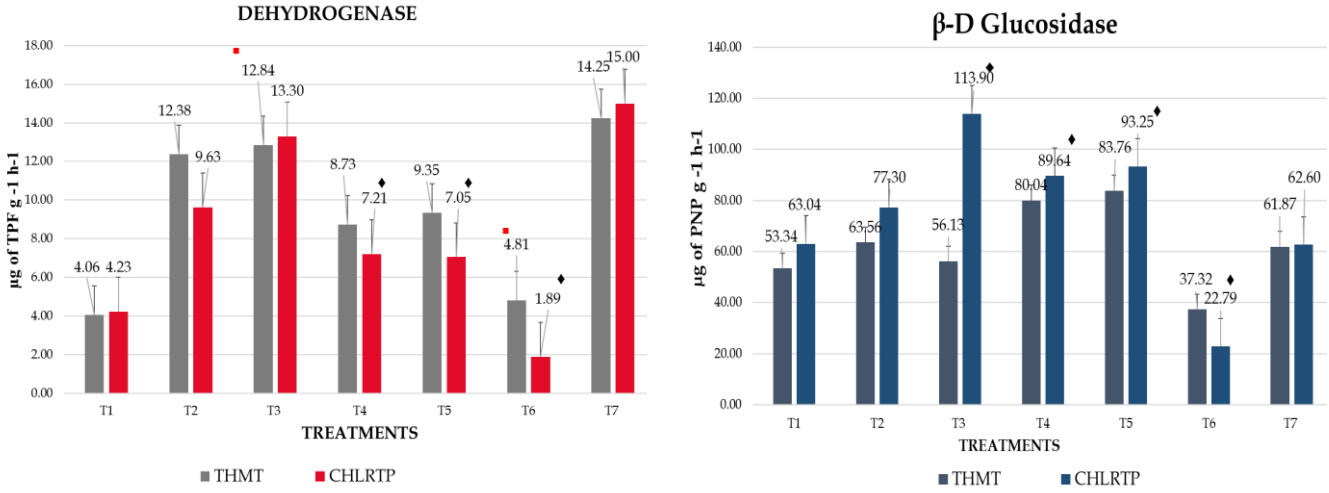
a) b)

Fig. 2: Chromatogram depicting the peaks of 1) thiamethoxam and 2) chlorantraniliprole in a) matrix match standard solution b) sample extracts

It was also observed that immediately after the application of thiamethoxam and chlorantraniliprole, there was a reduction in enzymatic activity, which later improved over the days. This could be attributed to the impact of pesticides on bacterial communities immediately after their application. The treatments with vermicompost amendments have enzymatic activity on par with the untreated controls, indicating that these treatments could recoup with microbial biomass over time. Vermicompost is enriched with various macro and micronutrients that are readily available for the soil microorganisms. These nutrient-enriched conditions might have stimulated the native as well as pesticide-degrading microbial population over time, resulting in their recoupment. These results are identical to the results of Romero et al. (2024) who reported enhanced enzymatic activity in vermicompost treatments in diuron-applied soils. Further, the same study reported variable enzymatic activity with time for soil dehydrogenase, β-glucosidase, protease, and acid phosphatase, as observed in the present study, indicating microbial adaptation. The DH activity in the treatments varied between 4.06 to 12.84 and 4.23 to 13.30 µg TPF g^{-1} h^{-1} for thiamethoxam and chlorantraniliprole, respectively (Fig. 3a). There is a significant difference in the dehydrogenase activity values in treated microcosms compared to uncontaminated controls ($p<0.05$) for both pesticides. Increased levels of enzymatic activity were observed in uncontaminated microcosms compared to non-augmented contaminated treatments (T6 - NA) throughout the 21-day experimental period**,** revealing thiamethoxam's negative effect on soil microcosms. The DH activity is more negatively influenced in chlorantraniliprole-treated microcosms than in thiamethoxam-treated soils.

β-D-glucosidases are key enzymes involved in the carbon cycle and conversion of cellobiose to disaccharides. Their activity ranged between 37.32 to 83.76 for thiamethoxam and 22.79 to 113.90 µg of pNP g^{-1} h^{-1} for chlorantraniliprole (Fig. 3b). Their activity is significantly higher in the combined treatments of *B.megaterium* with vermicompost or VAM. These high levels of BDG activity can be attributed to the high organic carbon content, which is readily available in amended treatments. Significantly lower ($p<0.05$) BDG activity was observed in soils treated with chlorantraniliprole under natural attenuated conditions compared to amended soils.

a) b)



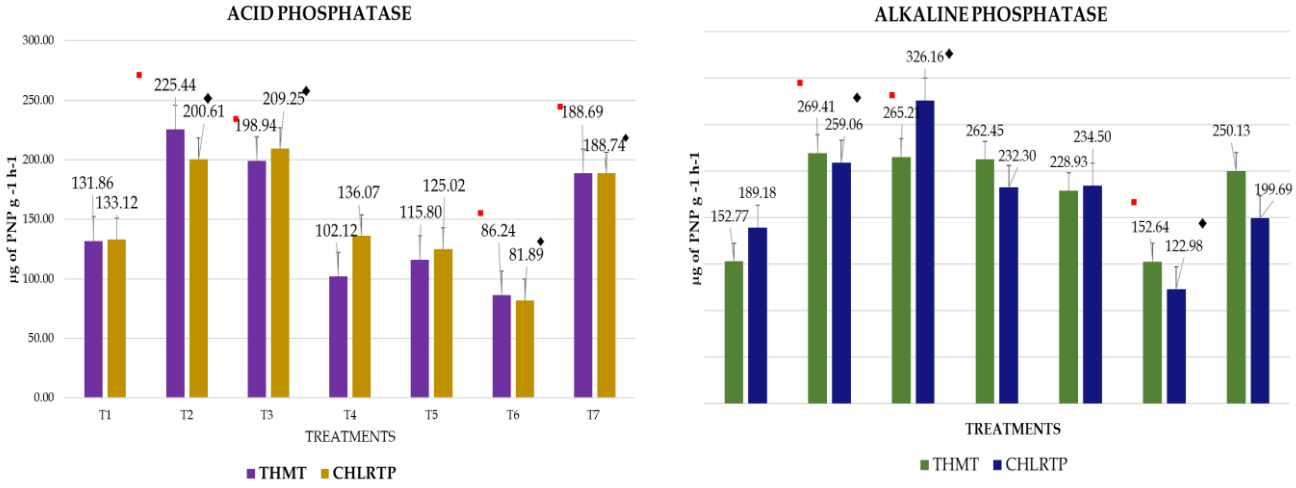
♦▪ indicate significant figures by ANOVA single factor (at $p<0.05$)

Fig. 3: Effect of thiamethoxam and chlorantraniliprole on the enzymatic activity of a) dehydrogenase and b) β-D Glucosidase

Similarly, the acid and alkaline phosphatase activity was significantly higher ($p<0.05$) in vermicompost-treated soils and untreated controls compared to natural attenuated soils (Fig. 4 a & b). Phosphatase activity is negatively related to the pH levels during the degradation process, organic P content or phosphorus fertilization due to the ready availability of phosphorus and positively influenced by the nitrogen fertilization (Sun et al. 2020, Margalef et al. 2021). Vermicompost contains high acid and alkaline phosphatase content contributed by the microbial populations and earthworm gut secretions. Further, it creates congenial conditions for the microbial population and phosphatase activity (Lv et al. 2020) .

The observed relatively higher AKP activity in the present study (122.98 to 326.16 µg of pNP g^{-1} h^{-1}) to that of ACP activity (81.89 to 225.44 µg of pNP g^{-1} h^{-1}) correlates with previous reports (Lv et al. 2020). Phosphatases are pH-dependent enzymes, and alkaline phosphatases can remain active over a neutral pH range than acid phosphatases. The neutral to alkaline pH range of soil in the present study (pH ~ 7.75) could have resulted in higher alkaline phosphatase activity.

a) b)



♦▪ indicate significant figures by ANOVA single factor (at $p<0.05$)

Fig. 4: Effect of thiamethoxam and chlorantraniliprole on the enzymatic activity of a) acid phosphatase and b) alkaline Phosphatase

4. CONCLUSION:

The present study suggests *B. megaterium* could grow in thiamethoxam or chlorantraniliprole-supplemented cultures in the range of 5-100 mg/L and has the potential for bioremediation. *B. megaterium* with the combined application of amendments could bring a reduction in pesticide concentration with observed residues as 2.16 mg kg-1 (thiamethoxam) and 2.58 mg kg^{-1} (chlorantraniliprole) soils. The biostimulated treatments have accelerated the half-life of both pesticides (11-18 days) compared to natural attenuation (29-35 days). The study also highlights that bio-stimulated treatments displayed increased enzymatic activity for all the enzymes assayed compared to natural attenuated treatments, correlating with the faster removal rates of pesticides. The initial decrease followed by an increase in enzymatic activity that is on par with natural soils for DH, BDG, ACP and AKP in vermicompost-applied treatments (20 g kg^{-1}) indicated a good recovery in the enzymatic activity of soil owing to the increased microbial population over time. However, metagenomic-based studies post the application of pesticides might indicate the changes in soil microbial functional diversity and effects on the microbes involved in nutrient transformation. Studies integrating metabolomic analysis for the identification of metabolized products may help in understanding the metabolic pathways for degradation. Thus, the study brings out the potential of *B. megaterium* for the remediation of thiamethoxam and chlorantraniliprole and can be combined with vermicompost amendments for enhanced and faster degradation rates. Future studies on the evaluation of their field application rates would bring practical applicability to the study for the effective removal of these pesticide contaminants besides improving soil and plant health.

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Conflict of Interest: The Authors declare no conflict of interest.

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