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Statistical optimization and ecotoxicological validation of a hydrolytic detoxification pathway for carbofuran using earthworm gut-associated Bacillus paralicheniformis PPH2

Puspendu Shit
Debra Thana Sahid Kshudiram Smriti Mahavidyalaya (Autonomous)
Harekrishna Jana
Raja Narendra Lal Khan Women's College (Autonomous)
Partha Pratim Chakravorty
parthapratimchakravorty@yahoo.in

Debra Thana Sahid Kshudiram Smriti Mahavidyalaya (Autonomous)
Priyanka Sarangi
Raja Narendra Lal Khan Women's College (Autonomous)
Payel Sur
Raja Narendra Lal Khan Women's College (Autonomous)
Samiran Sona Gauri
Indian Institute of Technology
Yuji Sakai
Kogakuin University

Research Article

Keywords: Carbofuran, Biodegradation, Bacillus paralicheniformis, RSM, Eisenia fetida, Detoxification

Posted Date: April 28th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9253134/v1

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Biodegradation on September 7th, 2026. See the published version at https://doi.org/10.1007/s10532-026-10370-2.

Abstract

The environmental persistence of carbofuran, a potent methylcarbamate neurotoxin, necessitates the development of advanced biotechnological strategies to safeguard soil biodiversity. This study investigated the bioremediation potential of *Bacillus paralicheniformis* PPH2, a specialized symbiont isolated from a unique ecological niche, the gastrointestinal tract of the indigenous paddy field earthworm *Glyphidrilus* sp. Unlike traditional soil isolates, strain PPH2 exhibited superior metabolic resilience, growing on carbofuran as the sole carbon and energy source. Through Central Composite Design with Response Surface Methodology, the catabolic efficiency was maximized at pH 7.1, 34.7°C, and 100 rpm rpm and a validated degradation efficiency of 62.07 ± 1.12 was realized within 120 h. Structural elucidation using the LC-MS/MS revealed carbofuran phenol as the main metabolite (m/z 164.36) and verified that PPH2 promotes the hydrolytic cleavage of the methylcarbamate ester linkage, which is the neurotoxic warhead of the molecule. The ecological relevance of this biotransformation was confirmed by use of in vivo ecotoxicological bioassays on sentinel earthworm, *Eisenia fetida*. The findings showed a large 1.66-fold decrease in acute systemic toxicity, and the 96-h LC_{50} of 5.458 mg/kg (parent compound) changed to 9.061 mg/kg (metabolite). This study offers a scientifically sound mechanism of restoring soil health and safeguarding terrestrial ecosystems against pesticide degradation by enhancing the gap between microbial kinetics, statistical optimization and quantitative ecotoxicology.

Introduction

Carbofuran (2,3-dihydro-2, 2-dimethyl-7- benzofuranyl methylcarbamate) is a broad-spectrum insecticide, acaricide, and nematicide that has recently posed major ecological hazards to the environment, including land and water (Cáceres et al. 2019). Although carbofuran is highly effective against pests in crops like rice and maize (Ariffin and Rahman 2020), it is described by a terrifying groundwater ubiquity score (GUS of 4.52) and high solubility (320–351 mg/L), and it is thus mobilized into non-target areas quickly (Ćwieląg-Piasecka 2023). Its major mode of action is that it irreversibly inhibits acetylcholinesterase (AChE), which causes acute neurotoxic effects in soil macrofauna, i.e. earthworms, which is an important bioindicator of soil health (Lan et al. 2019). Because carbofuran can remain in the soil matrix for up to one year, its use frequently results in long-term disturbances of the nutrient cycle and soil structure (Song 2014).

Traditional remediation methods, including thermal desorption for chemical oxidation, have mostly been ineffective because of their high operational cost and the potential to produce secondary pollutants (Mosa et al. 2016; Zhao et al. 2019). However, bioremediation has proven to be a better sustainable solution, as it is based on the metabolic versatility of microbial communities leading to total mineralization of xenobiotics (Mishra et al. 2020). Aerobic bacteria of the genus *Bacillus* have shown the highest levels of persistence and degradation (Mishra et al. 2020; Duc 2021). Nevertheless, microbial interventions in the field are frequently unsuccessful because of unfavorable environmental factors and insufficient knowledge of the toxicity of intermediate metabolites (Shit et al. 2023).

As biogeomorphogens, earthworms exert a transformative effect on soil structure and fertility due to their burrowing, feeding, and casting behaviors (Ojha and Devkota 2014). These geophagous detritivores serve as key agents of soil homeostasis through improved soil aeration, water percolation, and the redistribution of organic matter (Sharma 2024). Their ecological role is based on the central role of the earthworm gut, which is a specialized micro-bioreactor harboring a dense and metabolically diverse consortium of microorganisms (Biswas et al. 2018). This intestinal flora, comprising most common genera, including *Bacillus, Pseudomonas, Sphingomonas, and Chromobacterium*, plays a vital role in vital biochemical activities, such as cellulose degradation, nitrogen fixation, and phosphate solubilization (Drake and Horn 2007; Hussain et al. 2016; Dey et al. 2018). These symbiotic and related bacteria play important roles in nutrient conversion; however, their functionalities have long been explored in the context of the mineralization of organic matter and natural fertilization.

Although the role of earthworm gut bacteria in maintaining constant soil nutrient fluxes has been established, there is still a large knowledge gap regarding their contribution to the degradation of unbiodegradable pesticides. The metabolic pathways and bacterial species underlying the action of the identified relevant catabolic genes in the gut microbiome have not been fully characterized. The majority of current bioremediation studies use native soil isolates, leaving aside the gut microbiome as a pool of xenobiotic attenuation-competent, specialized enzymes. Moreover, the scanty data about the identification and ecological effect of byproducts generated in these microbial transformations is a challenge to determining the real safety of bioremediation. What is desperately required is the shift in a situation whereby natural decomposition is witnessed to the active utilization of specialized microbial resources in the targeted elimination of agricultural contaminants.

Against this background, we selected one of the resilient bacterial strains, *Bacillus paralicheniformis* PPH2, which is an isolate of the gastrointestinal tract of the ecologically important paddy field-dwelling earthworm, *Glyphidrilus* sp. It is a semi-aquatic endogeic species that inhabits the ecotones between land and aquatic ecosystems; therefore, it is a unique sentinel of pesticide-contaminated agricultural runoff. This work was broadly aimed at assessing the bioremediation capability of the given strain, PPH2, with respect to carbofuran, which addresses the gap between the isolation and environmental safety of microbes. We used a central composite design (CCD) under the response surface methodology (RSM) to maximize the catabolic activity of the strain under different pH, temperature, and agitation gradients. Combining structural elucidation of metabolites through liquid chromatography-tandem mass spectrometry (LC-MS/MS) with extensive ecotoxicological authentication through the use of *Eisenia fetida*, this study offers a conclusive, ecologically robust framework for the restoration of pesticide-affected soil.

Materials and Methods

Isolation and molecular identification of strain PPH2

The bacterial strain, PPH2, used in this study was previously isolated from the gut of a semi-aquatic earthworm belonging to the genus *Glyphidrilus* sp., collected from a submerged paddy field ecosystem in the Paschim Medinipur district, West Bengal, India (22°14′15.9″ N, 87°25′34.1″ E) (Supplementary Fig. S1). The strain was identified via 16S rRNA gene sequencing using the universal primers 8F and 1492R and phylogenetic analysis using the maximum-likelihood method, which confirmed its identity as *Bacillus paralicheniformis* (NCBI Accession no. OP781906) (Supplementary Fig. S2). The strain was used for subsequent carbofuran biodegradation experiments.

Chemicals, reagents, and media composition

Analytical standards and stock preparation

Analytical grade carbofuran (purity ≥ 99.5%; CAS No. 1563-66-2) was procured from Sigma-Aldrich (India). A primary stock solution (10,000 mg/L) was prepared in HPLC-grade methanol, sterilized via a 0.22 µm polytetrafluoroethylene (PTFE) membrane, and stored in amber glass vials at 4°C. To achieve an experimental concentration of 100 mg/L, 1.0 mL of the stock solution was added to sterile flasks, followed by complete solvent evaporation under a laminar airflow hood to prevent methanol-induced metabolic interference (Bailey et al. 1996). The residue was subsequently equilibrated in sterile MS medium at 150 rpm for 4 h to ensure complete substrate dissolution.

Cultivation and mineral salts medium (MSM)

A defined mineral salt medium (MSM) was used for isolation, cultivation, and carbofuran degradation experiments with *Bacillus paralicheniformis* PPH2, based on the protocol described by Gür et al. (2014). The MSM contained the following components per litre of deionized water: NH_4Cl, 2.0 g; KH_2PO_4, 5.0 g; $Na_2HPO_4 \cdot 12H_2O$, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4$,

0.2 g; $CaCl_2 \cdot 2H_2O$, 0.001 g; $FeCl_3$, 0.05 g; and $CoCl_2$, 0.001 g. Trace elements were supplemented as follows: $ZnCl_2$ (136 mg/L), $CuCl_2 \cdot 2H_2O$ (171 mg/L), and $NiCl_2 \cdot 6H_2O$ (24 mg/L).

- *Inoculum preparation*: For the initial biomass build-up, MSM was enriched with 0.2% (w/v) glucose and 0.05% (w/v) yeast extract as readily assimilable carbon and nitrogen sources (Malick et al. 2017).
- *Degradation assays*: To ensure that carbofuran served as the exclusive carbon and energy substrate, all biodegradation experiments utilized non-supplemented MSM, omitting additional organic carbon sources, such as glucose or peptone (Fareed et al. 2017).

Preliminary biodegradation assays and growth kinetics

The biodegradation potential of *B. paralicheniformis* PPH2 was tested using 100 mL of sterile MSM. For inoculum preparation, the isolate was revived in nutrient broth (NB) until the late-exponential phase at 30°C and 120 rpm. To eliminate residual nutrients, the microbial suspension was collected via sedimentation at 6000 × g for 5 min at 25°C. This pellet was then rinsed twice with sterile 0.85% physiological saline before being re-suspended in deionized water until reaching an optical density ($OD6_{00}$) of 1.0. Experimental flasks were enriched with 100 mg/L of analytical-grade carbofuran (Sigma-Aldrich, ≥ 99.5%), which served as the only organic nutrient, followed by the addition of a 5% (v/v) bacterial suspension. Biodegradation experiments were performed in triplicate and comprised (i) an experimental set (MSM + carbofuran + PPH2), (ii) an abiotic control (MSM + carbofuran, no bacteria) to assess non-biological losses, and (iii) a biomass control (MSM + PPH2, no carbofuran) to monitor background growth and endogenous metabolism. The experimental containers were containers were maintained in a temperature-regulated shaking apparatus operating at 30°C and 120 rpm, continuously away from light exposure for five days (Wang et al. 2010).

Growth kinetics and pesticide dissipation were monitored through daily aseptic sampling over a 5-day incubation period. A Systronics dual-beam instrument (model 2206) was used to measure the optical density at 600 nm. Quantification of the remaining carbofuran involved pelleting the microbial mass via sedimentation at 10,000 rpm for 10 min. The supernatant was then passed through a 0.22-µm PTFE membrane filter before injection into a high-performance liquid chromatography (HPLC) system. The entire procedure was conducted thrice per condition, and the results are presented as the average value along with the standard deviation to confirm statistical consistency. This dual-monitoring approach allowed for the correlation of microbial biomass expansion with the metabolic utilization of the pesticide, confirming the efficacy of *B. paralicheniformis* PPH2 in carbon-limited environments.

RSM-based statistical optimization of the degradation of carbofuran

Response surface methodology (RSM) was used to optimize the degradation of carbofuran by *B. paralicheniformis* PPH2 using a three-factor, five-level central composite design (CCD). Using this statistical model, we examined the effects of certain parameters on metabolic efficiency with the aim of increasing the degradation capacity of the strain above its natural level. Three independent variables-pH (X_1), incubation temperature (X_2, °C), and agitation speed (X_3, rpm)–were tested in ranges that had been previously determined by one-factor-at-a-time (OFAT) assays: pH 5.0–9.0, temperature 20–45°C, and agitation 80–140 rpm. The CCD was utilized to organize 20 distinct trials, comprising a mix of axial, factorial, and six replicated central points. A total of 20 different trials were organized using the CCD and included a combination of axial, factorial, and six replicated central points. The three-fold sets were performed in 250 mL flasks containing 100 mL of mineral salts medium. A 5% (v/v) homogeneous PPH2 culture (OD_{600} =1.0) was inoculated into this growth medium, which was enriched with a 100 mg/L concentration of carbofuran. All flasks were incubated under the designated conditions for 5 d, and residual carbofuran was measured via high-performance liquid chromatography (HPLC) following the protocols mentioned earlier (Bhandari et al. 2022).

Mathematical modelling and statistical validation

The empirical data were statistically calculated using Design-Expert (v13.0.5) to plot the mathematical correlation between the independent factors and the dependent response (Y). This reaction was measured in terms of the percentage of carbofuran destroyed during five days. The data were plotted against the following quadratic regression equation to estimate the conditions for peak performance:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \sum_{j}^{k} \beta_{ij} X_i X_j \# (1)$$

Y in this model indicates the projected outcome, with β_0 being the constant offset. β_i, β_{ii}, and β_{ij} are the linear, squared, and cross-product coefficients, respectively. To verify the reliability of the model, we conducted an analysis of variance (ANOVA) to test the predictive power using the coefficient of determination (R^2) and measure the adequacy of the model using lack-of-fit diagnostics. Moreover, 3D RSM was used to show the interrelationships among variables and identify the optimal conditions under which carbofuran is broken down by strain PPH2.

Analytical procedures for carbofuran quantification

To extract the remaining carbofuran and its decomposition products from the liquid culture broth, a modified two-phase liquid–liquid extraction (LLE) method was applied, following the protocol described by Wang et al. 2010. Next, 10 mL of culture supernatant was transferred into glass separatory funnels, and extraction was performed by splitting its contents (in equal parts) with acetone (1:1, v/v). The tube was vigorously vortexed (60 s) to allow phase equilibrium and maximize analyte transfer. NaCl was introduced to a final concentration of approximately 25% (w/v) to increase the recovery of the organic phase and to exert the salting-out effect. The system was then incubated at 100°C for 15 min in a precision water bath, and incubation was intended to enhance the partitioning of the analytes into the organic phase and simultaneously volatilize the remaining acetone. The resulting organic phase was pooled and filtered through an anhydrous sodium sulfate (Na_2SO_4) column to eliminate any traces of water. The dehydrated extract was further concentrated using a rotational evaporator at 40°C and reduced pressure. The dry residue, which contained the parent pesticide and possible metabolites, was resolubilized in 5 mL of HPLC-grade acetonitrile with the aid of ultrasonic treatment for 5 min to achieve full solubility. To further instrumentally determine it, a purification step in terms of traversing the extracts through a 0.22-m PTFE membrane was performed to remove fine suspended material. The final preparations were analyzed using an HPLC system to determine the carbofuran content after five days of incubation.

HPLC analysis and quantitative validation

Residual carbofuran was measured using an Agilent 1260 Infinity LC system (Santa Clara, CA, USA) with a quaternary pump, diode array detector (DAD), and an integrated degasser controlled using ChemStation software. Separation was carried out on a Zorbax SB-C18 column (4.6 x 150mm, 5um; agilent) at room temperature. The elution employed an isocratic mobile phase of HPLC grade acetonitrile and deionized water (70:30, v/v) at a constant flow rate of 1.0 mL/min. The sample injection volume was 50 µL, and the analyte was monitored at 280 nm (maximum absorbance). These steps were modified based on the methodology developed by Mustapha et al. (2020). To quantify, a six-point standard calibration curve was made (10–100 mg/L) in OriginPro 2024 (v10.1.0.178). This approach showed high linearity (R^2 > 0.999), as calculated using least-squares regression, enabling the accurate estimation of pesticide concentration based on the integration of peak areas.

Structural elucidation of transformation products via LC-MS/MS

To identify the metabolic intermediates of carbofuran, high-resolution mass spectrometry was coupled with liquid chromatography using a Waters 2695/Quattro Micro API LC-MS/MS system (Milford, MA, USA). Aliquots of 10 µL were harvested from the optimized RSM runs and injected onto an XBridge MS C18 column (2.1 × 100 mm, 2.5 µm; Waters) at 25°C. Binary gradient elution was employed at a flow rate of 0.3 mL/min, consisting of (A) 0.1% formic acid in water and

(B) LC-MS grade acetonitrile. The gradient profile progressed from 10% to 60% B over 10 min, followed by an increase to 90% B over 3 min and a 2 min re-equilibration to the initial conditions. Analytes were monitored via a photo-diode array (PDA) detector (230–360 nm) and subsequently introduced into the mass spectrometer via an electrospray ionization (ESI) source in both positive and negative polarity modes. The optimized source parameters included a capillary voltage of 3 kV, cone voltage of 30 V, and desolvation/source temperatures of 300 and 130°C, respectively. Full-scan MS data were obtained across a mass-to-charge ratio range of 50–999, and the structural elucidation of the degradation products was performed via collision-induced dissociation (CID) for targeted MS/MS fragmentation (Shit et al. 2025).

Acute ecotoxicological bioassays using *Eisenia fetida*

Test organism and artificial soil preparation

Age-synchronized mature earthworms (*Eisenia fetida*, 250–300 mg) were used as model organisms for ecotoxicological validation. The worms were reared in a 1:1 mixture of finely ground soil and farmyard manure, with moisture levels maintained at 50–60% and a regulated diet of livestock-derived organic fertilizer (Sanyal et al. 2015). For the bioassays, an artificial soil substrate was formulated according to the OECD guidelines, consisting of 10% pulverized rice husk, 20% kaolin clay, and 69% silica sand (w/w). The substrate pH was stabilized at 6.0 ± 0.2 using 1% calcium carbonate ($CaCO_3$). Prior to use, the growth matrix was desiccated, mechanically fragmented, and sieved to a size of ≤ 0.25 mm (De Silva et al. 2009).

Acute toxicity bioassay and LC_{50} determination

The comparative toxicity of carbofuran (commercial grade, 3% CG) and its primary biotransformation product, carbofuran phenol (technical grade, Sigma-Aldrich), was assessed using procedures modified from those established by Sanyal et al. (2015). Bioassays were conducted in rectangular polyethylene containers filled with 200 g of the prepared artificial soil. The experimental matrix included three different types of treatments: negative control, parent pesticide group, and byproduct group. The control group comprised artificial soil moistened with sterile deionized water and free from any chemical amendments. For the chemical treatments, five different concentrations were developed from the recommended agricultural dose (RAD) of 2.08 mg of the active ingredient (a.i.) per kg of soil recommended by the Central Insecticides Board and Registration Committee (CIBRC), India. The concentrations were 0.52, 1.04, 2.08, 4.16, and 6.24 mg a.i./kg soil, corresponding to 0.25 ×, 0.5 ×, 1.0 ×, 2.0 ×, and 3.0 × RAD, respectively. Ten size-matched earthworms were added to each unit after a 30 min equilibration time to ensure uniform distribution of the insecticides. All treatments and controls were maintained in triplicate at 28.0 ± 0.5°C and 50–60% relative humidity. Death was measured at 24-h intervals, and earthworms were considered nonviable when they could not respond to mechanical stimulation. Probit analysis (SPSS v.2513) was used to calculate the median lethal concentration (LC50) and the 95% fiducial limits after 96 h of exposure.

$$Detoxification\ Factor\ (DF) = \frac{LC_{50}\ of\ Metabolite}{LC_{50}\ of\ Parent\ Compound} \# (2)$$

Statistical analysis

Three distinct biological replicates (n = 3) of the experiments were completed, and the results are presented as the mean with standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post hoc test ($p < 0.05$) were used to determine the statistically significant depletion of carbofuran and concurrent microbial growth. Pearson 'scorrelation analysis (r) and coefficient of determination (R^2) were used to measure the metabolic relationship between the concentration of residual substrates and biomass accumulation.

Results and discussion

Metabolic profiling and growth kinetic analysis of strain PPH2

To determine the ability of *Bacillus paralicheniformis* PPH2 to use carbofuran as the sole source of carbon and energy, its metabolic plasticity was studied. Quantitative HPLC analysis (R^2 = 0.99; Supplementary Fig. S3) indicated that strain PPH2 has an extensive catabolic system for attenuating recalcitrant methylcarbamate.

The experimental group (CBF + PPH2) exhibited high substrate depletion, as illustrated in Table 1, with a reduction in carbofuran concentration from 98.33 ± 1.33 mg/L to 45.54 ± 2.89 mg/L after 120 h. This corresponds to a biodegradation efficiency of 53.69% and an average degradation rate of 10.56 mg/L/day. In contrast, the abiotic control recorded only a slight (3.4%) loss of substrate, and the biomass control (without CBF) showed no significant growth (OD600 = 0.002), confirming that the observed disappearance was a biologically mediated process and not spontaneous chemical hydrolysis or photodegradation of the substrate.

Table 1
Summary of carbofuran biodegradation and microbial growth parameters of *B. paralicheniformis* PPH2 after 120 h of incubation.

Treatment Group	Initial Carbofuran (mg/L)	Final Carbofuran (mg/L)	Degradation Efficiency (%)	Average Degradation Rate (mg/L/day)	Initial OD_{600}	Final OD_{600}
Experimental (CBF + PPH2)	98.33 ± 1.33	45.54 ± 2.89	53.69	10.56	0.05 ± 0.01	0.43 ± 0.01
Abiotic Control (CBF only)	99.47 ± 2.64	96.09 ± 1.36	3.4	0.68	-	-
Biomass Control (PPH2 only)	-	-	-	-	0.002 ± 0.001	0.002 ± 0.001

The kinetics of the degradation had a typical sigmoidal shape, with a short lag phase followed by an intensive catabolic phase of 24–72 h (Fig. 1a). During this exponential growth period, biomass proliferation (OD_{600} increased between 0.05 and 0.43) was perfectly correlated with pesticide consumption. This type of synchronous growth kinetics is specific to specialized degraders, in which the contaminant is the primary metabolic fuel (Mishra et al. 2020).

In order to further measure this metabolic dependency, Pearson correlation analysis was conducted (Fig. 1b). The findings indicated that the correlation between them was highly significant ($r = -0.963$, $p < 0.01$) and the coefficient of determination (R^2) was 0.928. This statistical data suggests that more than 92% of the change in microbial biomass can be directly traced to the catabolic use of carbofuran, which is an indication that the strain is not only tolerant of the pesticide but also actively mineralizes it for cellular synthesis.

The catabolic activity of *B. paralicheniformis* PPH2 was similar to that of other known *Bacillus* species. For instance, Nafeesa et al. (2017) described a Bacillus sp. that demonstrated the efficacy of a 17.71% reduction in carbofuran within the same period; however, PPH2 excelled at a three-fold rate. Mishra et al. (2020) showed an increase in absolute transformation (98%) but only over a longer period of 10 days. The rapid catabolic activity of PPH2, isolated from the unique gut environment of the earthworm *Glyphidrilus* sp., indicates a specialized evolutionary adaptation. The earthworm gut is a natural micro-bioreactor that concentrates organic contaminants and selects consortia of microbes with increased xenobiotic resilience (Dey et al. 2018; Patowary et al. 2023). The fact that PPH2 has a high degradation rate (10.56 mg/L/day) in MSM highlights its potential as a strong candidate for the bioaugmentation of pesticide-affected agricultural soils, where nutrient availability is commonly a limiting factor.

Multi-factorial optimization of degradation parameters via RSM

Model validation and predictive accuracy

A central composite design within response surface methodology was used to identify specific environmental optima to avoid bottlenecks encountered by *B. paralicheniformis* PPH2 metabolism. The efficiency of degradation varied greatly, with a minimum of 11.39% and a maximum of 63.45%, as shown in Table 2. The large range of this variation reflects the vulnerability of the bacterial catabolic apparatus to physicochemical changes and the need to adopt a multifactorial strategy to exploit the full bioremediation capabilities of the strain.

Table 2
Experimental design and response of carbofuran degradation optimization.

Run	A (pH)	B (Temperature, °C)	C (Agitation speed, rpm)	Response Degradation %
1	7.00	32.50	110.00	63.45
2	5.00	20.00	140.00	23.39
3	9.00	45.00	80.00	38.83
4	7.00	32.50	59.55	55.24
5	7.00	32.50	110.00	61.5
6	5.00	45.00	140.00	22.57
7	7.00	32.50	160.45	40.77
8	5.00	45.00	80.00	33.66
9	9.00	20.00	80.00	20.19
10	3.64	32.50	110.00	13.33
11	7.00	53.52	110.00	24.59
12	7.00	32.50	110.00	62.51
13	7.00	32.50	110.00	62.9
14	10.36	32.50	110.00	11.64
15	5.00	20.00	80.00	19.63
16	9.00	45.00	140.00	18.84
17	7.00	11.48	110.00	11.39
18	7.00	32.50	110.00	62.5
19	9.00	20.00	140.00	17.3
20	7.00	32.50	110.00	60.35

Table 3
Statistical model fit summary.

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	21.55	0.0568	-0.1200	-0.3570	10687.11	
2FI	23.64	0.0777	-0.3480	-1.1926	17267.12	
Quadratic	0.9082	0.9990	0.9980	0.9967	25.76	Suggested
Cubic	1.04	0.9992	0.9974	0.9893	84.38	Aliased

A second-order quadratic polynomial model was used to map landscape degradation. The resulting empirical relationship between the degradation efficiency (Y) and the independent variables pH (A), temperature (B), and agitation speed (C) is expressed by the following equation:

$$Y = 62.19 - 0.51A + 4.07B - 3.99C + 0.87AB - 1.94AC - 3.99BC - 17.51A^2 - 15.56B^2 - 4.95C^2 \# (3)$$

The model showed outstanding correlation statistics and a coefficient of determination (R^2) and adjusted R^2 of 0.9990 and 0.9980, respectively (Table 3). The predicted R^2 of 0.9967 was highly consistent with the adjusted R2 (difference < 0.2), confirming that the model was not overfitted and had high predictive power for new observations. Moreover, the adequate precision ratio value of 83.18 (far higher than the desirable level of 4.0) demonstrated a high signal-to-noise ratio. The assumptions of the statistical analysis were also met, as diagnostic plots (see Supplementary Fig. S4) ensured that the residuals were normally distributed.

ANOVA was used to rigorously test the statistical validity of the model (Table 4). The model F-value of 1059.71 (p < 0.0001) confirmed that the model was highly significant. The analysis revealed that all linear (B and C), interaction (AB, AC, and BC), and quadratic (A^2, B^2, and C^2) terms were significant ($p < 0.05$). Most importantly, the F-value of 0.34 ($p = 0.8686$) under Lack of Fit was not significant, indicating that the quadratic model was a strong fit to the experimental data and could make high-fidelity predictions.

Table 4
Analysis of variance (ANOVA) for the quadratic response surface model.

Source	df	Sum of Squares	Mean Square	F-value	p-value	
Model	7867.08	9	874.12	1059.71	< 0.0001	significant
A-pH	3.52	1	3.52	4.27	0.0658	
B-Temperature	226.28	1	226.28	274.32	< 0.0001	
C-Agitation speed	217.86	1	217.86	264.11	< 0.0001	
AB	6.07	1	6.07	7.36	0.0218	
AC	30.23	1	30.23	36.64	0.0001	
BC	127.6	1	127.6	154.69	< 0.0001	
A^2	4417.68	1	4417.68	5355.64	< 0.0001	
B^2	3490.1	1	3490.1	4231.11	< 0.0001	
C^2	353.14	1	353.14	428.11	< 0.0001	
Residual	8.25	10	0.8249			
Lack of Fit	2.1	5	0.4195	0.341	0.8686	not significant
Pure Error	6.15	5	1.23			
Cor Total	7875.33	19				

Interaction effects of environmental variables

The 3D response surface analysis (Fig. 2) indicates that there is a complex mechanistic interaction among the environmental factors.

pH and temperature (AB): The quadratic effect of pH (A^2, $p < 0.0001$) and its interaction with temperature were significant, even though the linear effect of pH was insignificant ($p = 0.065$). This implies that *B. paralicheniformis* PPH2 requires a well-regulated neutral to slightly alkaline pH (approximately 7.1) to maintain the integrity of its extracellular enzymes, especially at high temperatures.

pH and agitation (AC): pH and agitation are significantly interacting at a significantly lower p ($p < 0.0001$), which indicates that the bioavailability and solubility of carbofuran are optimized in an ideal pH. The mass transfer of the substrate to the bacterial cell surface is facilitated by agitation, however, only under an ionic environment (pH) that favors the enzymatic stability.

Temperature and agitation (BC) are important factors affecting microbial survival. We observed that degradation at low frequency (> 140 rpm) and high temperature resulted in considerable degradation reduction. This is probably because of the synergistic stress of both mechanical shear forces and thermal denaturation, which can damage fragile microbial membranes and inactivate the hydrolytic enzymes catalyzing the cleavage of carbamate. Other Bacillus optimization studies have also reported similar agitation inhibition (Zhang et al. 2020).

The degradation efficiency was optimized using numerical optimization, which allowed the determination of the global maximum. The model estimated the optimum efficiency of 63.48 per cent at pH 7.1, temperature 34.7 o C and the speed of agitation was 100rpm. A triplicate validation experiment was conducted under these specific conditions to validate the correctness of this in silico prediction. The resulting degradation of 62.07 ± 1.12% exhibited a small error difference with

the predicted degradation of 2.22% and a relative increase in carbofuran of 15.61% in contrast to the unoptimized initial assay. This close correspondence strictly justifies the CCD model as a valid and effective model to harmonize the bioremediation of carbamate pesticides at dynamic agricultural sites.

Structural characterization of metabolites and proposed biotransformation pathway

Comparative LC-MS/MS analysis was used to explain the metabolic fate of carbofuran upon its interaction with *B. paralicheniformis* PPH2. The resulting total ion chromatograms (TICs) indicated a change in the structure of the parent molecule during the 120 h incubation period. As shown in Fig. 3, the main peak, carbofuran (R_t 10.89 min), showed a marked decrease in the intensity in the experimental extracts relative to the intensity in the abiotic control. This loss was accompanied by the appearance of a prominent, more polar metabolic peak at a lower retention time (R_t 8.05 min), which is indicative of the formation of a more hydrophilic transformation product.

The identity of the parent compound was established by mass spectrometric analysis in positive electrospray ionization (ESI+) mode at m/z 221.25 $[M + H]^+$. A protonated molecular ion of the degradation product was observed at m/z = 164.36 $[M + H]^+$. Mass spectrometric analysis of the MS/MS fragmentation patterns and mass measurements revealed this metabolite to be carbofuran phenol ($C_{10}H_{12}O_2$, also known as 2,3-dihydro-2,2-dimethyl-7-benzofuranol). This identification was also confirmed by cross-referencing with the PubChem database and was in agreement with the metabolic profiles of other carbamate-degrading *Bacillus* strains (Mishra et al. 2020; Patowary et al. 2023).

The identification of carbofuran phenol as the primary metabolite indicates that the initial biotransformation by PPH2 proceeded via enzymatic hydrolysis of the carbamate ester bond. Hydrolytic cleavage is a critical step in environmental detoxification. In terms of toxicology, the methylcarbamate structure of carbofuran is considered a warhead that binds as an acetylcholine analog, resulting in the irreversible inactivation of acetylcholinesterase (AChE) in nontarget soil organisms (Lan et al. 2019; Gupta et al. 2019). PPH2 can disable the pesticide at the molecular level by promoting the loss of the methylcarbamate group.

Our results are consistent with the activities of hydrolysis observed in highly efficient degraders, including Enterobacter sp. Z1 and other Bacillus species, which degrade using hydrolase enzymes (e.g., mcd or cehA genes) to circumvent the formation of more toxic oxidative intermediates, such as 3-hydroxycarbofuran (Zhang et al. 2020; Duc 2021). The trend in retention time (10.89 to 8.05 min) confirms a change in the polar phenolic intermediate, which is capable of further microbial ring-opening or is a precursor to mineralization (Mishra et al. 2020).

It is important to note that this particular hydrolytic machinery is present in gut-associated earthworm isolates. This implies that *B. paralicheniformis* PPH2 has adapted to provide a symbiotic detoxification service to its host, *Glyphidrilus* sp., by swiftly transforming an acute neurotoxin into a much less toxic phenolic compound. Such mechanistic understanding provides the biochemical basis for the decrease in mortality recorded in the course of our ecotoxicological validation using *Eisenia fetida*.

Comparative ecotoxicological assessment: parent vs. transformation product

The acute systemic toxicity (LC50) of carbofuran and its main metabolite, carbofuran phenol, was compared to confirm the relevance of *B. paralicheniformis* PPH2-mediated biotransformation. This evaluation, based on the sentinel earthworm *Eisenia fetida*, is an important step in bridging the critical gap between microbial catabolism and ecosystem safety.

The mortality data (Table 5) strongly fit the probit regression model, as the chi-squared values were not significant ($\chi^2 < 0.7$; $p > 0.05$), indicating high experimental reliability.

Table 5
Comparative 96-h acute toxicity of carbofuran and its metabolite in *Eisenia fetida*.

Treatment	96-Hour LC_{50} (mg a.i./kg soil)	95% Confidence Limits	χ^2 (df = 3)	*p*-value
Carbofuran	5.458	3.513–18.363	0.207	0.976
Carbofuran phenol	9.061	5.042–5886.930	0.627	0.890

The 96-h LC_{50} of carbofuran, as summarized in Table 5, was found to be 5.458 mg a.i./kg soil. Conversely, the LC_{50} of the microbial metabolite carbofuran phenol was 9.061 mg a.i. / kg soil. This numerical change represents a 1.66-fold decrease in the acute systemic toxicity of *E. fetida*. This is denoted by the high rightward movement of the dose-response curve (Fig. 4), which implies that the earthworms were capable of enduring even higher levels of the transformation product than the original insecticide.

The structural disassembly of carbofuran molecules is essentially connected with the toxicological attenuation observed. The neurotoxicity of carbofuran is due to the presence of an acetylcholine (ACh) molecular mimic, the methylcarbamate side chain. This moiety interacts with the active site of the enzyme acetylcholinesterase (AChE) and causes the persistence of cholinergic crisis, paralysis, and death in soil organisms (Lan et al. 2019; Park et al. 2022). PPH2 breaks the particular neurotoxic warhead off the molecule by promoting the hydrolytic breakage of this carbamate bond to carbofuran phenol.

Carbofuran phenol does not have the targeted lethal neurotoxicity of the parent compound but retains some inherent toxicity, presumably through non-selective phenolic effects, including membrane disruption or oxidative stress (Gupta et al. 2019). This conversion of a highly potent neurotoxin into a less toxic phenolic intermediate is a characteristic of successful bioremediation. Our findings align with those of *Enterobacter* sp. Z1 and other degraders with high efficiency, in which microbial hydrolysis results in an immediate increase in the safety margin of non-target fauna (Zhang et al. 2020).

Ecologically, the conservation of ecosystem engineers (earthworms) is crucial for the preservation of soil porosity, nutrient circulation, and the redistribution of microbes (Sharma et al. 2017). The 1.66-fold attenuation of toxicity by PPH2 was proposed to be a specialized symbiotic adaptation that probably safeguards the host earthworm *Glyphidrilus* sp. against pesticide-induced death in its natural paddy field environment. Therefore, the use of PPH2 is a biologically verified concept for the rehabilitation of pesticide-stressed agricultural landscapes, which aligns with the goals of SDG 15 (Life on Land).

Conclusion

This study identified *Bacillus paralicheniformis* PPH2, a gastrointestinal tract-specific isolate of the paddy field earthworm, *Glyphidrilus* sp., as a powerful biotechnological agent for the directed control of carbofuran contamination. Through effective optimization of the catabolic performance of PPH2 by means of response surface methodology, we achieved a peak degradation efficiency of 62.07%, reflecting that the earthworm gut-associated microenvironment is a key site for storing microbial strains with high metabolic plasticity. Mechanistically, hydrolytic cleavage of the methylcarbamate ester bond was identified as the ultimate detoxification gateway, effectively disarming the neurotoxic potential of the pesticide. The resulting 1.66-fold decrease in acute systemic toxicity (LC_{50} shift from 5.458 to 9.061 mg/kg) in *Eisenia fetida* provided the quantitative "proof-of-safety" needed to restore the ecology.

This study has broader consequences than a laboratory victory, which is directly related to the United Nations Sustainable Development Goals (SDGs). This study contributes to SDGs 15 (Life on Land) and 2 (Zero Hunger) by making agricultural land self-sufficient in the long term through the restoration of soil integrity and protection of vital ecosystem engineers.

Eventually, this work is one of the pioneers to demonstrate a model of circular bioremediation, in which the individual relies on its own symbiotic microbial resources to remediate the habitat it creates. Although future studies must utilize multi-omics to describe the distinct hydrolase genes and assess the competitive stability of the strain in complex soil matrices, *B. paralicheniformis* PPH2 can be a good candidate for the sustainable restoration of soil. This combined system of statistical optimization and ecotoxicological verification offers a scientifically sound roadmap for global success in restoring pesticide-affected landscapes and ensuring the existence of terrestrial biodiversity.

Declarations

Ethical approval:

The study involving *Eisenia fetida* was conducted following OECD guidelines for toxicological testing. Ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC).

Conflict of interest:

The authors declare that there are no conflicts of interest that could be perceived to influence or bias this work.

Author Contribution

P.Shit, P.Sarangi, P.Sur, S.S.Gauri., Y.Sakai: Formal analysis, investigation and validation; P.P.Chakravorty and H.Jana: Resources and Supervision; P.Shit: Conceptualization, Methodology, Writing—original draft preparation, review and editing. All authors read and approved the final manuscript.

Acknowledgement

Puspendu Shit acknowledges the receipt of financial assistance in the form of a Junior and Senior Research Fellowship (JRF and SRF) from the Council of Scientific & Industrial Research (CSIR), New Delhi, India (File No: 08/745(0001)/2020-EMR-I).

Data Availability

All the raw data in this research can be obtained from the corresponding authors upon reasonable request.

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Figures

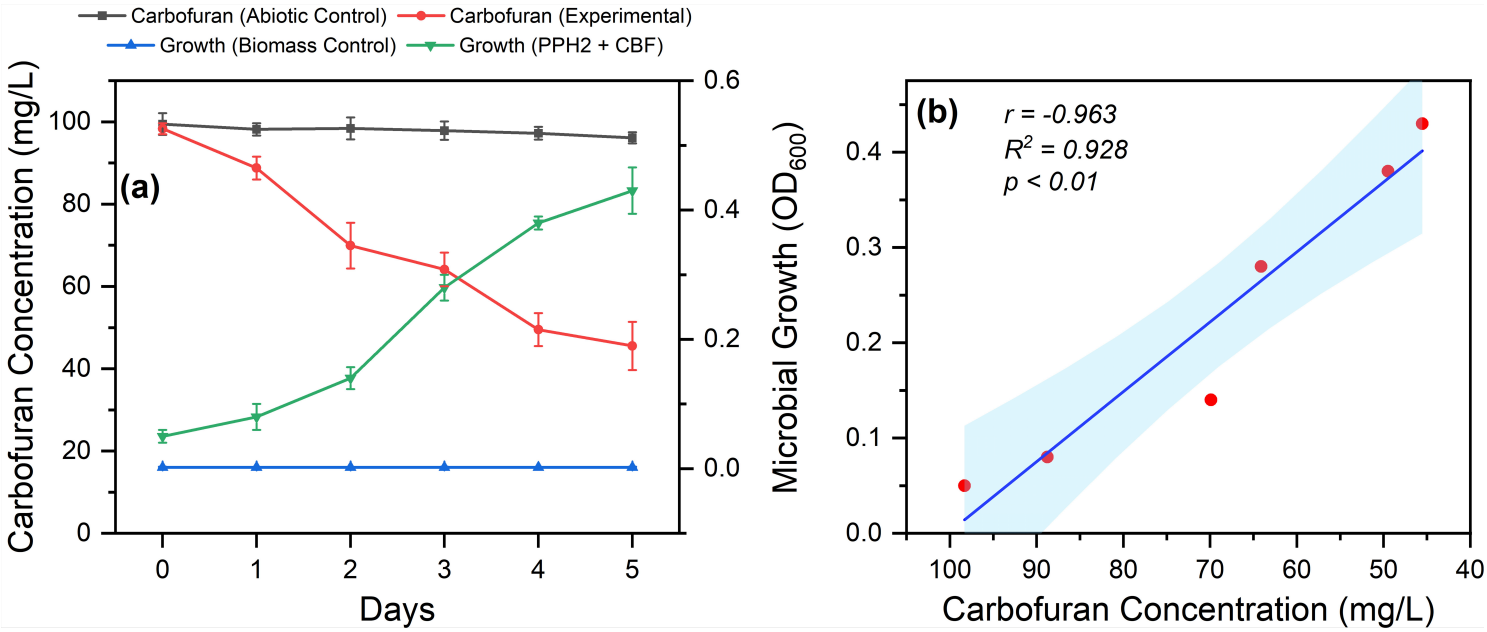


Figure 1

Biodegradation kinetics and metabolic correlation of carbofuran by *Bacillus paralicheniformis* PPH2. (a) Kinetics of carbofuran depletion and concomitant growth of *B. paralicheniformis* PPH2 over 120 h. Error bars denote the standard deviation of the independent biological replicates (*n* = 3). (b) Pearson correlation analysis between residual carbofuran concentration and microbial biomass.

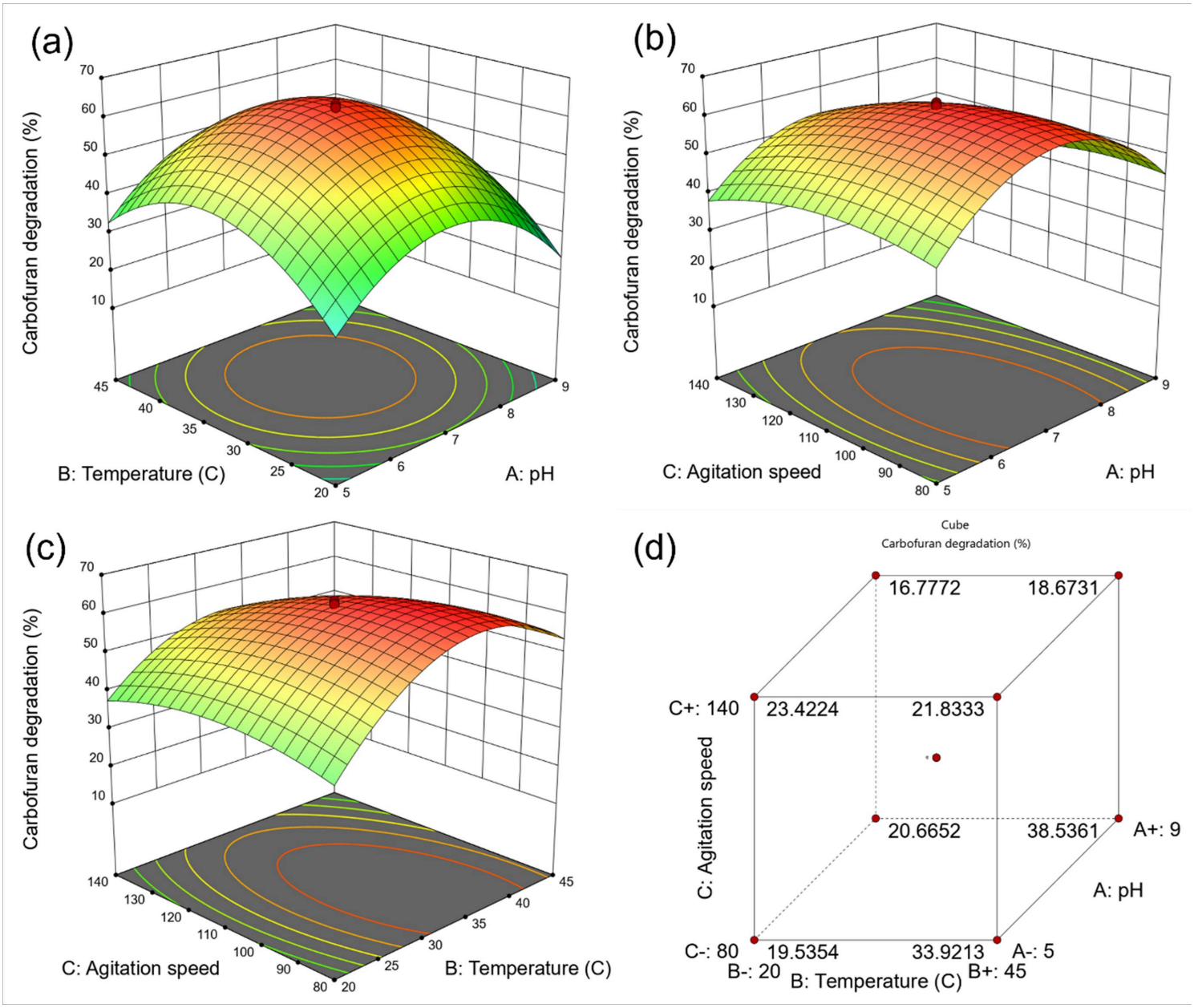


Figure 2

Three-dimensional (3D) response surface plots illustrating the interactive effects on carbofuran biodegradation: (a) pH and temperature; (b) pH and Agitation Speed; (c) Temperature and Agitation Speed; (d) cube representing the interaction of the three critical factors in a combined form.

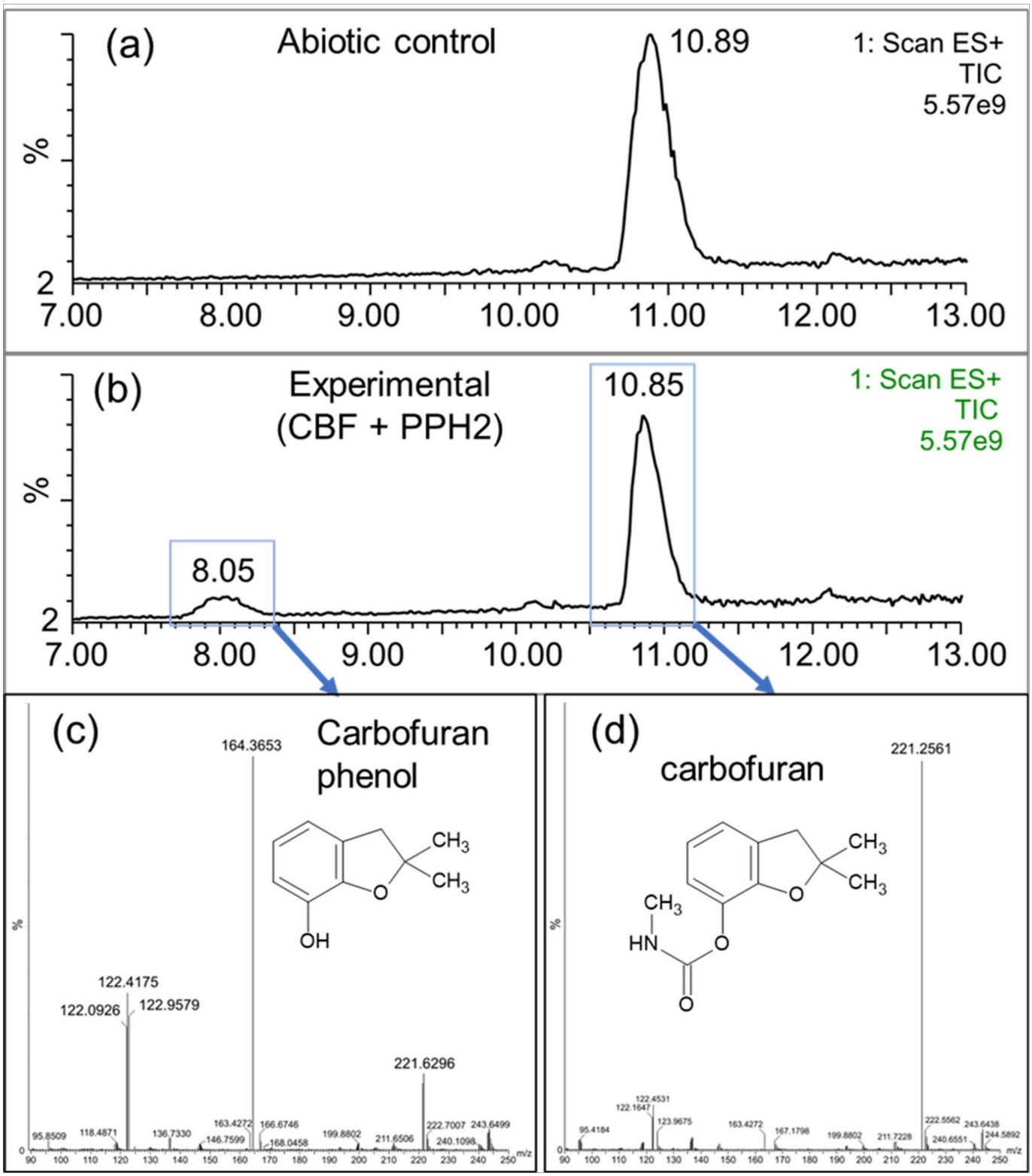


Figure 3

LC-MS/MS identification and structural characterization of carbofuran biotransformation products. (a) Total ion chromatogram (TIC) of the abiotic control, (b) TIC of the *B. paralicheniformis* PPH2 culture extract after 120 h, (c) mass spectrum of a more polar metabolic peak at R_t 8.05 min, and (d) mass spectrum of the parent compound (carbofuran).

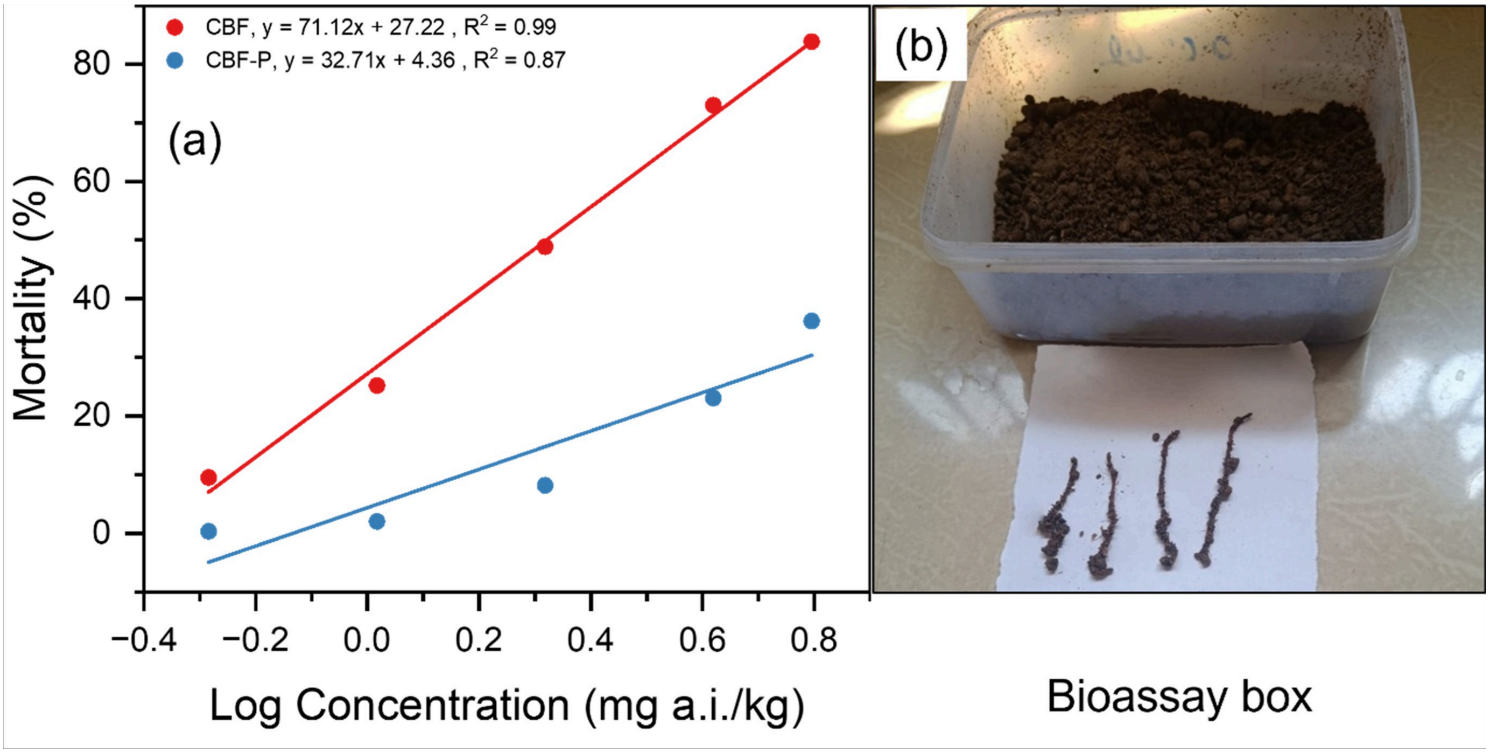


Figure 4

(a) Dose-response relationship showing the mortality (%) of *Eisenia fetida* versus the log concentration (mg a.i. /kg) of carbofuran (CBF, red) and carbofuran phenol (CBF-P, blue) after 96 hours of exposure. Linear regressions and R^2 values are indicated in the legend. (b) Image of the bioassay setup, showing the treated soil matrix and the earthworms used for toxicity testing.

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