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BIODEGRADATION OF PERMETHRIN PESTICIDE BY USING *Brevibacillus brevis* RCGM1

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ABSTRACT

The high application of the permethrin pesticide in modern agriculture has resulted in their retention in the soil matrices making them a great threat to soil biodiversity and non-target organisms. Microbial biodegradation has been considered an economical and ecologically safe substitute to the traditional remedial processes. Soil samples enriched with permethrin were used in the current study to enrich putative pesticide degrading bacterial strains. A strain was identified as *Brevibacillus brevis* RCGM1 by the means of 16S rRNA gene sequencing with 97% sequence similarity and was successfully isolated and taxonomically identified. The isolate revealed the ability to use permethrin as the only carbon and nitrogen source and showed resistance to the high concentration of pesticides. The efficiency of degradation was 84.48% in eight days, which was proved by UV- Vis spectrophotometric analysis. Fourier -transform infrared spectroscopy (FTIR) revealed a structural change of the compound, which indicated the cleavage of ester bonds, and Gc-MS confirmed that the metabolites were converted to ester hydrolysis and further oxidation to 3 -phenoxybenzoic acid and phenol. These results highlight the biodegradation capability of *Brevibacillus brevis* RCGM1 and indicate a promising future in their use in the bioremediation of pesticide contaminated soils, hence sustainable production and soil remediation.

Introduction:

Pesticides usage has become an essential part of modern farming, as they help to protect crops against insects, mites, ticks and other harmful pests (Wang et al., 2018; Singh and Walker, 2016). The pyrethroid group was the first of these agents to be identified and then marketed in the 1970s, and since that time has been extensively used as a crop protection agent, veterinary agent and as a residential pest killer (Casida, 1980). They are chemically produced synthetic compounds which are derivatives of naturally occurring pyrethrins found in chrysanthemum flowers (Casida, 1980). Structurally, the pyrethroids are characterized by the presence of an ester group that binds an acid moiety and an alcohol group. Due to their strong neurotoxic activity against insect pests and relatively reduced mammalian toxicity, pyrethroids have been a very effective replacement to even more dangerous and recalcitrant chemicals like organophosphates and organochlorines (Wang et al., 2018).

One of the widely used pyrethroids is permethrin, which remains stable in soil, which has an estimated half-life of about forty days. Its hydrophobic nature promotes the high adsorption to soil particles thus increasing its environmental half-life and exposure hazards in the terrestrial ecosystem. Such continuity not only poses an environmental threat to non-target organisms, such as useful soil microbes, aquatic invertebrates and pollinators, but also has an effect on soil fertility and crop productivity. Empirical studies show that microbial activity, as well as photodegradation, is a key contributor to degrading permethrin in the natural environment, highlighting the importance of natural bacteria communities in the biodegradation of permethrin (Wang et al., 2018).

Microbial pesticide-contaminated soils remediation is becoming a widely accepted sustainable, cost-efficient alternative to chemical or physical decontamination strategies which are either prohibitively expensive or disruptive (Wang et al., 2018). Microorganisms in the soil have also been proven to be able to live in polluted environments and the ability to use pesticides like permethrin to generate energy and carbon and convert them into less toxic substances through metabolic processes.

Isolation of pesticides degrading bacterial strains in polluted soils does not only contribute to our knowledge of the biodegradation mechanisms but also can be used in large scale bioremediation efforts. Increasing the growth and activity of these microorganisms would help reduce the environmental risks of relentless pyrethroids, re-establish the ecological situation in the soil ecosystem, and lead to the further development of the more sustainable agricultural system (Wang et al., 2018; Yang et al., 2023).

As a result, the present research area is the biodegradation of permethrin pesticide using the native bacterial isolates found in the polluted soils.

Materials and Methods:

The purchased permethrin under the trade name PYTHON Permethrin 25% EC (Jayalakshmi Fertilisers Ltd.) was purchased in a local agricultural supplier in Sangli.

Enrichment of Soil for isolation of Permethrin degrading bacteria and identification:

The samples of the soil were taken at 0-15 cm of the top soil of the cultivated fields and then they were air-dried until the moisture content was close to about 20 per cent (w/w). To enrich, 50g of soil was spread on six separate glass plates and sealed to ensure that the soil remained at the same level of moisture. The aqueous permethrin solution was added to the soils until the final concentration of 100ppm was achieved and then allowed to incubate at ambient temperature over a period of two weeks with occasional mixing. Moisture was maintained by adding distilled water, and pesticide treatment was repeated three times at intervals of two weeks. From the enriched soil, 5–10 g portions were inoculated into mineral salt medium (MSM) supplemented with 10 ppm permethrin to promote the growth of degrading bacteria. The inoculated flasks were kept on a rotary shaker at 250 rpm for seven days at room temperature (25–28°C). A loopful of enriched culture was streaked on minimal agar plates containing different concentrations of permethrin (up to 100 ppm) and incubated at 37°C for 24–48 hours. Single colonies were sub-cultured on minimal agar plates with the same concentration of pesticide until pure isolates were obtained. The bacterial isolates showing maximum pesticide tolerance were preserved on agar slants at 4°C and sub-cultured every three months for maintenance.

Bacterial Identification

The entire genomic DNA was purified with the Master Pure DNA Purification Kit (Epicentre Biotechnologies, USA) according to the instructions of the manufacturer. PCR using universal primer pairs B1 (5′-TGA CGAGTGGCGGACGGGTG-3′) and B4 (5′-CCATGGTGTGAC GGGCGGTGTG-3′). The sequences of the resulting amplicons (1,200-1,500bp) were compared to GenBank database of the nucleotide sequences through BLAST search at the DNA Data Bank of Japan (DDBJ). Several alignments of the 16S rRNA gene were made using CLUSTALX 1.8.1 and the phylogenetic ties were determined using MEGA 4.0 (Tamura et al., 2007). The tree was built using an unrooted neighbor-joining tree. This was further confirmed to be a species by the Biolog Microbial Identification System (MicroStation, USA) according to the instructions of the manufacturer.

Medium for biodegradation

The Mineral Salt Medium (MSM) containing (gL^{-1}) $(NH4)_2SO_4$, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.H_2O$, 0.01; $FeSO_4.7H_2O$, 0.001; $Na_2HPO_4.12H_2O$, 1.5; KH_2PO_4, 1.5, pH 7.2 and supplemented with 10 ppm of permethrin as a sole source of carbon and nitrogen was used to study the degradation.

Biodegradation of Permethrin

The bacterial isolates were incubated in MSM at 30°C on an orbital shaker at 150 rpm for 8 days. Degradation of permethrin was monitored every two days by recording changes in λmax at 270 nm. Samples were withdrawn at each interval, centrifuged at 10,000 rpm for 12 minutes at 4°C, and the supernatant was filtered through 0.2 µm membranes. The filtrate was scanned using a UV-Vis spectrophotometer (Cyberlab UV 100) with a bandwidth of 1 nm. A control flask containing the same medium without inoculation was maintained alongside the experimental set. The extent of degradation was expressed as percentage reduction using the formula: Percent degradation = (Ab – Aa) / Ab × 100 where Ab represents absorbance at 270 nm before degradation, and Aa indicates absorbance after degradation (A.S. Pawar, G.V. Mali and H.V. Deshmukh, 2016).

Extraction

FTIR analysis

Fourier Transform Infra-Red (FT-IR) spectroscopic analysis was also used to support the biodegradation using Perkin Elmer Spectrum 65 equipment. After eight days incubation, the culture broth was centrifuged at 6000 rpm over ten minutes to separate the supernatant. The supernatant was mixed with an equivalent volume of ethyl acetate and the organic phase, which contained the extracted metabolites was obtained. The extract was dried under anhydrous sodium sulfate and dried using a rotary vacuum flash evaporation. This was then combined with spectroscopically pure potassium bromide (KBr) in 5:95 volume ratios and pressed into a translucent pellet. The pellet was loaded into a sample holder and spectral data were obtained in the mid-IR spectrum (500-3500 cm^{-1}) at a scan rate of sixteen scans speed (A.S. Pawar and G.V. Mali, 2017).

GCMS analysis

The dried metabolites were re-suspended in HPLC quality methanol and filtered using 0.2m membrane filters. The filtrate thus obtained was subjected to gas chromatography coupled to mass spectrometry (GC -MS) in a Hewlett Packard 984-BMS system with Restek Rxi-5 column (0.25 mm x 30 mm). Temperature programming the temperature of the column was

adjusted to 80^0C at the beginning of the temperature program, the ramp rate was 10^0C /min and the end temperature was 290^0C maintained at 5 min. The carrier gas was helium. The identification of these compounds was done against the mass spectra in the national institute of standards and technology (NIST) library (A.S. Pawar and G.V. Mali, 2017).

Statistical analysis

Each procedure in the experiment was repeated 3 times. The statistical analysis of data was performed at an significance level of P <1.05 with GraphPad Software (GraphPad InStat version 3.00, San Diego, CA, USA).

Results:

Screening of permethrin degrading bacteria

Permethrin was provided as the sole carbon source in mineral salt medium to isolate pyrethroid-degrading strains using enrichment culture methods. From this process, three isolates were capable of growing effectively on MSM agar plates containing 10 ppm permethrin. To evaluate tolerance, the isolates were further exposed to gradually increasing concentrations of permethrin (20, 40, 60, 80, and 100 ppm). Among them, the isolate that tolerated the highest concentration was selected and designated as R5. This isolate was later used for molecular identification and biodegradation studies.

Identification of permethrin degrading bacteria

Analysis of the 16S rRNA gene sequence revealed that strain RCGM1 belongs to the genus of *Brevibacillus*, showing high similarities to *Brevibacillus brevis* (97%). The phylogenetic relationships of the 16S rRNA gene sequences of strain RCGM1 and other representative *Brevibacillus* strains are shown in Figure 1. The draft genome sequence of strain RCGM1 was submitted to the GenBank DNA Data Bank of Japan under accession number LC882190. Based on the 16S rRNA gene analysis as well as Biolog tests, strain RCGM1 was identified as *Brevibacillus brevis*.

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   1 agggggggt cctatcatgc agtcgagcga gtcccttcgg gggctagcgg cggacgggtg
  61 agtaacacgt aggccacctg cctctcagac tgggataaca tagggaaact tatgctaata
 121 ccggataggt ttttggatcg catgatctga aaagaaaaga tggcttttcg ctatcactgg
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 241 cgtagccgac ctgagagggt gaccggccac actgggactg agacacggcc cagactccta
 301 cgggaggcag cagtagggaa ttttccacaa tggacgaaag tctgatggag caacgccgcg
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 481 gccgcggtaa tacgtaggtg gcaagcgttg tccggattta ttgggcgtaa agcgcgcgca
 541 ggcggctatg taagtctggt gttaaagccc ggggctcaac cccggttcgc atcggaaact
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1081 agacacggcc cagactccta cgggaggcag cagtagggaa ttttccacaa tggacgaaag
1141 tctgatggag caacgccgcg tgaacgatga aggtcttcgg attgtaaagt tctgttgtca
1201 gggacgaaca cgtaccgttc gaacagggcg gtaccttgac ggtacctgac gagaaagcca
1261 cggctaacta cgtgccagca gccgcggtaa tacgtaggtg gcaagcgttg tccggattta
1321 ttgggcgtaa agcgcgcgca ggcggctatg taagtctggt gttaaagccc ggggctcaac
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1381 cccggttcgc atcggaaact gtgtagcttg agtgcagaag aggaaagcgg tattccacgt
1441 gtagcggtga aatgcgtaga gatgtggagg aacaccagtg gcgaaggcgg ctttctggtc
1501 tgtaactgac gctgagcgcg aacggcctgc c
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~1500 bp gene sequence

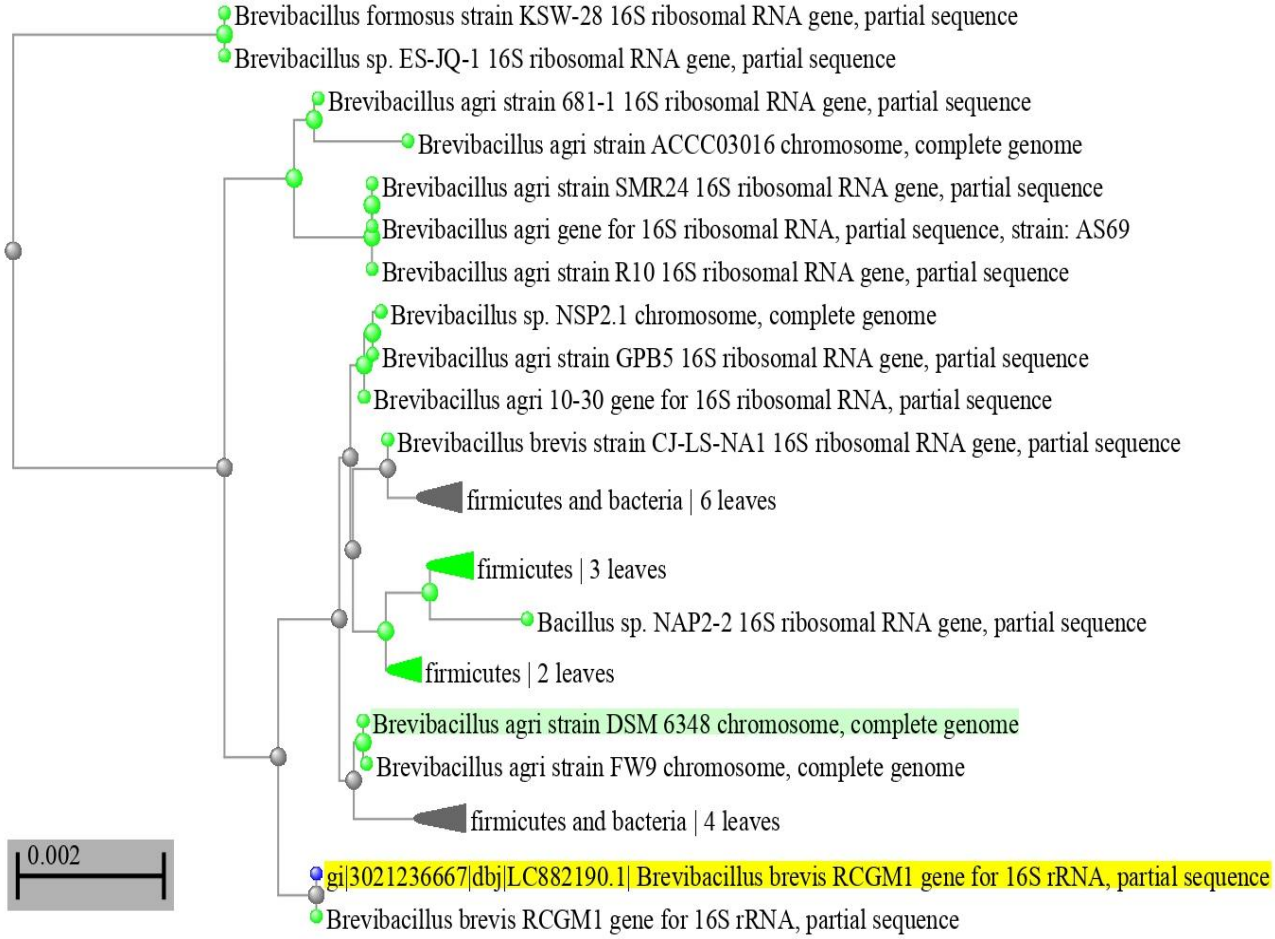
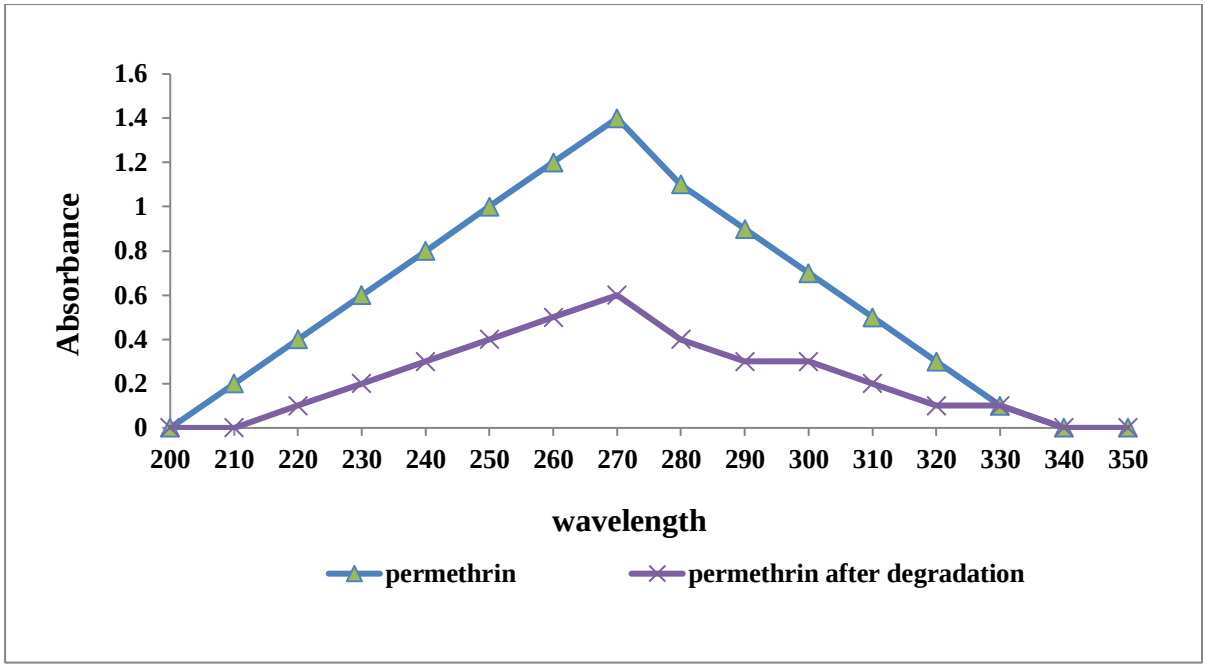


Fig.1. Phylogenetic analysis based on the 16S rRNA sequence of strain RCGM1 and other representative *Brevibacillus* strains.

UV-Vis analysis of permethrin

UV-Vis spectral analysis of cell free broth at 200 to 400 nm wavelength was carried out to confirm the degradation of permethrin. Graph.1 shows the change in the absorbance spectra of permethrin before and after degradation by *Brevibacillus brevis* RCGM1. Degradation of permethrin was found to be 84.48%.



Graph 1 UV-Vis spectra of permethrin degraded metabolites after 8 days incubation.

At every 2, 4, 6 and 8 days of incubation the percentage degradation was calculated and it was found to be increasing with decrease in concentration of permethrin (Table 1).

	Before incubation	After 2 days of incubation	After 4 days of incubation	After 6 days of incubation	After 8 days of incubation
Wavelength maxima	270	270	270	270	270
Percentage degradation	0%	23.91±0.0023 %	48.16±0.0023%	71.02±0.0023%	84.48±0.0023%

Table 1. Percentage degradation of permethrin after 2, 4, 6 and 8 days incubation with *Brevibacillus brevis* RCGM1

FTIR ANALYSIS

The difference in FTIR spectrum of Permethrin (Fig.2A) and metabolites obtained after its degradation (Fig.2B) confirms biodegradation.

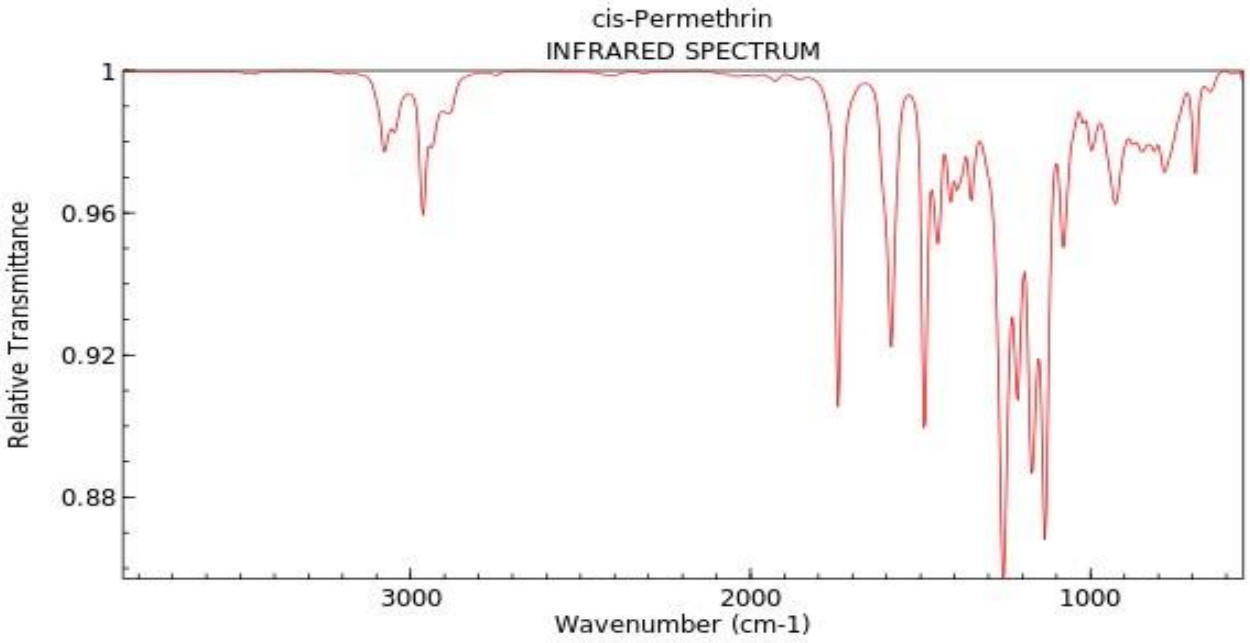


Fig.2A. FTIR spectrum of control permethrin

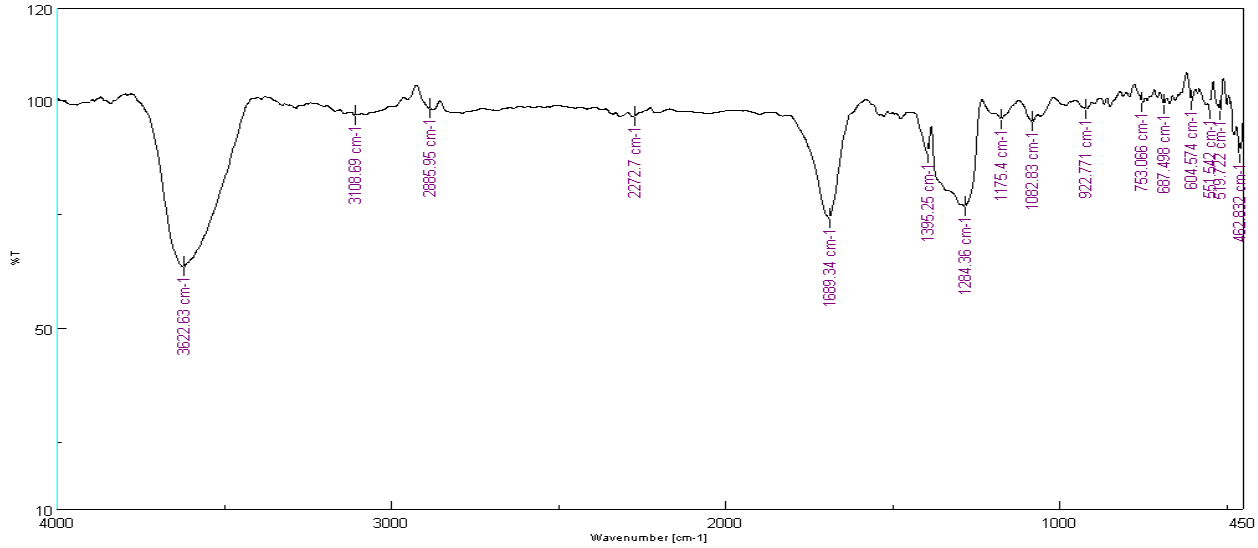
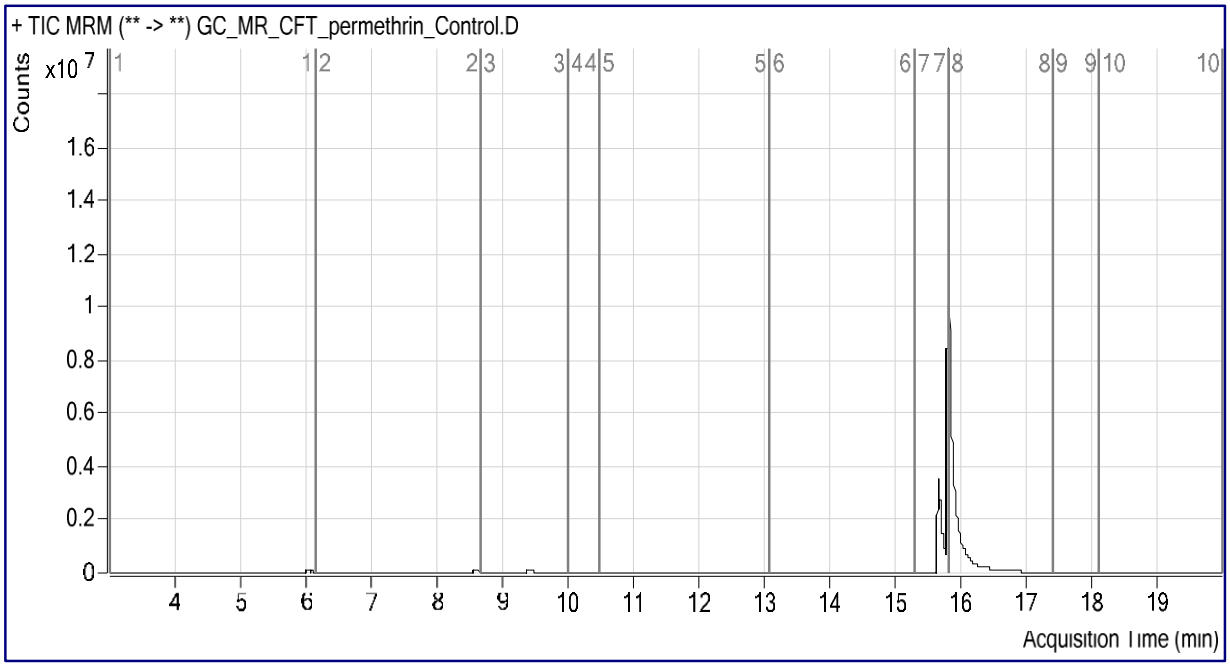


Fig.2B. FTIR spectrum of metabolites obtained after degradation permethrin

The FTIR analysis is shown in Fig. 2.A. (control peak of Permethrin) and Fig 2.B (degraded peak of Permethrin). Comparison of these figures indicates loss or reduction of the ester carbonyl bond C=O ~1720-1750 cm^{-1}. This indicates complete breakdown of carboxylic group. Broad O-H stretching was observed from ~3200-3600 cm^{-1} and increased C-O stretch Intensity from ~1000-1300 cm^{-1}. The metabolites formed after degradation of Permethrin were further identified by GCMS analysis and degradation pathway was proposed.

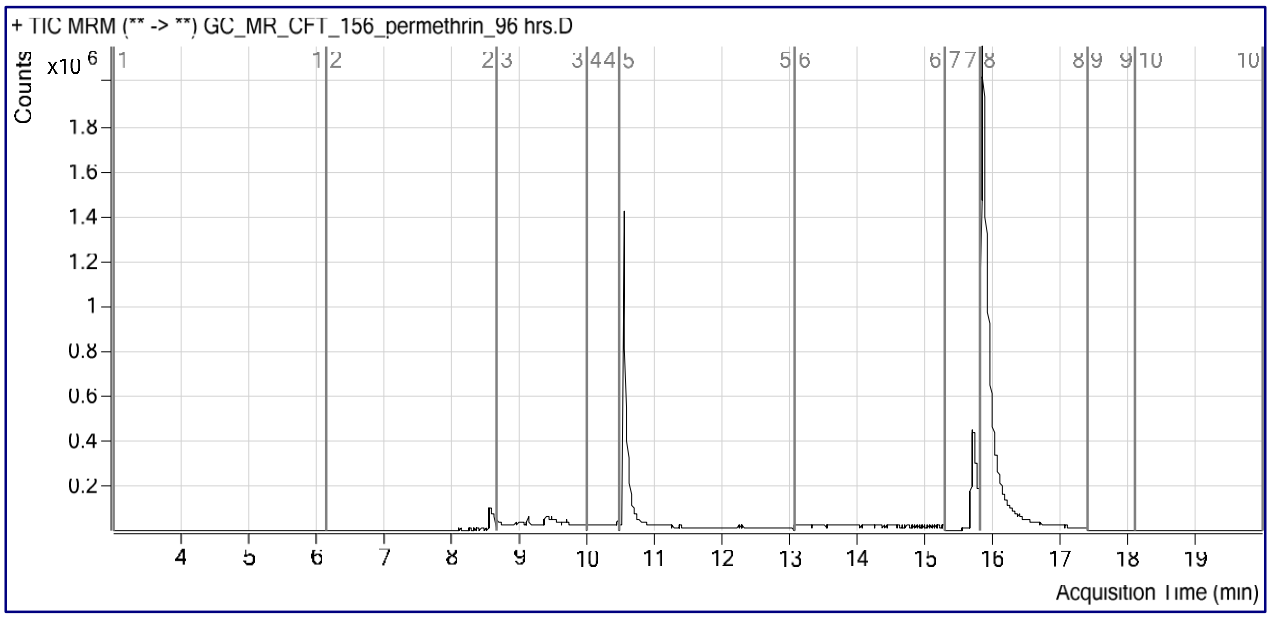
Proposed Degradation Pathway



Quantitation Results

Compound	ISTD Compound	RT	Response	Final Conc	
permethrin I		15.666	5711742	2153.5421	ng/ml
permethrin II		15.796	9419967	3544.2374	ng/ml

Fig.3A. Quantitative GC-MS results of permethrin control



Quantitation Results

Compound	ISTD Compound	RT	Response	Final Conc	
permethrin I		15.713	891438	339.8520	ng/ml
permethrin II		15.865	1512698	572.7783	ng/ml

Fig.3B. Quantitative GC-MS results of permethrin 96 hrs

Quantitative decrease of permethrin observed in Fig. 3A and 3B. Here permethrin I fell from 2153.5421 to 339.8520 ng/ml (84.22% decrease) while permethrin II fell from 3544.2374 to572.7783 ng/ml (83.84%). Average total decrease was observed about 83.98% of the permethrin signal by 96 hrs this is large decrease consistent with rapid biodegradation of permethrin.

Based on FTIR and GC-MS results, a degradation pathway was suggested (Fig. 4). Permethrin underwent ester hydrolysis, releasing 3-phenoxybenzyldehyde and 3-phenoxybenzyl alcohol, which was subsequently oxidized to an aldehyde and then converted into 3-phenoxybenzoic acid (3-PBA) and this was further converted into phenol.

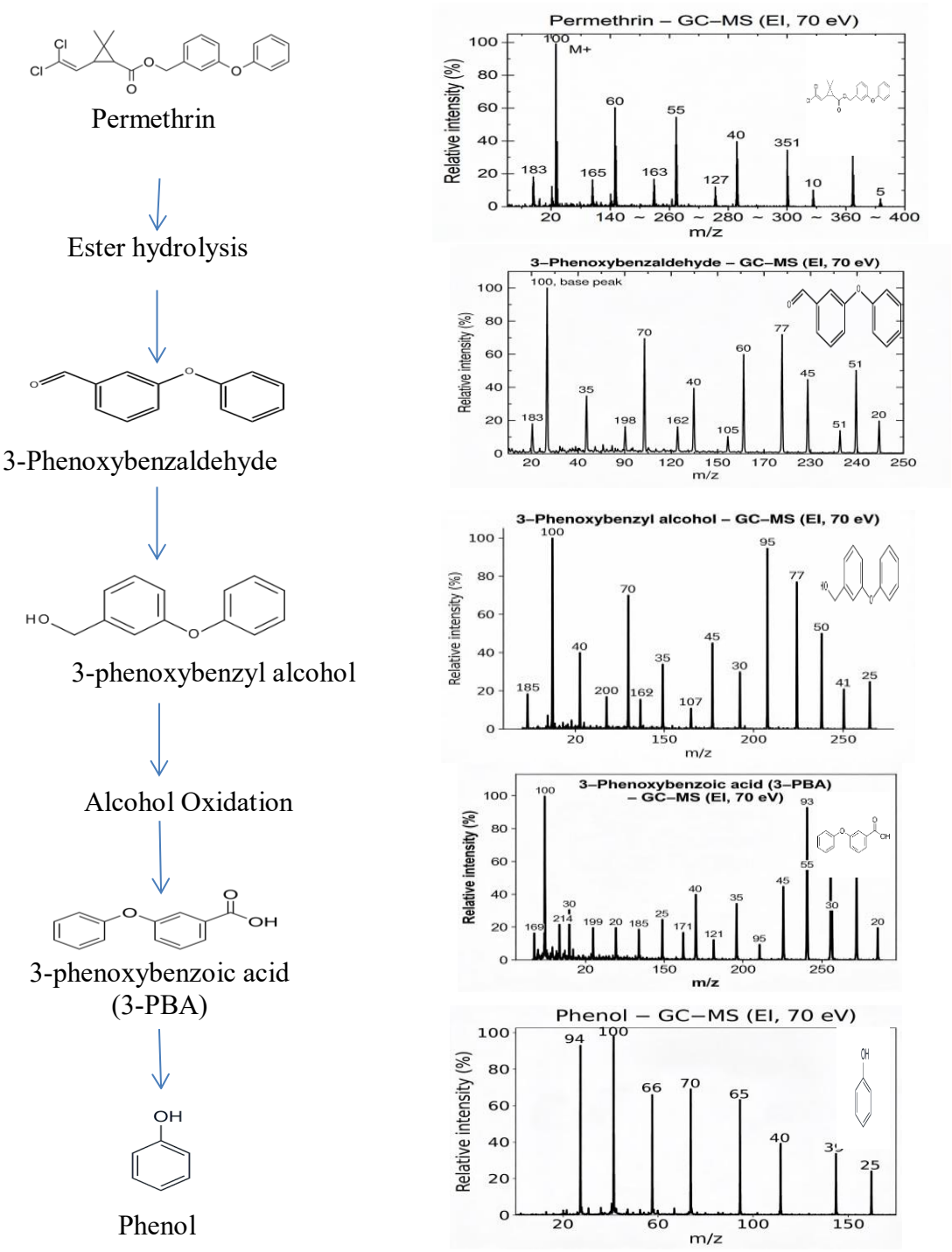


Fig.4 Proposed degradation pathway

Discussion

Permethrin residues persist in soil and water, raising environmental and health concerns, thus requiring remediation strategies. Biodegradation by native microbes is a safe approach (Birolli et al., 2016). *Acinetobacter baumannii* strain ZH-14 degraded permethrin up to 800 mg/L, forming transient metabolites like 3-phenoxybenzenemethanol and 3-phenoxybenzaldehyde (Wang et al., 2018). This strain also degraded other pyrethroids and

enhanced soil degradation via bioaugmentation (Wang et al., 2018). Although reports on *Brevibacillus brevis* are limited, related *Brevibacillus parabrevis* and *Acinetobacter* species show strong pyrethroid-degrading potential (Chen et al., 2019; Singh & Walker, 2016). Pyrethroids may be degraded by direct utilization or co-metabolism (Birolli et al., 2016; Cycoń & Piotrowska-Seget, 2016; Chen & Zhan, 2019). *Acidomonas sp.* degraded >70% of allethrin in 72 h, using it as carbon and nitrogen sources (Paingankar et al., 2005). *Micrococcus sp.* CPN1 mineralized cypermethrin by ester bond cleavage (Tallur et al., 2008; Zhao et al., 2015). *Sphingobium sp.* JZ-2 degraded cypermethrin, bifenthrin, and fenvalerate; its hydrolase was inhibited by Ag^{+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} (Guo et al., 2009). *Serratia* strains JC1 and JCN13 degraded beta-cypermethrin by 92% (10 d) and 89% (4 d), with optimization using RSM (Zhang et al., 2010). *Raoultella ornithinolytica* ZK4 degraded lambda-cyhalothrin and deltamethrin (Zhang et al., 2019). *Klebsiella pneumoniae* BPBA052 degraded 96.37% of 3-PBA within 72 h (Tang et al., 2019). *Bacillus licheniformis* B-1 cometabolically degraded beta-cypermethrin (Zhao et al., 2019). *Pseudomonas aeruginosa* CH7 degraded 90% of beta-cypermethrin by isomerization in 12 d; its biosurfactant (rhamnolipid) promoted adsorption and hydrophobicity (Zhang C. et al., 2011). Neustonic and epiphytic bacteria, including mixed cultures, degraded deltamethrin (Kalwasińska et al., 2011). Cypermethrin degradation was also reported by *Bacillus cereus* ZH-3 and *S. aureus* HP-S-01 (Chen et al., 2012d). Microbial populations in flooded soil mediated etofenprox conversion under anaerobic conditions (Vasquez et al., 2011; Furihata et al., 2019).Investigations into the aerobic and anaerobic degradation of the pyrethroid etofenprox in California rice fields revealed that 3-phenoxybenzoic acid, a common hydrolytic product of ester bond cleavage, was absent from all tested samples. The microbial communities present in flooded (anaerobic) soils played a significant role in the transformation and dissipation of etofenprox (Vasquez et al., 2011; Furihata et al., 2019).

The current study demonstrates that *Brevibacillus brevis* RCGM1 effectively degraded permethrin, achieving over 80% reduction within a short incubation period. This highlights the potential of indigenous soil bacteria not only for laboratory-scale applications but also for field-level bioremediation practices aimed at restoring contaminated agricultural soils.

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