



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, has been exploited as a biofertilizer to increase crop yield. Thiamethoxam and chlorantraniliprole are novel insecticides that are applied as granular and foliar formulations for insect pest control. The present study evaluated the potential of *B. megaterium* for the bioremediation of these novel pesticides in natural and amended soils. The survivability of *B. megaterium* was studied in a 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, vermicompost and Vesicular Arbuscular Mycorrhiza (VAM). The impact of pesticides was evaluated by calculating the enzymatic activities of dehydrogenase, phosphatase, and β-D-glucosidase. The experimental results revealed that *B. megaterium* could survive in pesticide-supplemented conditions, with maximum optical densities of 0.734 AU and 0.965 AU at 100 mg.kg⁻¹ for thiamethoxam and chlorantraniliprole, respectively. Furthermore, these *B. megaterium* cultures also exhibited colony-forming units when plated on nutrient agar supplemented with thiamethoxam (21 × 10⁵) and chlorantraniliprole (43 × 10⁵) at the end of 21 days, indicating their adaptability. The soil application of *B. megaterium* combined with vermicompost or VAM 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 rapid degradation. The observed half-life values in these combined treatments were 11-12 days (thiamethoxam) and 11 and 15 days (chlorantraniliprole), which were on par with each other and significantly differed (two-factor ANOVA, p<0.05) when compared to natural attenuation (29–35 days). The enzymatic activity was negatively impacted for all enzymes under study. However, vermicompost amendments can recover 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 the revival of contaminated soils. Soil microbiota help degrade many xenobiotic compounds and maintain soil fertility and health. Natural attenuation is a natural degradation process of toxic pesticides by native soil microorganisms, including bacteria, fungi, and actinomycetes. They produce various extracellular enzymes that aid in the degradation metabolic pathway. The chemical nature of pesticides varies widely, as do the requirements of various enzymes and microbes to degrade pesticides at various phases. Native microbial communities of actinobacterial species, including *Azotobacter*, *Pseudomonas*, *Bacillus*, and *Klebsiella*, have been employed to degrade a wide range of pesticides to recover the soil (Bokade et al. 2021). *Ochrobactrum thiophenivorans* and *Sphingomonas melonis* degraded 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 was attributed to the participation of the soil native microbiota.

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

Pesticides have diverse effects on the established soil microbial community structure, which participates in various biological processes in the soil (Prashar & Shah 2016). Organochlorine-contaminated soils have been reported to be dominated by certain types of proteobacteria and Mortierella fungi (Egbe et al. 2021). Numerous enzyme exudates from microorganisms play a key role in various biogeochemical processes of chemical elements and nitrogen, phosphorus, sulfur, and carbon cycles (Mahdi et al. 2017). Pesticide-affected soils with altered microbial community structures exhibit reduced enzymatic activity 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, including biological nitrogen fixation, phosphorus and potassium solubilization, and hydrolytic enzymes (Khatoon et al. 2020). In addition to their growth-promoting effects, PGPRs can degrade pesticides. Previous studies have reported 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-solubilizing bacterium 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, and fumaric acids, to enable the mineralization of phosphorus with the subsequent release of OH⁻ ions (Castagno et al. 2021). This leads to ion chelation, affecting the adsorption and desorption of pesticides. In addition, *B. megaterium* has been reported to absorb toxic contaminants in soil through various mechanisms, including biosorption, exopolysaccharide, and siderophore production (Saeed et al. 2021). Nitroreductase genes from *B. megaterium* have been reported to aid in the enzymatic degradation of mesotrol (Carles et al. 2021).

Previous studies on bioremediation have primarily 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 new classes of pesticides. Similarly, the sublethal effects of novel pesticides on beneficial microbes are not well understood. Studies on the prospects of a proven PGPR, such as *B. megaterium*, for pesticide degradation would provide the added advantage of removing pesticide contaminants, in addition to fulfilling the nutrient requirements of plants. In this study, the degradation potential and survivability of *B. megaterium* were evaluated through laboratory studies for 21 days for the removal of supplemented concentrations 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⁻¹) when *B. megaterium* is used along with organic amendments, vermicompost, and Vesicular Arbuscular Mycorrhiza (VAM). The Degradation half-lives were estimated based on their residual concentrations over 21 days in soil samples using HPLC-PDA. Furthermore, the effect of the pesticides on soil dehydrogenase, β-D-glucosidase, acid, and alkaline phosphatase activities was determined to analyze the microbial activity in the natural attenuation, bioaugmentation, and biostimulation treatments.

MATERIALS AND METHODS

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%) and 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 Sulfate (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.

Standard Solutions

Individual stock solutions of thiamethoxam and chlorantraniliprole were prepared in methanol to obtain a pesticide concentration of 10000 mg.L⁻¹. The individual standard solutions were serially diluted in the range of 2.5–100 mg.L⁻¹ in a 25 mL volumetric flask.

Preparation of Pure Cultures

B. megaterium cultures from soil were isolated on Pikovskaya's 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 a phosphate solubilizer. The bacterial cultures were re-inoculated and incubated at 28 ± 2°C for 24 h to obtain a pure culture on the nutrient agar medium.

Acclimatization of *B. megaterium*

The cultures of *B. megaterium* were revived in nutrient broth for 24 h. To acclimatize to the presence of pesticides, the bacteria were cultured in 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 was composed of (NH₄)₂ SO₄ (4.99 g), KH₂PO₄ (1.36 g), Na₂HPO₄ (2.13 g), MgSO₄·7H₂O (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 minimal medium supplemented with thiamethoxam or chlorantraniliprole (5 mg.kg⁻¹), replacing the carbon source (L-tryptophan) to establish whether the bacterium could utilize pesticides as a carbon source. The cultures were incubated for 48 h 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 in 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 replicates along with a control to understand the inhibitory

concentrations and degradation efficiency in nutrient-limited conditions.

Bacterial cultures were sampled on 0 (18 h), 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. This 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 determined. 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.

Preparation of Cultures of *B. megaterium* for Bioremediation Experiments

B. megaterium cultures for bioremediation experiments were prepared from 72-h incubated, pesticide-spiked, half-strength nutrient broth cultures of *B. megaterium*. The cells were harvested by centrifuging at 10,000 rpm at 4°C for 10 min. The biomass was washed twice 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 10⁸ CFU/mL. The soil treatments under microcosm experiments are mixed with this solution at 2 mL.kg⁻¹ of soil (Raimondo et al. 2019)

Experimental Design for Evaluation of Bioremediation Potential of *B. megaterium* in Soil Microcosms

Bioremediation experiments were conducted in rectangular trays measuring 30 × 15 × 5 cm (L × W × H) filled with soil collected at a depth of 5–15 cm from pesticide contamination-free areas and analyzed for physicochemical properties. The collected soil samples were shade-dried and sieved through a 2 mm sieve. The bio-stimulants used for the experiment were vermicompost and VAM, which 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 fortified with thiamethoxam or chlorantraniliprole at 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 replicates 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, amendments, or inoculum served as a negative

control. The experiment was set up under room conditions, and observations were made for 21 days. The soil moisture content was 20–30 % of the water holding capacity, and the room temperature varied between 30–34°C. The soil was mixed daily to ensure uniform distribution and aeration of the microcosms. Soil samples were collected at 0 (18 h), 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 attenuation 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

Quantitative Estimation of Residual Content in Culture Media and Soil Samples

High-performance liquid chromatography (Shimadzu model: Nexara) with a binary pump and photodiode array (PDA) detector was used to quantify the pesticide residues of thiamethoxam and chlorantraniliprole along with a 250 × 4.6 mm, 5 µm particle reverse phase (C₁₈) column. The pesticide residues in the liquid bacterial cultures were determined at 0 (18 h), 3, 7, 14, and 21 d by taking 5 mL of culture into a centrifuge tube. The extract was centrifuged at 10,000 rpm for 10 min at -10°C to separate the biomass and filtered (0.22 µm) to quantify the residues using HPLC-PDA. Residue quantification was performed using linear calibration standards (2.5–100 mg.L⁻¹).

The residual thiamethoxam content in the soil microcosms was estimated at 0 (18 h), 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 mixed with 5 mL of water, followed by 10 mL of extraction solvent, acetonitrile with 1 % acetic acid. The samples were hand shaken vigorously for 5 min and vortexed for approximately 3 min, followed by the addition of extraction salts, that is, 4 g MgSO₄ and 1 g NaCl. The contents were mixed properly for 1 min by hand shaking and centrifuged at 3000 rpm for 3 min. After centrifugation, the supernatant (6 mL) was transferred to a 15 mL centrifuge tube containing 150 mg of Primary Secondary Amine (PSA) and 900 mg of MgSO₄ for clean-up to remove the co-extracts.

The extract was filtered through a 0.22 µm membrane filter into an injection vial to quantify the residues according to the Analytical Conditions (Table 1).

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). The specificity of the method, which is the ability to quantitatively measure the analyte in the presence of components that may be expected to be present in the sample matrix, was evaluated by ensuring that there was no interference at the retention time of the analyte from the 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 the detector responses. The LOD and LOQ of the method were evaluated from this linear calibration curve, and the standard deviation of the y-intercept and slope was determined using regression analysis at a 95% confidence level. LOD was calculated as 3 × (standard deviation of intercept/slope), and LOQ was calculated as 10 × (standard deviation of intercept/slope). Fortification experiments were conducted to determine the accuracy of the method by spiking the control or blank samples with known concentrations of pesticides (n=5). The precision was evaluated by calculating the relative standard deviation (RSD %) between different measurements (n=5) for residue estimation.

Degradation Kinetics

The half-life ($t_{1/2}$) of the pesticides, which is 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 number of days of pesticide application, and C_t is the recovered concentration of pesticides over 21 days in the different treatments. The half-life of the pesticides in the different treatments was determined as $t_{1/2} = \ln(2)/k$.

Determination of Soil Enzymatic Activity

A UV-Vis spectrophotometer (Shimadzu) was used to measure the color intensity of the substrate-enzyme complex formed at different wavelengths during the evaluation

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 [min]
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

of soil enzymatic activity. Dehydrogenase activity was determined as per the method outlined in ISO 23753-1:2005 by estimating reduced triphenyl formazan (TPF) in terms of μg of TPF $\text{g}^{-1} \cdot \text{h}^{-1}$. The activity of β -D-glucosidase, acid, and alkaline phosphatase was evaluated by measuring the absorbance of the yellow-colored extract at 400 nm using a p-nitrophenol standard, and the results were expressed as μg of pNP $\text{g}^{-1} \cdot \text{h}^{-1}$ (Deng & Popova 2015, Eivazi & Tabatabai 1988, Acosta-Martínez & Ali Tabatabai 2015, Eivazi & Tabatabai 1977).

Statistical Analysis

The experiments were performed in triplicate, and the results are expressed as average values. The recovered log concentrations 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. Significant differences among the treatments in the observed half-lives of thiamethoxam and chlorantraniliprole were analyzed using a two-factor ANOVA ($p < 0.05$). The results of enzymatic activity over 21 days were analyzed using One-way ANOVA with a two-sample t-test ($p < 0.05$) to compute significant differences among the treatments.

RESULTS AND DISCUSSION

Survivability and Biodegradability of *B. Megaterium* in Pesticide-Supplemented Liquid Media

The results of the evaluation of the growth of *B. megaterium* in 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 0.734 AU and 0.965 AU at 100 mg.kg^{-1} of thiamethoxam and chlorantraniliprole, respectively, demonstrating the adaptability of the bacterium to both pesticides (Table 2). Growth was more prominent in the treatments supplemented with chlorantraniliprole than with thiamethoxam. This could be attributed to the variation in the molecular structures and degradation products of these pesticides, which could act

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 <i>B. megaterium</i>	
	Thiamethoxam	Chlorantraniliprole
5	0.418 \pm 0.09	0.847 \pm 0.18
10	0.453 \pm 0.08	0.878 \pm 0.12
25	0.456 \pm 0.11	0.877 \pm 0.10
50	0.503 \pm 0.13	0.905 \pm 0.09
100	0.734 \pm 0.21	0.964 \pm 0.17

*Data are the mean of three replicates \pm SD.

as energy sources. Carbon sources taken up by 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. The differential growth rates of *B. megaterium* in the presence of thiamethoxam and chlorantraniliprole as carbon sources could be due to variations in the metabolized products, which are utilized as carbon sources (Wang et al. 2019). At the end of 21 days, the liquid cultures of *B. megaterium* were plated on nutrient broth, and the colony-forming unit (CFU) count was determined (Fig. 1d). *B. megaterium* exhibited colony-forming units 21×10^5 and 43×10^5 in cultures with thiamethoxam and chlorantraniliprole, respectively, correlating with the intensity of optical density in these cultures and confirming its survivability.

Furthermore, 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 the retarding effect of the pesticides compared to the control with no pesticide (Fig. 1a, b, and c).

Method Validation

The method validation results evaluating the suitability of the method to estimate the concentration of pesticides indicated that there was no interference from co-extractives of the sample components at the retention times of the principal

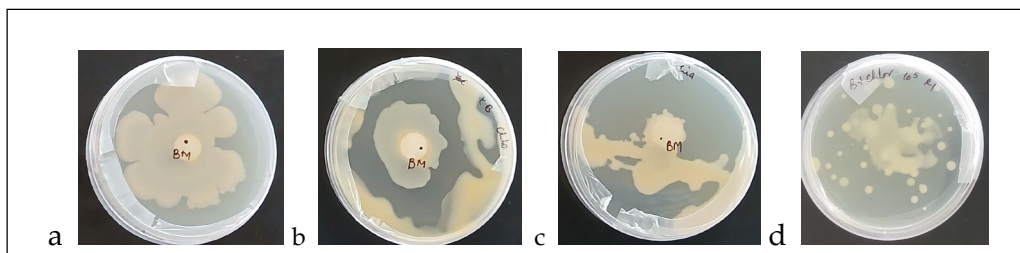


Fig. 1: Relative spread of *B. megaterium* on agar plates with a) no pesticide addition, b) chlorantraniliprole, c) thiamethoxam, and d) CFU of *B. megaterium* on NA.

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 2.5–100 mg.kg⁻¹ using a matrix-matched calibration curve with a coefficient of regression value $R^2 \geq 0.99$, exhibiting good linearity. The LOD and LOQ of the method were determined as 1.0 mg.kg⁻¹ and LOQ as 3.0 mg.kg⁻¹, respectively 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, respectively. 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 1.03–6.04 %. The results were found to be satisfactory and were as per the SANTE guidelines for specified limits of mean recovery in the range of 70-120% and less than $\pm 30\%$ RSD between measurements.

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 below the detection limit 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, is transformed to fumarate, and enters the Krebs' cycle to yield energy (Pang et al. 2020). Similarly, amide hydrolysis by amidases has been reported to cause the degradation of chlorantraniliprole (Gao et al. 2019). Thus, it can be inferred that the

amidases produced by *B. megaterium* may have aided in chlorantraniliprole degradation in the supplemented cultures. The higher growth rate (0.847-0.964 AU) and CFU (43×10^5) 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. Although the observed OD and CFU were lower in the thiamethoxam-supplemented cultures (0.418-0.734 AU, 21×10^5), 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 was no significant difference between chlorantraniliprole and thiamethoxam treatments at higher concentrations (50-100 mg.L⁻¹), exhibiting a percentage reduction that was 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).

Degradation and Half-Life Estimation for Thiamethoxam and Chlorantraniliprole in Soil Microcosms

The degradation rates of thiamethoxam and chlorantraniliprole were studied under natural, bioaugmented, and biostimulated conditions using soil microcosm experiments in the laboratory. The experimental trays were filled with silt-clay loam textured soil with a pH of 7.75. The kinetics of degradation of thiamethoxam and chlorantraniliprole were evaluated by quantifying their residual concentrations in soil samples at 0 (18 h), 3, 7, 14, and 21 d using 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 2.5–100 mg L⁻¹. The dissipation patterns of thiamethoxam and chlorantraniliprole were fitted to a first-order kinetic equation (Table 4), and half-lives were determined by $t_{1/2} = \ln(2)/k$, where the half-

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 [mg.L ⁻¹]	Per cent reduction in conc.	Residual Conc. at 21 days ¹ [mg.L ⁻¹]	Per cent reduction in conc.
T1	5	ND \pm 0.53	~ 100	3.05 \pm 0.13	39.00
T2	10	ND \pm 0.65	~ 100	5.70 \pm 0.46	43.00
T3	25	9.62 \pm 0.54	61.52	14.55 \pm 0.83	41.80
T4	50	20.48 \pm 0.10	59.04	13.59 \pm 1.08	72.82
T5	100	32.98 \pm 3.50	67.02	19.75 \pm 3.64	80.25
T6	10*	6.93 \pm 0.03	30.7	7.52 \pm 0.18	24.80

¹ Data are the mean of three replicates, each \pm SE of the regression.* Uninoculated control supplemented with pesticide at 10 mg/L

life is independent of the initial concentration of pesticides. The treatments amended with vermicompost combined with *B. megaterium* has exhibited shorter half-lives for both pesticides (11-18 days) compared to natural attenuation (29-35 days). The pesticide degradation rate is dependent on various factors, such as soil pH, organic matter content, clay content, pesticide characteristics, temperature, and moisture content in the soil. The faster degradation rates of pesticides under amended conditions than under natural attenuation conditions may be attributed to the high nutrient availability and carbon content, which could have aided in the establishment of augmented microorganisms. Furthermore, 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 phosphorus mineralization with subsequent release of OH⁻ ions may 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. The release of organic acids by *B. megaterium*, leading to the acidification of the rhizosphere, may have affected the desorption of chlorantraniliprole, causing rapid degradation in augmented and biostimulated soils. Furthermore, the release of hydroxyl groups from organic acids may have caused the metabolic transformation of thiamethoxam to its hydroxyl and urea derivatives (Xiang et al. 2024). The production of enzymes such as nitroreductases by *B. megaterium* may have also contributed to the rapid

degradation of thiamethoxam. Vermicompost with a high nutrient content and enriched microbial diversity (Raza et al. 2022) may 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, in addition to improving the quality.

Evaluation of Soil Enzymatic Activity in Microcosms

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

It was also observed that immediately after the application of thiamethoxam and chlorantraniliprole, there was a reduction in enzymatic activity, which improved over time. This could be attributed to the immediate impact of pesticides on bacterial communities after application. The vermicompost amendment treatments had enzymatic activity comparable to that of the untreated controls, indicating that these treatments could recover microbial biomass over time. Vermicompost is enriched with various macro-and micronutrients that are readily available to soil microorganisms. These nutrient-enriched conditions might have stimulated the native and pesticide-degrading microbial populations over time, resulting in their recoument. These results are identical to those of Romero et al. (2024), who reported enhanced enzymatic activity in vermicompost treatments in diuron-applied soils. Furthermore, the same

Table 4: Degradation of thiamethoxam and chlorantraniliprole in Soil microcosms.

Treatment	Thiamethoxam			Chlorantraniliprole		
	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
<i>B. megaterium</i>	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
<i>B. megaterium</i> + 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
<i>B. megaterium</i> + 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.

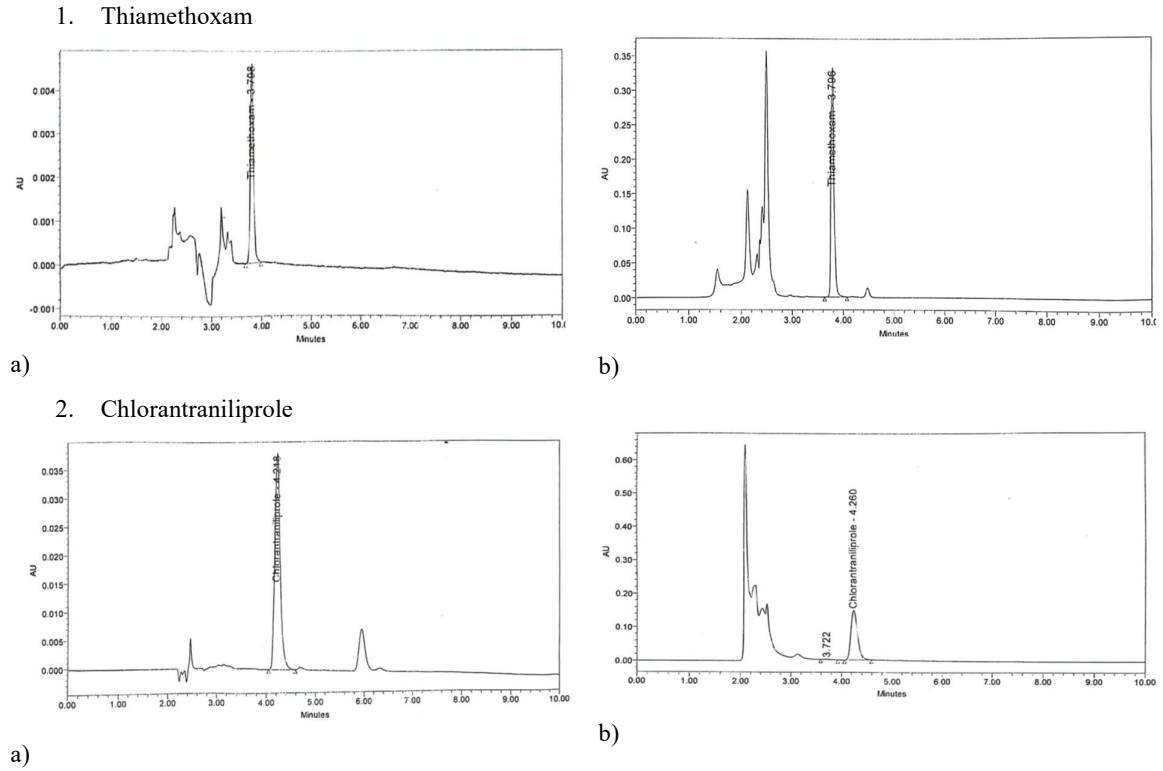
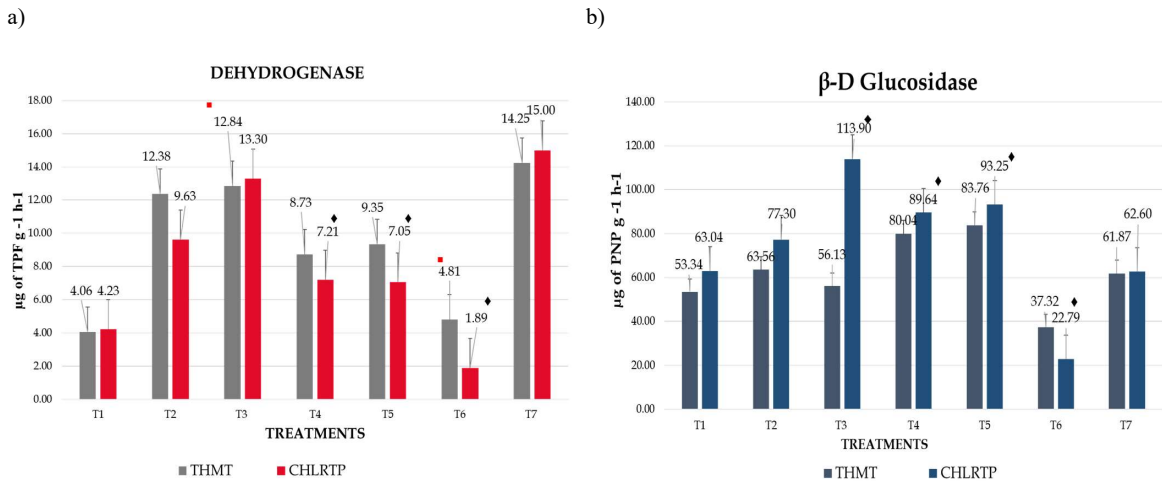


Fig. 2: Chromatogram depicting the peaks of 1) thiamethoxam and 2) chlorantraniliprole in a) matrix match standard solution, b) sample extracts.



• 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.

study reported variable enzymatic activity over 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 and 12.84 and 4.23 to 13.30 $\mu\text{g TPF g}^{-1}\text{h}^{-1}$ for thiamethoxam and chlorantraniliprole, respectively (Fig. 3a). There was a significant difference in

the dehydrogenase activity values in the treated microcosms compared to the 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 the negative effect of thiamethoxam on soil microcosms. DH activity was

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 and 83.76 for thiamethoxam and 22.79 to 113.90 μg of pNP $\text{g}^{-1}\text{h}^{-1}$ for chlorantraniliprole (Fig. 3b). Their activity was significantly higher in the combined treatments of *B. megaterium* with vermicompost or VAM. These high BDG activity levels can be attributed to the high organic carbon content, which was readily available in the amended treatments. Significantly lower ($p < 0.05$) BDG activity was observed in soils treated with chlorantraniliprole under natural attenuation conditions compared to amended soils.

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

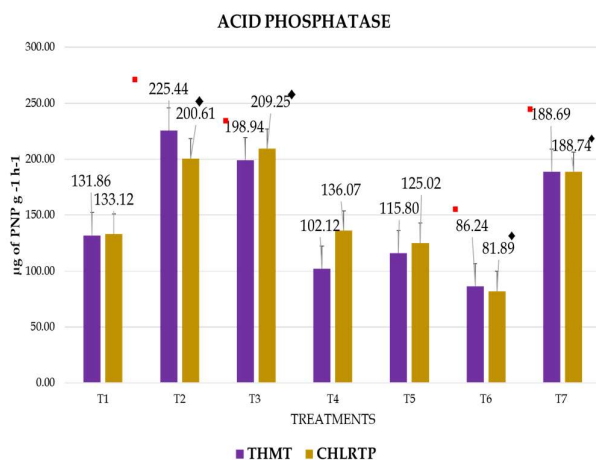
The relatively higher AKP activity observed in the present study (122.98 to 326.16 μg of pNP $\text{g}^{-1}\text{h}^{-1}$) compared to ACP activity (81.89 to 225.44 μg of pNP $\text{g}^{-1}\text{h}^{-1}$) correlates with previous reports (Lv et al. 2020).

Phosphatases are pH-dependent enzymes, and alkaline phosphatases remain active over a wider 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.

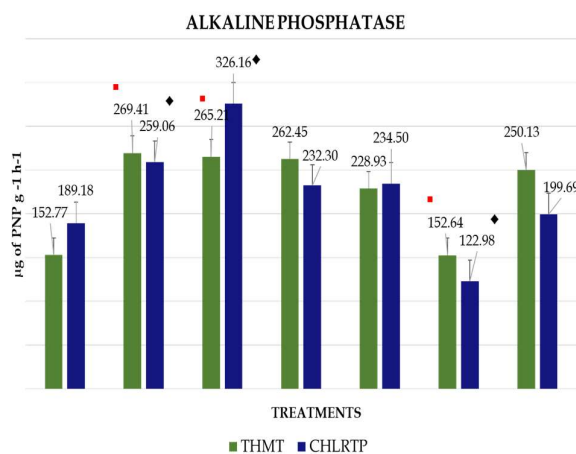
CONCLUSIONS

The present study suggests that *B. megaterium* can grow in thiamethoxam or chlorantraniliprole-supplemented cultures in the range of 5-100 $\text{mg}\cdot\text{L}^{-1}$ 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 $\text{mg}\cdot\text{kg}^{-1}$ (thiamethoxam) and 2.58 $\text{mg}\cdot\text{kg}^{-1}$ (chlorantraniliprole) in soils. The biostimulated treatments accelerated the half-life of both pesticides (11-18 days) compared to natural attenuation (29-35 days). The study also highlighted 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 $\text{g}\cdot\text{kg}^{-1}$), indicated a good recovery in the enzymatic activity of soil owing to the increased microbial population over time. However, metagenomic-based studies after the application of pesticides might indicate 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 of degradation.

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.

Thus, this study highlights the potential of *B. megaterium* for the remediation of thiamethoxam and chlorantraniliprole, which 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, in addition to improving soil and plant health.

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