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Journal of Biological Research and Biotechnology
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Biodegradation of Cypermethrin and its metabolic products by *Bacillus flexus* strain PC5 isolated from cypermethrin-treated agricultural soil

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ABSTRACT

Cypermethrin (CPM) are frequently used in agricultural farmlands to control pest infestation in crops. However, its toxicity towards human and aquatic organisms requires its complete degradation in the environment. In this study, agricultural soil with extensive history of CPM application was used to prepare enrichment cultures using CPM as sole carbon source for isolation of CPM degraders. GC-MS method was used to detect the CPM degradation metabolites in the culture medium. Strain PC5 was found highly efficient in degradation of CPM and its metabolic products and was identified as *Bacillus flexus* (GenBank accession number LC218817) using 16S rRNA gene sequences. Strain PC5 degraded 75.74 % of CPM (100 mg L⁻¹) within 6 days of incubation in CPM minimal salts medium (MSM) with utilization rate of 0.532 mg L⁻¹h⁻¹. *Bacillus flexus* strain PC5 had specific growth rate of 0.04 h⁻¹ during exponential growth phase in MSM. GC-MS analysis of the culture extract showed presence of 3-Phenoxybenzaldehyde, 3-Phenoxybenzoic acid, 3-Phenoxyphenol and phenol as major metabolites. The optimal pH for CPM degradation by strain PC5 was 7.0, with degradation percentage of 75.74 % within 6 days. Degradation of CPM at different concentrations (150 mg L⁻¹ and 200 mg L⁻¹) by strain PC5 was 64.76 % and 34.13 % respectively after 6 days of growth. Decrease in CPM concentration (89.10 % degradation) over a period of 30 days was recorded in soil microcosms using strain PC5. Based on the metabolites detected in the culture extract, CPM degradation pathway was proposed for strain PC5. The efficient biodegradation potential and utilization of major metabolites of CPM suggests that strain PC5 can be a valuable strain for the development of bioremediation strategies for CPM polluted ecosystem.

Keywords: Cypermethrin; Biodegradation; *Bacillus flexus*; Bioremediation; GC-MS; Soil microcosm

Received: 30th December 2025 / Accepted: 11th April 2026 / **Published online:**30th April 2026

© The Author(s), under exclusive license to Bio-Research-Journal-of-Biological-Research-and-Biotechnology (JBRB), ISSN: 2705-3822; print-ISSN: 1596-7409 (Online); Open Access This article is available at <https://bio-research.com.ng/index.php/home>, 2026.

DOI: <https://dx.doi.org/10.4314/br.v24i1.10>

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INTRODUCTION

Cypermethrin (CPM) is a synthetic pyrethroid insecticide widely used in agriculture to control pest insects in vegetable crops, fruit, paddy, cereal, cotton, ornamental plants and treatment of cattle diseases. In the environment, including households, it is used to control ants, fleas, termites and cockroaches (Indratin et al., 2019; Zhan, et al., 2020). CPM belongs to the fourth generation of synthetic pyrethroids and constitutes a lipophilic ester, with an α -cyano-3-phenoxybenzyl alcohol and a dichlorovinylcyclopropane-carboxylic acid moiety (Diegelmann et al., 2015). During the last four decades, CPM has been used extensively as a replacement for the more toxic and environmentally persistent organochlorine, carbamate, and organophosphate compounds (Shafer et al., 2005; Indratin et al., 2019). The rate of CPM usage in agriculture will continue to increase due to high demand of agricultural produce to meet the food consumption of growing population. Application of CPM in agricultural farmlands by Nigerian farmers will continue to increase due to its effectiveness in pest control and this will lead to more contamination of the soil and aquatic ecosystem. CPM interacts with sodium channels to cause a hyperexcitable condition in the central nervous system (CNS) of insects (Akbar et al., 2015a). It has been identified as a potential human carcinogen, but it is also extremely hazardous to fish, bees, and aquatic invertebrates (Lin et al., 2011). In humans, it produces allergic skin reactions, eye irritation, and negative effects on the central nervous system (Zhan et al., 2020). Research has demonstrated that CPM exhibits both estrogenic action and the ability to cause cancer and co-carcinogenesis (Anozie et al., 2017). There have been reports of ongoing CPM and its residue pollution of the downstream water bodies and sediments from runoff and erosion from agricultural areas and non-agricultural applications (Eneyi et al., 2021). Previous studies have also demonstrated that CPM is typically adsorbed on the soil surface upon application because of its non-polar character and low water solubility (Chen et al., 2012a; Eneyi et al., 2021). Depending on the physicochemical characteristics of the soil, the half-life of CPM in the environment might vary from 14.6 to 76.2 days (Akbar et al., 2015a). A key factor in influencing the fate and behavior of CPM in the soil ecosystem is the biodegradation of pesticides in the soil. The primary pathway for the breakdown of CPM in the natural environment is the hydrolysis of the ester bond, which results to the release of the metabolites such as 3-Phenoxybenzaldehyde (3-PBA), 3-Phenoxybenzoic acid, and cyclopropanecarboxylic acid (DCVA) in the environment. According to Chen et al. (2012a), the CPM metabolites are organic acids that are transportable in soil. Meeker et al. (2009) reported that 3-phenoxybenzoic acid is categorized as an endocrine disruptive agent due to its antiestrogenic activity and potential for toxicity. However, extensive use of CPM has resulted in its widespread occurrence as a pollutant of global concern. The frequent and uncontrolled use of CPM

has led to unintentional exposure to humans (Sharma et al., 2019; Malla et al., 2022a). This emphasizes the need to develop strategies for removal of CPM and its toxic residues from the environment. Various bioremediation techniques have been used to remove pesticides and its residues from the environment. The most successful way to clean up pesticide-contaminated soils is bioremediation, which uses bioaugmentation and biostimulation and is both economical, high efficiency, selective destruction, and environmentally friendly (Cycon & Piotrowska-Seget, 2016; Raj et al., 2021; Malla et al., 2022b) compared to other remediation strategies. Many bacterial species such as *Micrococcus* sp. (Tallur et al., 2015), *Pseudomonas* sp. (Indratin et al., 2019), *Streptomyces* sp. (Lin et al., 2011), *Serratia* sp. (Zhang et al., 2010); Tyagi and Prashar, 2015), *Sphingomonas* sp. (Akbar et al., 2015a), *Ochrobactrum* sp. (Wang et al., 2011), *Methylobacterium* sp. (Diegelmann, et al., 2015), *Rhodococcus* sp. (He et al., 2022), *Comamonas* sp. (He et al., 2022) and *Bacillus* sp. (Gangola et al., 2018) have been studied at physiological and molecular level for CPM degradation in the environment. To develop an effective strategy for the biodegradation of CPM and its metabolites and to achieve high removal efficiency is necessary, but the stability of the microorganism under different environmental conditions is also important. Hence, the purpose of the present study is to investigate and screen the capability of isolated bacterial strain from CPM-treated agricultural soil for the biodegradation of CPM. The present research is unique and have no resemblance to other CPM biodegradation studies. Therefore, in this study, we investigated the degradation potential of CPM-degrading *Bacillus flexus* strain PC5 isolated from CPM-treated agricultural soil and proposed possible biodegradation pathway for CPM degradation based on detected metabolites.

Materials and Methods

Soil sample collection

Soil samples were collected from three different agricultural fields of Ewuowa in Ikorodu Local Government Area in Lagos State, Nigeria which had history of CPM application for more than 15 years. Samples were collected from 5 to 15 cm depth, pooled together, homogenized and immediately placed in sterile universal bottles and transported to the laboratory. Samples for chemical analysis were stored at -2 °C while soil samples for microbiological analysis were stored at 4 °C for future use. All microbiological analysis was performed within two days of sampling.

Chemicals and Media

Analytical grade CPM, 3-phenoxybenzoic acid (C₁₃H₁₀O₃, CAS NO: 3739-38-6, EC NO: 223-121-2), 3-phenoxybenzaldehyde (CAS NO: 39515-51-0), phenol and catechol were purchased from Sigma Aldrich Chemical Company, South Africa. All the chemicals were analytical grade, $\geq 98\%$ purity. Stock solution of CPM (100 mg) was

<https://dx.doi.org/10.4314/br.v24i1.10>

made by dissolving the pesticide in 1 ml of analytical grade acetone. The stock solution was stored in the bottle from the manufacturer and sealed to avoid volatilization. From this stock solution, different concentrations of CPM were prepared for standard curve.

Enrichment, Isolation and Screening of CPM-degrading strain PC5

Stock solution of CPM (at a final concentration of 100 mg L⁻¹) was aseptically dispensed into sterile 250 mL Erlenmeyer flask placed in a laminar flow for 24 h to achieve complete evaporation of acetone. Soil sample (5 g) was added into 90 mL of sterile minimal salts medium (MSM) containing (g/l) Na₂HPO₄·2H₂O, 3.5; KH₂PO₄, 1.0; (NH₄)₂SO₄, 0.5; MgCl₂·6H₂O, 0.1; Ca(NO₃)₂·4H₂O, 50 mg and 0.5 ml of trace element solution in Erlenmeyer flask supplemented with CPM. This mixture was incubated at 28 °C on a rotary shaker at 180 rpm for one week. Thereafter, 100 µl of enriched cultures were transferred into 99 ml of MSM supplemented with CPM and incubated under the same condition for another one week. This transfer was repeated seven times to ensure selection of competent CPM-degrading bacteria. Pure cultures were subsequently isolated from the enrichment culture by spread-plate technique. From the last enrichment cultures, aliquots (0.1 ml) from 10⁻¹ to 10⁻⁷ of enrichment culture were plated on CPM-MSM agar plates and incubated at 28 °C for seven days. Predominant colonies which appeared on the plates after incubation were selected and purified on CPM-MSM agar and residual CPM degradation was detected by GC-FID. Biodegradation of CPM into metabolites was confirmed by Gas chromatography - mass spectrometry (GC-MS) (Lin et al., 2011).

Inoculum preparation

Pure bacterial cultures were maintained in glycerol: Luria Bertani (LB) medium (50:50). Before each experiment, the bacterial isolates were thawed and grown in 250 mL Erlenmeyer flasks containing 100 mL of LB medium. Cultures were grown in LB medium for 24 h, harvested by centrifugation at (12,000 rev min⁻¹ for 5 min) and washed twice with sterile water and finally re-suspended in sterile MSM. The optical density was adjusted to 0.5 and used for biodegradation studies (Moxley & Schmidt, 2010).

Morphological, physiological and biochemical characterization

Identification of bacterial isolate was carried out using standard cultural, morphological and biochemical characteristics according to the method of Cowan and Steel (Barrow & Feltham, 1995). The identification of the isolate was complemented using molecular biology techniques.

Sequencing of PCR amplified 16S rRNA gene and phylogenetic analysis

Genomic DNA of strain PC5 was extracted using a Wizard Genomic DNA purification kit (Promega A1120, USA). The

16S rRNA gene sequence was amplified using the forward primer 27F (5'- AGAGTTTGATCMTGGCTCAG -3') and reverse primer 1525R (5'- AAGGAGGTGWTCCARCCGCA -3'). PCR reaction was carried out by subjecting 2.0 µl of the genomic DNA (10 ng/µl) to PCR in a final reaction volume of 10 µl. The PCR mixtures contained 0.5 µl of each primer (5 pMol); 1.0 µl of MgCl₂ (25 mM); 0.8 µl of dNTP (2.5 mM); 1.0 µl of 10x PCR buffer (KapaTaq Hotstart Buffer); 0.1 µl of Taq polymerase (5 U/µl); 3.1 µl of MilliQ water and 2.0 µl of template DNA. Amplification conditions were 94 °C for 5 min, followed by 36 cycles of denaturation at 94 °C for 30 sec, annealing at 56 °C for 30 sec, extension at 72 °C for 45 sec, and a final extension cycle at 72 °C for 7 min in a thermocycler (Eppendorf Mastercycler). The temperature within the thermocycler was held at 4 °C until the tubes were stored in a refrigerator at -20 °C for future use. The DNA that had been purified was sequenced at Inqaba Biotechnologies in Pretoria, South Africa. The sequence was compared to small-subunit (SSU) sequences deposited with NCBI GenBank (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (www.rdp.cme.msu.edu) after being aligned using the Basic Local Alignment Search Tool (BLAST, www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). Using type strain sequences from the RDP and resampling for 1000 repeats, MEGA version 11 was used to create a phylogenetic tree based on the neighbour joining approach (Tamura et al., 2011).

Biodegradation Studies

Biodegradation and extraction of residual CPM in liquid medium

To investigate the ability of strain PC5 to degrade CPM, stock solution of CPM (final concentration of 100 mg L⁻¹) was aseptically transferred into sterile 250 mL Erlenmeyer flasks, placed in a laminar flow for 24 h to achieve complete evaporation of the acetone. Thereafter, 50 ml of MSM was dispensed into the flasks and pre-grown washed cells (1 % v/v) were aseptically inoculated into the medium in triplicates and incubated on bench top orbital shaker (180 rev min⁻¹) at 28 °C for 24 h. For growth studies, after, 0, 1, 2, 3, 4, 5 and 6 days, 1 ml aliquot of the utilizing culture was aseptically withdrawn and total viable count was estimated by plating out appropriate dilutions of the cultures onto nutrient agar. After 0, 1, 2, 3, 4, 5, and 6 days, 50 ml of the culture from control and treated flasks were removed and extracted with dichloromethane by vigorous shaking with separatory funnel for 15-20 min. The dichloromethane layer was separated, dehydrated with anhydrous sodium sulfate and evaporated to dryness at room temperature. The dried residue was reconstituted in 1 mL chromatographic grade n-hexane and an aliquot of 5 µL was used for residual CPM using GC-FID while metabolites of cypermethrin degradation were assayed by Gas chromatography – mass spectrometry (Indratin et al., 2019). Control flasks containing cypermethrin and sterile MSM were used to monitor abiotic degradation of

<https://dx.doi.org/10.4314/br.v24i1.10>

cypermethrin while cultures incubated in the absence of cypermethrin as the utilizable carbon source served to demonstrate that biomass formation depended on the utilization of cypermethrin as carbon source.

Biodegradation of 3-Phenoxybenzaldehyde (3-PBA)

Bacteria cells were pre-grown in MSM supplemented with 3-PBA (at a final concentration of 100 mg L⁻¹) and incubated on bench top orbital shaker (180 rev min⁻¹) at 28 °C for 24 h. After incubation, the isolates were harvested and washed twice (12,000 rev min⁻¹ for 5 min) and re-suspended in sterile MSM. The cell suspension (1 % v/v) was inoculated into 50 ml of sterile MSM in 250 mL Erlenmeyer flasks and 100 mg L⁻¹ of 3-PBA was added to the flasks. The flasks were incubated on a bench top orbital shaker (180 rev min⁻¹) for 144 h. The growth of the isolate was monitored at 24 h interval by optical density measurement at 600 nm. Incubation without active cells served as controls. The entire content of the flasks were extracted and the extract obtained was used for quantification of residual 3-PBA and metabolites of degradation were assayed by gas chromatography – mass spectrometry (GC-MS).

Gas Chromatography Mass Spectrometry (GC-MS) analysis of CPM metabolites

The residual CPM was extracted from the culture medium using a modified method of [Lin et al. \(2011\)](#). To improve the extraction efficiency of the samples, the metabolites of CPM in cell free filtrates of strain PC5 culture cultured in CPM-MSM were extracted using dichloromethane (100 mL X 3). The samples were vigorously shaken for 15-20 minutes in a separating funnel. After the solvent layer was removed, it was dried at room temperature (26 °C ± 2 °C) by evaporating it with sodium sulfate. Hexane was used to dissolve the residue, and 2 mL was the final volume needed for GC-MS analysis. The GC-MS (Agilent Technologies 5975C VL MSD, USA) was used to examine the extracts. GC-MS analyses were carried out using an Agilent gas chromatography system with an MS detector in electron ionization (EI) mode (70 eV). With a temperature program of 80 °C for 1 minute, 200 °C at 8 °C/min and held for 2 minutes, and finally 260 °C at 15 °C/min and held for 10 minutes, an HP-5MS capillary column (30 m length x 0.320 mm id x 0.25 µm film thickness) was employed. The carrier gas, helium, was employed at a steady flow rate of 1.0 ml/min. The samples were scanned in the mass range of 50 m/z to 450 m/z while being analyzed in splitless mode at an injection temperature of 260 °C and an EI source temperature of 230 °C ([Huang et al., 2018](#)). Documented information from the National Institute of Standards and Technology (NIST) library database was used to identify the metabolites.

Extraction and GC-MS analysis of 3-Phenoxybenzaldehyde from culture medium

3-Phenoxybenzaldehyde (3-PBA) was extracted from culture medium using a modified technique from [Huang et al. \(2018\)](#).

This was accomplished by vigorously shaking 50 mL of cell-free media in a separatory funnel for 15-20 minutes, along with 100 mL of methanol and dichloromethane (2:1, v/v) and 60 µl of strong hydrochloric acid. After the solvent layer was removed, it was dried at room temperature (26 °C ± 2 °C) by evaporating it with anhydrous sodium sulfate. After being dried, the residue was reconstituted in hexane of chromatographic grade and subjected to GC-MS analysis (Agilent, USA). The column was maintained at the following beginning temperature of 90 °C for 2 minutes, at 6 °C min⁻¹ to 150 °C for 1 minute, at 10 °C min⁻¹ to 180 °C for 4 minutes, and at a final temperature of 20 °C min⁻¹ to 260 °C for 10 minutes for the GC-MS operating conditions. The ionization energy was 70 eV, and the temperatures corresponding to the transfer line and the ion trap were 280 and 230 °C, respectively. At 250 °C, splitless sampling was used with an injection volume of 1.0 µL. A flow rate of 1.5 mL min⁻¹ of helium was employed as the carrier gas. The metabolites were identified using documented data from National Institute of Standards and Technology (NIST) library database ([Huang et al., 2018](#)).

Determination of inorganic chloride

The following reagents were added to 1 ml of culture sample placed in 1.5 ml microcentrifuge tubes; 100 µl of 0.25 M FeNH₄(SO₄)₂.12H₂O in 9 M nitric acid, followed by addition of 100 µl of saturated solution of Hg(SCN)₂ in ethyl alcohol. The solution was thoroughly mixed and centrifuged for 1 min. The supernatant was placed in a 1.5 ml glass cuvette and the absorbance was determined via UV-VIS spectrophotometry at a wavelength of 460 nm. A measurement was performed against a reagent blank containing MSM. A standard curve which represents correlation between absorbance and chloride concentration was constructed using standard solutions of NaCl at concentration range of 0.005 to 1.0 mM ([van der Woude et al., 1995](#)).

Effect of CPM concentration on degradation rate by strain PC5

The growth of strain PC5 on different concentrations of CPM (150 – 200 mg L⁻¹) as the sole carbon source were performed in 250 mL Erlenmeyer flasks containing 50 ml of sterile MSM. Pre-grown washed culture of the isolate (1% v/v) was inoculated into the flasks and incubated on a bench top orbital shaker for 144 h. Control flasks contained CPM and MSM only. Residual CPM concentration was analyzed at 24h interval using GC-FID ([Tallur et al., 2015](#)). Also, 1 ml of the culture medium was withdrawn and optical density at 600 nm was measured spectrophotometrically in a UV-VIS spectrophotometer to monitor growth of bacterial strain.

Effect of pH on CPM Degradation by Strain PC5

The pH of the MSM (50 mL) was adjusted with a pH meter to pH 4, 7 and 11 before autoclaving the flasks. Pre-grown washed cultures (1% v/v) were inoculated into MSM flasks containing CPM (at a final concentration of 100 mg L⁻¹) and

<https://dx.doi.org/10.4314/br.v24i1.10>

incubated on a bench top orbital shaker (180 rev min⁻¹) at 28 °C for 144 h. Control flasks contained CPM and MSM only. CPM residual concentration was analyzed after 144 h using GC-FID (Tallur et al., 2015).

Biodegradation of Cypermethrin in Soil microcosms

Microcosms were set up according to the method described by Sundaram et al. (2013) with some modifications. Soil samples were obtained from uncontaminated site from the botanical garden at the University of Lagos. Soil sample was dried at 50 °C for 3 days, sieved through a 2-mm sieve and sterilized in small foil packets by autoclaving at 121 °C for 30 min for three consecutive days. Triplicate soil samples (10 g) were placed in 150 mL beakers and their water contents were adjusted to 60 % of water holding capacity. Each of the soil samples was amended with CPM dissolved in acetone to obtain a final concentration of 100 mg kg⁻¹ of dry soil. After mixing, the acetone was allowed to evaporate for 24 h and strain PC5 was inoculated in triplicate using sterilized soil samples (SSSi). Also, non-sterilized soil samples (NSS) was mixed with CPM to determine the degradation ability of indigenous microbes. Sterilized soil samples (SSS) without inoculation of isolate served as abiotic control. All the beakers were covered with perforated aluminum foil and incubated at room temperature (26 °C ± 2 °C) for 30 days. Soil moisture content was maintained throughout the experimental period by addition of 10 ml of sterile distilled water. At time intervals of 0, 5, 10, 20 and 30 days, the entire content of soil samples from each beaker were extracted with 50 mL of hexane/acetone mixture (1:1, v/v). After vigorous shaking for 20-30 min, the solvent layer was filtered, dried with dehydrated with sodium sulfate and allowed to evaporate at room temperature (26 °C ± 2 °C). The extract was dissolved with 1 ml of acetone and analyzed using GC-FID.

Data analysis

All statistical analysis were done using SAS statistical software version 9.2 (Statistical Analysis Software Inc. 1990) and Prism computer software programmed (Graph pad Software, San Diego, CA. USA) version 5.03. The significance ($p < 0.05$) differences was treated statistically using one-way ANOVA.

Results and Discussion

Enrichment, Isolation and Screening of CPM-degrading strain PC5

Cypermethrin has been in use for over four decades for treatment of insect pests especially in agricultural fields but its extensive use have posed serious problem to aquatic organisms and humans. Thus, its removal from the environment is necessary. A total of twelve (12) bacterial isolates were obtained from the final CPM-MSM enrichment culture based on their colony and morphological

characteristics. Out of which only strain PC5 showed comparatively high growth rate and greater CPM degradation efficiency. Strain PC5 degraded 75.74 % of CPM (100 mg L⁻¹) within 144 h of incubation in MSM. Hence, strain PC5 was selected for further study to determine the optimal conditions and degradation pathway of CPM by this isolate. Many studies reported the isolation of CPM degrading microorganisms from pesticide contaminated soils, ground water and waste water sludge (Siddiqui & Kamal, 2025). For example, *Ochrobactrum anthropic* JcM1, *Bacillus megaterium* JcM2, *Sphingomonas* sp., *Brevibacillus* sp. JcM4, *Rhodococcus* sp., JcM5, *Acinetobacter* sp., were isolated from CPM-contaminated agricultural fields (Akbar et al., 2015a). Similarly, *Aspergillus niger* ZD11 and *Micrococcus* sp. were isolated from pesticide polluted environment (Kannan et al., 2024). In another report, *Rhodopseudomonas palustris* GJ-22 was isolated from waste water sludge (Yin et al., 2012) while *Pseudomonas fluorescens* and *Serratia plymuthica* which lived in sheep dipping facility and garden soils were also isolated (Gür Özdal and Algur, 2020). Some bacterial isolates such as *Methylobacterium* sp. A-1, *Aspergillus niger* ZD11, *Micrococcus* sp. can mineralize pesticides and be able to utilize them as a sole carbon and energy source (Diegelmann, et al., 2015).

Identification of Strain PC5

Strain PC5 formed creamish spreading colonies on nutrient agar plate. The cells were Gram-positive, motile rods with oval centrally located spores. It was catalase positive but negative for oxidase, urea, indole and citrate. It liquefied gelatin and hydrolysed starch and fermented glucose, lactose, sucrose and mannitol but not maltose. The nucleotide sequences obtained from strain PC5 was compared to the GenBank database for similarity analyses. The phylogenetic analysis (Figure 1) based on the 16S rRNA analysis using MEGA 6.0 showed that strain PC5 had 98 % sequence similarity with *Bacillus flexus* CSIRCH03 (JQ408383). The sequence obtained has been deposited in the GenBank under accession number LC218817. Based on the results of morphological, physiological, biochemical test and 16S rRNA gene sequence, strain PC5 was identified as *Bacillus flexus* PC5. At present, many pyrethroid degrading strains have been isolated and identified in different ecosystem contaminated with pesticides that possess similar morphological and biochemical characteristics with the *Bacillus* sp. isolated in this study that has the capacity to break down cypermenthrin. Many of the isolates are different species of *Bacillus* including *Bacillus licheniformis* B-1 (Liu et al., 2014; Zhao et al., 2016), *Bacillus cereus* ZH-3 (Chen et al., 2012), *Bacillus megaterium* JcM2 (Akbar et al., 2015a) and *Bacillus* sp. strain SG2 (Pankaj et al., 2016).

Growth and Biodegradation of CPM in MSM

Bacillus flexus strain PC5 was able to utilize CPM (100 mg L⁻¹) as sole source of carbon and energy (Figure 2). Significant differences ($p < 0.05$) were observed between the experimental and control medium during biodegradation of

<https://dx.doi.org/10.4314/br.v24i1.10>

CPM by the isolated bacterial strain. The population of strain PC5 increased from 5.3×10^5 to 3.5×10^7 cfu ml⁻¹ after 144 h of incubation. The generation (based on total viable count) time of strain PC5 at 28 °C was 14.99 h with specific growth rate of 0.04 h⁻¹ during exponential growth phase. Cultures incubated without CPM had no increase in biomass formation.

Microbial degradation plays a major role in the removal of pesticides from the environment. Among other approaches used to eliminate pesticides, the use of microbes is highly efficient, environmentally friendly and cost effective (Ruan et al., 2013; Huang et al., 2018). Strain PC5 did not show any lag phase during CPM degradation and 55 % of CPM was degraded within 48 h. The degradation of CPM by strain PC5 was 75.74 % (Figure 2) with corresponding utilization rate of 0.532 mg L⁻¹h⁻¹ after 144 h of incubation. Cell free cultures had no decrease in concentration of CPM over time thus

showing that the loss of CPM was not due to abiotic factors. Chloride released in the culture medium by strain PC5 is 2.9 mg after 144 h of growth on CPM. The minimal chloride ion production by strain PC5 indicates that growth was primarily as a result of metabolism of the non-chlorinated 3-phenoxybenzoic acid. The genera to which strain PC5 was affiliated have been documented as potential pesticide degraders. For example, *Bacillus megaterium* JCM2 have been studied for their role in degradation of CPM and other pyrethroids in the soil ecosystem (Gangola et al., 2018). Similarly genus *Brevibacillus* sp. strain KB2 and *Bacillus cereus* strain PU (Chen et al., 2012), *Rhodopseudomonas palustris* GJ-22 (Yin et al., 2012) and *Acinetobacter radioresistens* USTB-04 (Kansal et al., 2023) have been shown for pesticide degradation. Increase in cell biomass corresponded with the depletion of CPM by strain PC5.

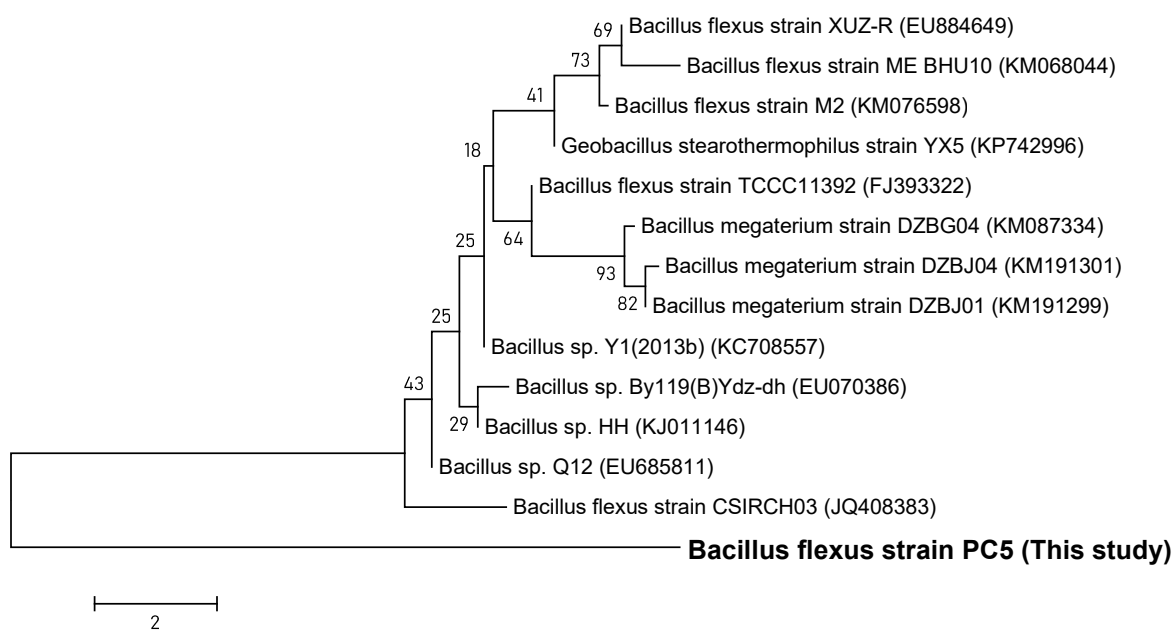


Figure 1: Neighbour-joining tree indicating the phylogenetic affiliation of *Bacillus flexus* strain PC5 based on selected 16S rRNA gene sequences for type strains obtained from the Ribosomal Database Project.

Table 1: Influence of CPM concentrations (150 mg L⁻¹ and 200 mg L⁻¹) on degradation by *Bacillus flexus* strain PC5 after 144 h of incubation.

Concentration (mg L ⁻¹)	Degradation (%)	Rate of degradation (mg L ⁻¹ h ⁻¹)
150	64.76	0.77
200	35.20	0.474

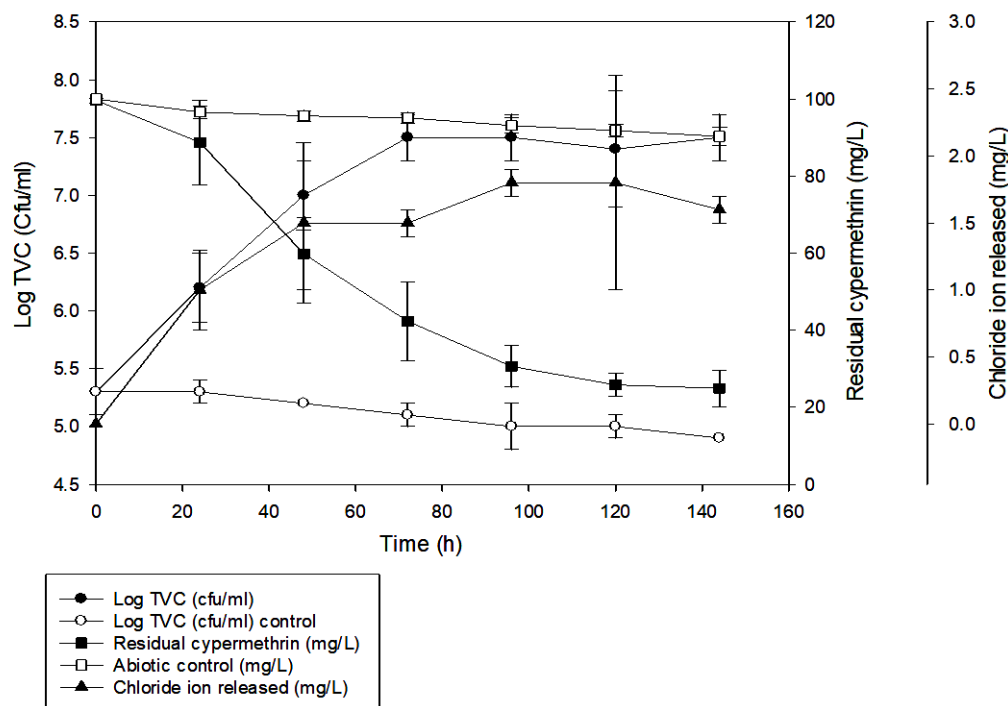


Figure 2: Growth and utilization of cypermethrin by *Bacillus flexus* strain PC5 at concentration of 100 mg L⁻¹. Error bars represent SD and values are mean of three replicates.

Effects of CPM Concentration on Degradation Rate by Strain PC5

It has been demonstrated in previous studies that the pyrethroid concentration has an important effect on their degradation rate in the environment and culture media. In general, higher concentration of CPM reduced the rate of degradation of the insecticide (Xiao et al., 2015). The effect of CPM concentrations (150 mg L⁻¹ and 200 mg L⁻¹) on the ability of *Bacillus flexus* strain PC5 to degrade CPM is presented in Figure 3a and 3b, respectively. The percentage degradation of CPM (150 mg L⁻¹) after 144 h of incubation by strain PC5 was 64.76 % with corresponding utilization rate of 0.778 mg L⁻¹h⁻¹. After 144 h of incubation in 200 mg L⁻¹ of CPM, the degradation rate of 0.474 mg L⁻¹ h⁻¹ was recorded and 35.20 % of CPM was utilized by *Bacillus* sp. strain PC5 (Table 1). Comparison of the growth of PC5 in

different concentrations of CPM showed that strain PC5 grows faster at lower concentration of 150 mg L⁻¹ compared to 200 mg L⁻¹. However, at higher concentration of CPM (200 mg L⁻¹), growth significantly decreased with an increase in the lag phase, followed by a decrease at the percentage and rate of degradation. Our results aligned with the findings of Jilani (2013) that increased concentrations of CPM had a marked effect on the growth of *Pseudomonas* sp., with a prolonged lag phase. Similar finding was reported by Xiao et al. (2015) with different concentrations of CPM using *Bacillus subtilis* strain BSF01. In that study, about 94, 89 and 85 % of CPM at concentrations of 25, 50 and 100 mg L⁻¹ were degraded within seven days respectively. Higher concentration of CPM decreased microbial growth and this result to the decrease in the degradation of CPM.

Table 2: Chromatographic properties of the metabolites formed by *Bacillus flexus* strain PC5 during growth on 3-Phenoxybenzaldehyde (100 mg L⁻¹).

Compound name	GC/MS retention time (min)	Molecular (weight)	Ions fragment
3-Phenoxyphenol	8.544	186	186, 157, 129
3-Phenoxybenzoic acid	9.255	214	214, 169, 141
Phenol	8.427	94	39, 66, 94

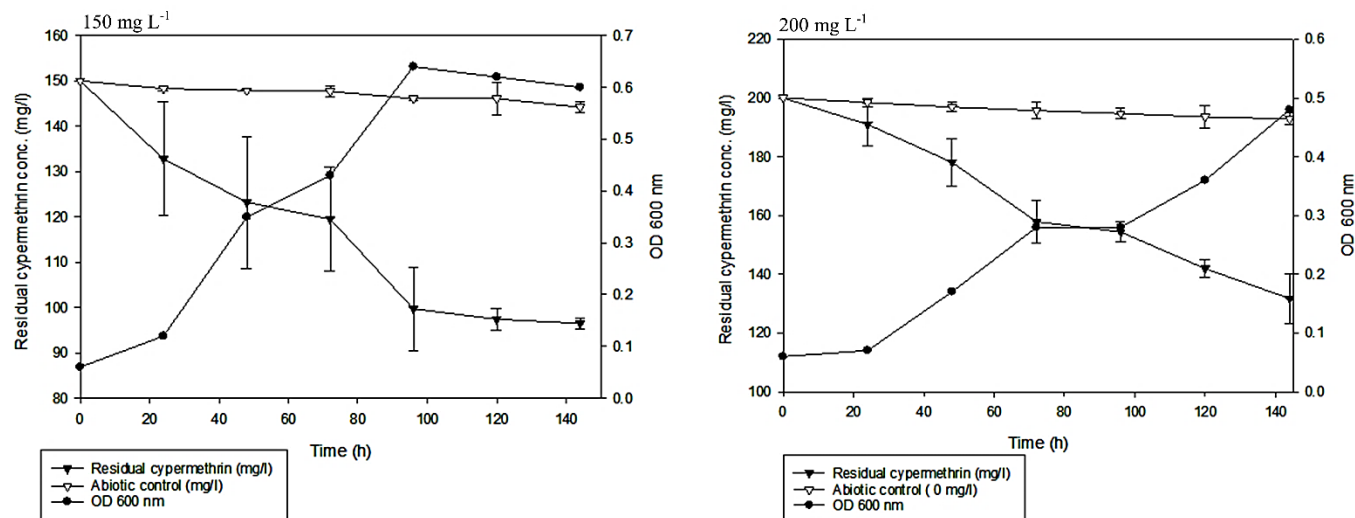


Figure 3: Degradation of CPM during growth of strain PC5 in MSM at concentration of 150 mg L⁻¹ and 200 mg L⁻¹. Error bars represent SD and values are means of three replicates.

Analysis of CPM and 3-PBA Biodegradation Metabolites

Degradation of CPM by *Bacillus flexus* strain PC5 after 24 h of incubation yielded 3-PBA as a major metabolite (Figure 4). 3-Phenoxybenzaldehyde (3-PBA) concentration decreased over time after 144 h of incubation with strain PC5 (Figure 4). The metabolite (3-PBA) eluted at retention time of 17.701. GC-MS mass spectra of the metabolite showed major fragment ions at *m/z* values of 198, 169, 115, 51 (Figure 5). GC-MS analysis of 3-Phenoxybenzaldehyde extracts obtained from growing cells of *Bacillus flexus* strain PC5 showed that strain PC5 was able to produce three different metabolic intermediates (Table 2). The peaks at retention times of 9.255, 8.544 and 8.427 min corresponded to 3-phenoxybenzoic acid, 3-phenoxyphenol and phenol respectively as detected by GC-MS analysis (Figure 6) with the major ion products at *m/z* of 214, 169, 141; 186, 157, 129 and 39, 66 and 94 respectively (Figure 7A, B and C). It is evident from the GC-MS results that strain PC5 degraded CPM via reductive dechlorination to yield 3-PBA. Oxidation of 3-PBA produced 3-phenoxybenzoic acid which was further transformed to 3-phenoxyphenol. Afterwards, 3-phenoxyphenol was metabolized to phenol which is a low molecular weight compound and was easily mineralized to muconic acid by strain PC5. This result is consistent with other research findings. For example, Chen et al. (2012) reported that a coculture of *Bacillus cereus* ZH-3 and *Streptomyces aureus* HP-S-01 resulted in the hydrolytic breakage of the ester link in CPM to generate 3-PBA. A

similar outcome was noted by Lin et al. (2011) who found that *Streptomyces* sp. HU-S-01 likewise converted CPM into 3-PBA and that no additional CPM degradation was seen in that investigation. Similar to the pyrethroids permethrin and deltamethrin, CPM is also catabolized by bacteria through the hydrolysis of ester linkages which results in the cleavage products that correspond to them (Pankaj et al., 2016). Given that the majority of pyrethroid insecticides have the ability to produce additional hazardous metabolites throughout the biodegradation process, research into the biodegradation pathway is crucial to the development of a bioremediation approach for pyrethroid-contaminated ecosystems. 3-PBA contaminates soil widely and is more mobile than its parent chemicals. Additionally, because of its anti-estrogenic properties, 3-PBA is categorized as an endocrine disruptor (Li et al., 2022). Due to its antibacterial properties, it may not undergo accelerated biodegradation and instead accumulate in the media or soils (Chen et al., 2011). It is therefore more hazardous to the environment than the parent chemicals. The results clearly show that the *Bacillus flexus* strain PC5 was able to efficiently breakdown CPM and also changed the metabolites that were created during this process (Table 2). The isolated bacterial strain can also be used as seed culture (bio-augmentation) for complete detoxification of CPM in contaminated soil or water ecosystem. On the basis of the results obtained from GC-MS analysis, the metabolic pathway for CPM degradation by strain PC5 was proposed (Figure 8).

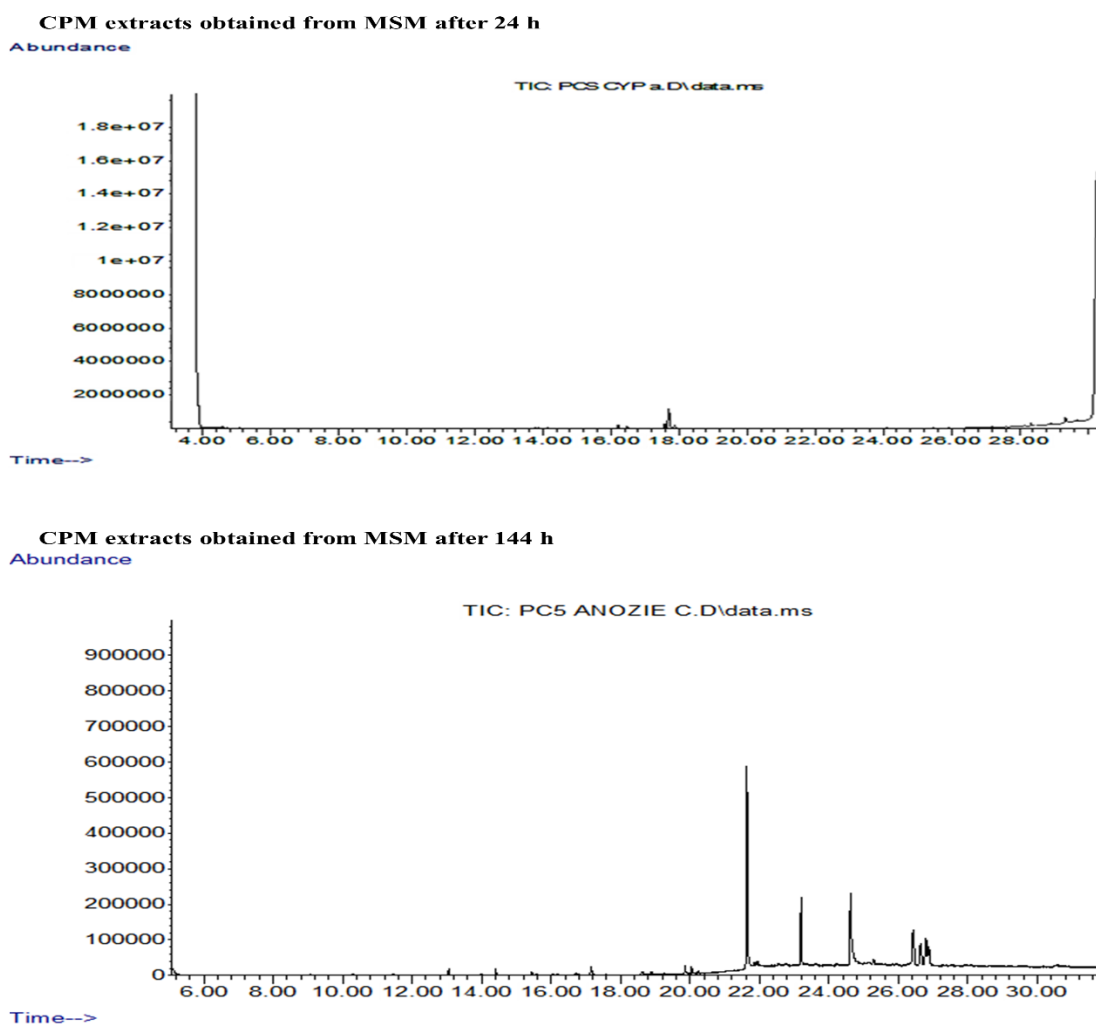


Figure 4: Gas chromatogram of CPM extracts obtained from MSM after 24 h and 144 h of incubation with *Bacillus flexus* strain PC5.

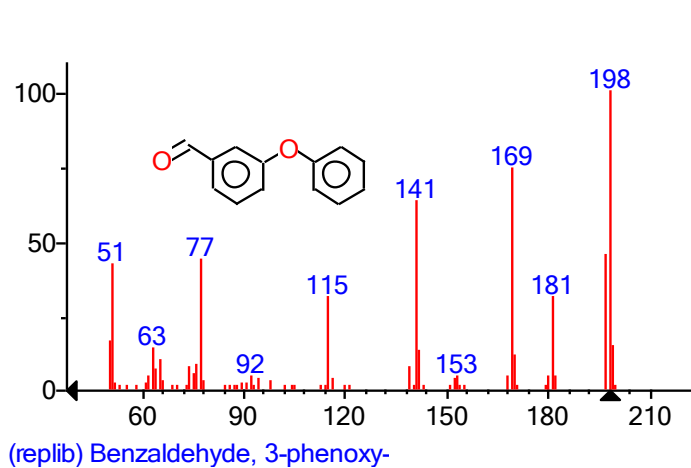


Figure 5: GC-MS spectra of the metabolite produced during CPM degradation by *Bacillus flexus* strain PC5.

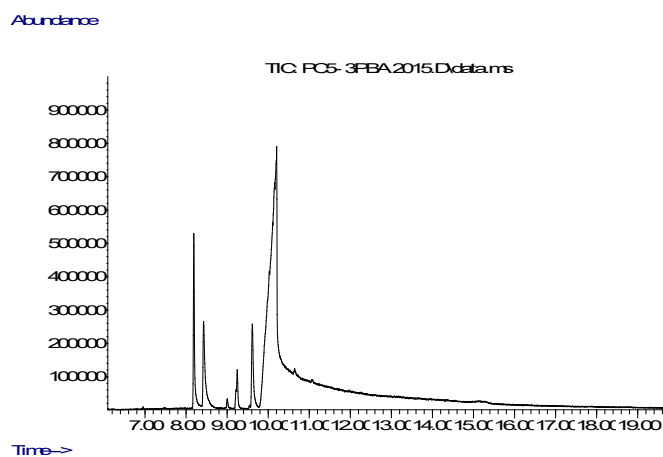


Figure 6: Gas chromatogram of 3-phenoxybenzaldehyde extracts obtained from MSM after 144 h of incubation in 3-PBA by *Bacillus flexus* strain PC5.

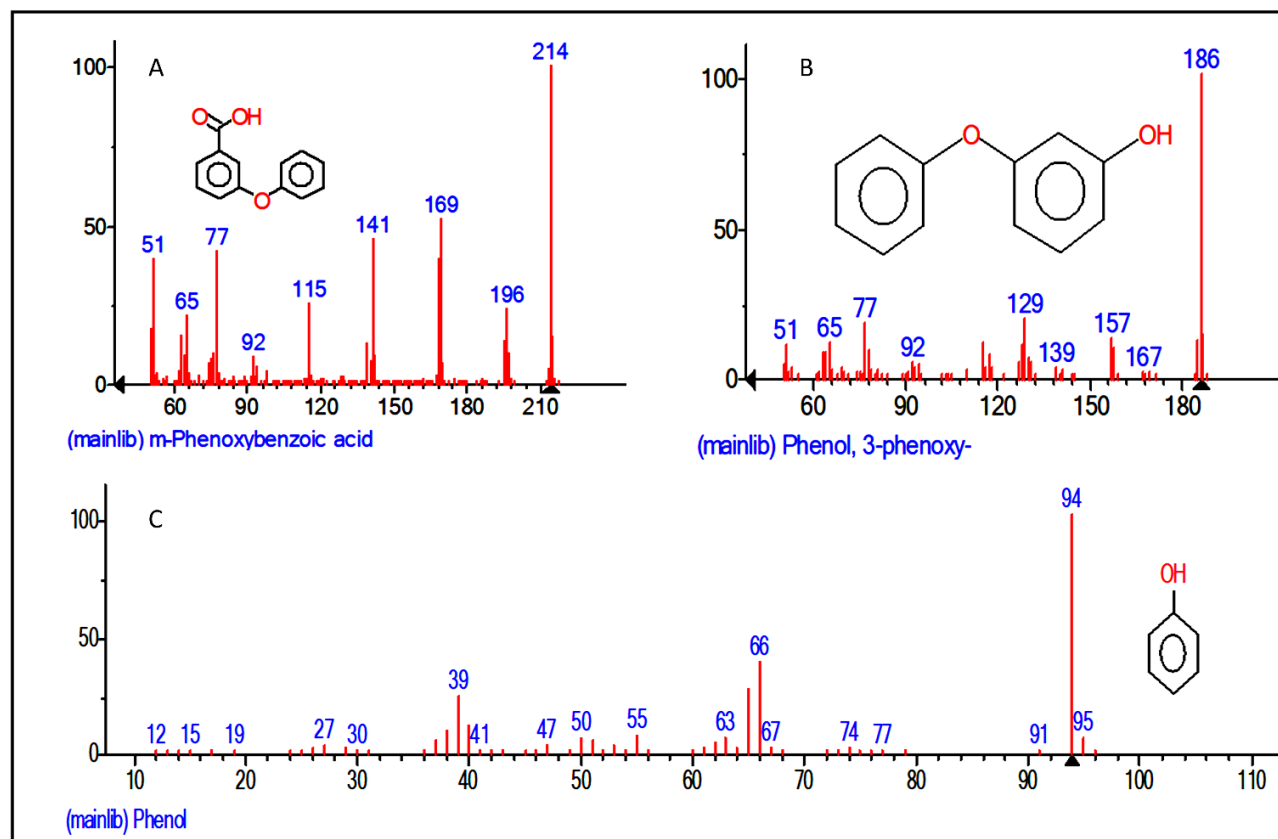


Figure 7: GC/MS spectra of metabolic products produced during 3-PBA degradation by *Bacillus flexus* strain PC5. (a) 3-phenoxybenzoic acid; (b) 3-phenoxyphenol; (c) phenol.

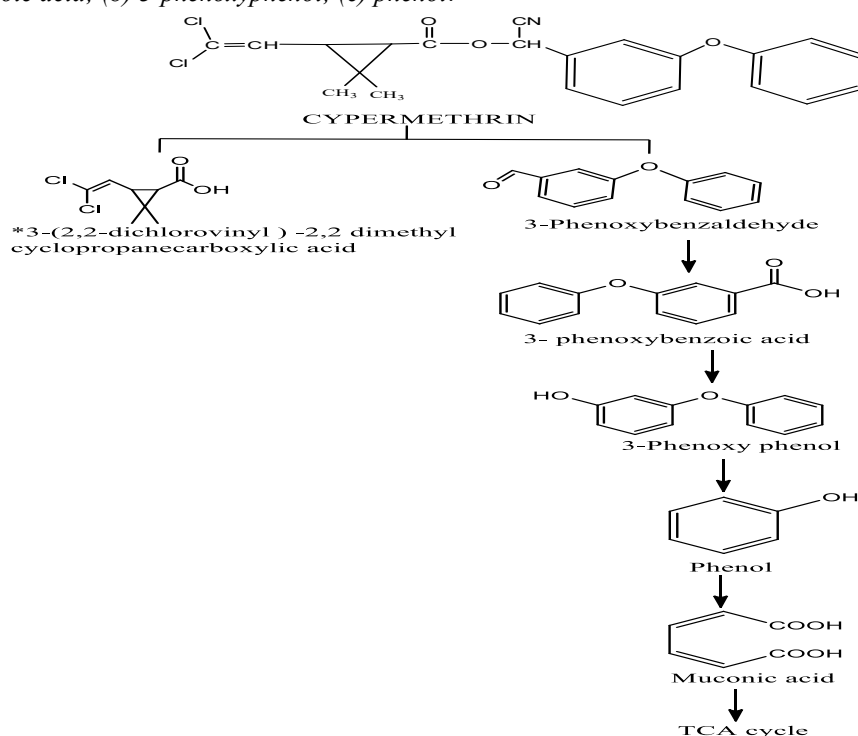


Figure 8: Proposed pathway for the degradation of cypermethrin by *Bacillus flexus* strain PC5

Optimum pH for CPM degradation by Strain PC5

One of the critical factors that determine the biodegradation of pyrethroids is the pH of the culture medium. The effects of pH on degradation of CPM by strain PC5 is presented in Figure 9. At pH values of 4 and 11, *Bacillus flexus* strain PC5 degraded 14.87 % and 32.70 % of CPM with corresponding utilization rate of 0.103 mg L⁻¹ h⁻¹ and 0.224 mg L⁻¹h⁻¹ respectively after 144 h of growth. The optimal pH for CPM degradation was at neutral (pH 7). The percentage degradation of CPM was 75.74 % at optimal pH range and utilization rate of 0.525 mg L⁻¹ h⁻¹ was recorded after 144 h of growth by strain PC5. Abiotic control flasks did not show decrease in the concentration of CPM at different pH values. There was significant difference at 5 % probability level in the utilization of CPM at different pH values by strain PC5 when compared with the control. The effect of pH on pyrethroid degradation was illustrated by the work of Zhao et al. (2013) who reported that 97 % of CPM was degraded at a pH of 7 by *Catellibacterium* strain CC-5 after 7 days of incubation. Similar findings was observed by Yin et al. (2012) in which the highest degradation rate of CPM by *Rhodopseudomonas palustris* GJ22 was achieved at neutral pH when compared with degradation rate at pH values of 5.0 and 9.0.

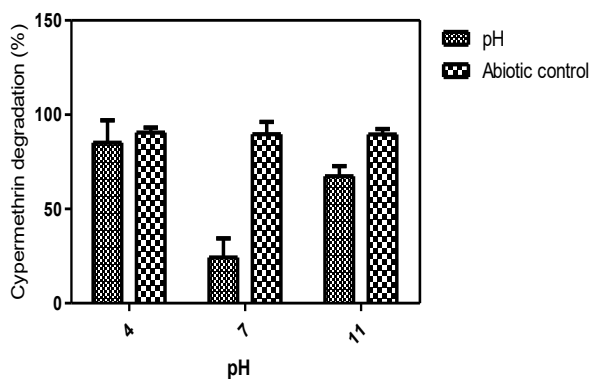


Figure 9: Effect of pH on the degradation of cypermethrin by *Bacillus flexus* strain PC5 after 144 h of incubation. Symbols are the means of three replicates and error bars represent SD.

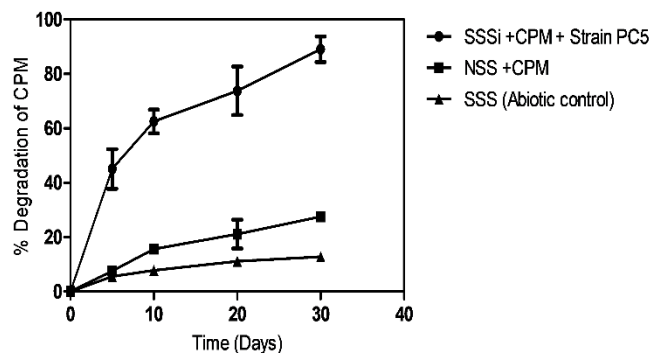


Figure 10: Degradation of cypermethrin (100 mg kg⁻¹) in agricultural soil inoculated with strain PC5. Symbols are the means of three replicates and error bars represent SD.

Bioremediation of cypermethrin in soil microcosms

Biodegradation of CPM in soil microcosms revealed significant differences ($p < 0.05$) in CPM degradation between strain PC5 and indigenous isolates (Figure 10). The percentage utilization of CPM by *Bacillus flexus* strain PC5 was 45.10 % after 5 days of incubation in soil. However, at the end of the experiment, strain PC5 degraded 89.10 % of applied CPM in the soil sample. The percentage degradation of CPM was much slower in sterilized soil samples (12.80 %) while non-sterilized soil containing only the indigenous microbial community degraded 27.50 % of CPM in the soil after 30 days of incubation.

Biodegradation studies of agricultural soils polluted with commercial CPM revealed that CPM (100 mg kg⁻¹) was degraded in soils inoculated with *Bacillus flexus* strain PC5 as compared to sterilized and non-sterilized soil samples. Inoculation of soil samples contaminated with CPM with CPM-degrading organisms enhances the utilization of CPM in the environment. In a similar research, within 42 days of incubation, Akbar et al., (2015a) found that soil inoculated individually with *Acinetobacter calcoaceticus* MCm5, *Brevibacillus parabrevis* FCm9, or *Sphingomonas* sp. consumed CPM (200 mg kg⁻¹) more quickly than non-inoculated, non-sterilized soil. Hence, the initial smaller size of the community of CPM-degrading bacteria that grew in number after employing CPM and increased degradation rates in following weeks can be the reason for the slower rate of CPM-degradation in non-sterilized soil (Sundaram et al., 2013; Indratin et al., 2019).

Conclusion

An efficient CPM-degrading bacterium strain *Bacillus flexus* PC5, was isolated from agricultural farmland which had a history of extensive use of CPM for insect control. Strain PC5 degraded CPM at optimum pH range of 7. High concentration of CPM (200 mg L⁻¹) decreased the utilization rate of CPM by strain PC5. GC-MS results revealed no accumulation of the degradation metabolites in the culture medium by strain PC5. Results obtained from this study showed that strain PC5 with a high CPM tolerance could be a good candidate for bioremediation of pyrethroid contaminated sites.

Acknowledgement

The authors are grateful to the Departments of Microbiology and Chemistry, University of Lagos, Akoka, Lagos State for extending the laboratory facilities to complete this research work.

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