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Biodegradation of Carbofuran by Bacillus subtilis Isolated from Agricultural Soils of Lahore, Pakistan

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Abstract

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate), a broad-spectrum N-methylcarbamate insecticide, constitutes a persistent organic contaminant of significant ecological and toxicological concern in intensively cultivated agricultural landscapes. The present study delineates the carbofuran biodegradation capacity of Bacillus subtilis isolated from contaminated agricultural soils in Lahore, Pakistan, under rigorously controlled in vitro conditions. Composite soil samples were obtained from five carbofuran-impacted cultivation sites; two morphologically distinct bacterial isolates (B1 and B2) were recovered by serial enrichment culture, with isolate B1 unambiguously assigned to B. subtilis through complete concordance across a 12-parameter biochemical identification matrix. Residual carbofuran concentrations were quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) at UV 230 nm using a validated five-point linear calibration function ($R^2 > 0.998$; working range: 0.5–8.0 µg/mL). Biodegradation experiments were conducted across a full factorial design encompassing three initial substrate concentrations (1.5, 3.0, and 4.5 µg/mL), three incubation temperatures (25, 35, and 45°C), and three pH conditions (2.5, 6.5, and 4.5) over five consecutive days. Under thermodynamically optimal conditions (35°C, pH 6.5), isolate B1 achieved near-complete elimination of carbofuran (99.9%) at the lowest tested concentration (1.5 µg/mL) within four days of incubation. Two-way analysis of variance identified both temperature ($F = 30.68$, $p < 0.001$) and initial substrate concentration ($F = 5.11$, $p = 0.011$) as statistically significant determinants of degradation efficiency, with temperature accounting for the dominant fraction of total variability. Duncan's Multiple Range Test confirmed 35°C as the unequivocal thermal optimum and established substrate inhibition as the mechanistic basis for the observed concentration-dependent attenuation of degradation. Collectively, these findings provide quantitative evidence for the utility of B. subtilis as a tractable biological agent for the remediation of carbofuran-contaminated agricultural soils under conditions representative of South Asian cultivation environments.

INTRODUCTION

Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a systemic, broad-spectrum N-methylcarbamate insecticide deployed globally to control soil-dwelling insects, plant-parasitic nematodes, and foliar arthropod pests across economically critical crops including rice, maize, and sugarcane (Mishra et al., 2020). Its agronomic utility rooted in systemic translocation via root absorption and vascular distribution, coupled with high acute pesticidal efficacy and comparatively low unit-area application cost has sustained its widespread use across South and Southeast Asian agricultural systems despite escalating regulatory scrutiny. At the biochemical level, carbofuran exerts acute neurotoxicity through reversible inhibition of acetylcholinesterase at cholinergic synapses, a mechanism that, while effective against target arthropods, engenders substantial hazard to mammalian non-target species (LD_{50} rat, oral: 8–14 mg/kg) and non-target invertebrate communities (Kumar et al., 2022). Its high aqueous solubility (700 mg/L at 25°C) further amplifies environmental risk by facilitating subsurface leaching to groundwater aquifers and lateral transport to surface water bodies, rendering it among the more concerning carbamate contaminants in agricultural catchments (Mishra et al., 2020).

The environmental persistence of carbofuran is governed by a complex, multivariate interplay of soil physicochemical parameters including pH, texture, organic carbon content, and cation exchange capacity with biotic and abiotic transformation processes encompassing microbial catabolic activity, alkaline and acid hydrolysis, and direct photolysis. Reported soil half-lives span 30 to > 120 days contingent on these site-specific determinants (Ataikiru and Odesiri-Eruteyan, 2021). The U.S. Environmental Protection Agency and the European Food Safety Authority have implemented cancellation or severe restriction of carbofuran registrations on the basis of documented acute avian toxicity and groundwater contamination risk; nevertheless, the compound persists as an actively marketed and applied insecticide throughout much of South Asia, including Pakistan, where regulatory enforcement capacity remains uneven (Bhatt et al., 2021). Residue surveillance in Lahore and the broader Punjab agricultural region has detected carbofuran in soil matrices, irrigation water, and food commodity samples at concentrations exceeding permissible thresholds, underscoring an urgent requirement for cost-effective, locally applicable remediation technologies (Jaffar et al., 2022).

Microbial biodegradation constitutes the principal biogeochemical pathway for carbofuran dissipation in agricultural soil ecosystems and provides the mechanistic foundation for microorganism-mediated bioremediation of carbamate pesticide contamination. A phylogenetically diverse array of bacterial taxa has been characterized as metabolically competent carbofuran degraders, encompassing genera including Pseudomonas, Enterobacter, Sphingomonas, Chryseobacterium, and Bacillus (Sopiah et al., 2022; Park et al., 2022; Umar Mustapha et al., 2020). Within this functional consortium, Bacillus subtilis occupies a particularly advantageous position from an applied bioremediation perspective, by virtue of its numerical dominance and ubiquitous distribution in agricultural soil microbiomes, extraordinary metabolic plasticity enabling utilization of structurally diverse xenobiotic substrates, capacity for endospore formation that confers exceptional desiccation tolerance and long-term viability in formulated bioinoculant preparations, formal GRAS (Generally Recognized As Safe) designation supporting unrestricted agricultural deployment, and an extensively characterized repertoire of secondary metabolite biosynthetic pathways with ancillary soil-health benefits (Omeiri et al., 2022; Jamali et al., 2020). Prior investigations have documented effective carbofuran biodegradation by Bacillus species in both planktonic liquid culture and immobilized-cell soil slurry configurations, achieving maximum efficiencies exceeding 90% under optimized physicochemical conditions (Duc and Huy, 2022; Duc, 2022).

The principal physicochemical determinants of microbial carbofuran catabolism substrate concentration, incubation temperature, and culture medium pH exert decisive influence on catabolic enzyme kinetics, membrane-associated transport efficiencies, and bacterial growth dynamics, thereby dictating overall biodegradation performance. Notwithstanding their central importance, systematic, factorial-design evaluation of these parameters employing a validated quantitative analytical platform remains conspicuously underrepresented in the published literature, particularly for bacterial isolates recovered from Pakistani agricultural environments. Moreover, the preponderance of extant degradation studies has relied upon spectrophotometric quantification methods that are inherently susceptible to matrix interference and exhibit inadequate selectivity for carbofuran in complex biological matrices containing co-eluting metabolites and pigments (Carvajal and Veglia, 2021). Reverse-phase HPLC with

UV detection addresses these analytical limitations by providing the selectivity, sensitivity, linearity, and reproducibility requisite for regulatory-grade carbofuran residue quantification and has been endorsed as the reference analytical method in international monitoring frameworks (Bakanov et al., 2023).

The present investigation was designed to: (i) isolate and phenotypically characterize B. subtilis from carbofuran-contaminated agricultural soils in Lahore, Pakistan; (ii) systematically evaluate its carbofuran biodegradation capacity across a matrix of physiologically relevant substrate concentrations, incubation temperatures, and pH regimes; (iii) quantify residual carbofuran by validated RP-HPLC; and (iv) apply inferential statistical modelling to partition the relative contributions of key physicochemical variables to biodegradation variance. The resultant data are intended to furnish a mechanistically grounded scientific basis for the rational design of bioremediation strategies appropriate to the specific agroclimatic and soil-physicochemical conditions of intensively cultivated agricultural districts in Punjab, Pakistan.

MATERIALS AND METHODS

Study Area and Soil Sample Collection

Composite soil samples were systematically collected from five intensively cultivated agricultural sites distributed across Lahore district, Punjab, Pakistan, each with a documented multi-season history of carbofuran application and confirmed proximity to actively treated or recently fallowed crop fields. At each sampling location, subsurface composite samples were obtained from the 0–15 cm A-horizon using a stainless-steel auger; each composite was derived from five equidistant sub-sampling points arrayed within a demarcated 10 m × 10 m plot, thereby minimizing spatial heterogeneity bias. Samples were immediately transferred to sterile polypropylene collection bags, maintained at 4°C during transport, and processed within 24 hours of field collection to preserve microbial community integrity.

Bacterial Isolation and Enrichment Culture

Sequential serial enrichment culture was employed as the primary selection strategy to preferentially amplify carbofuran-catabolizing microorganisms from background soil microbiota. Ten-gram soil subsamples were homogenized in 90 mL of sterile mineral salt medium (MSM) supplemented with technical-grade carbofuran (98.5% purity) as the exclusive carbon and energy source at a concentration of 50 mg/L. Enrichment flasks were maintained at 37°C on a temperature-controlled rotary shaker at 150 rpm for 7 days. Following three successive transfer cycles under selective pressure, enriched cultures were serially diluted and plated on nutrient agar; morphologically distinct colony types exhibiting Bacillus-consistent macroscopic characteristics were selected for further characterization. All procedural steps were executed under strict aseptic conditions within a Class II biological safety cabinet.

Pure Culture Isolation and Gram Staining

Presumptive Bacillus isolates were subcultured to axenic purity through iterative single-colony transfer on nutrient agar. Macroscopic colony morphology encompassing diameter, elevation, form, margin architecture, pigmentation, and surface texture was systematically characterized following 24 and 48 hours of incubation at 37°C. Gram staining was performed on mid-logarithmic-phase cultures according to the canonical four-step protocol employing crystal violet primary stain, Gram iodine mordant, acetone-ethanol decolorizer, and safranin counter-stain, with microscopic examination conducted at 1000× magnification under immersion oil on a compound light microscope. Endospore formation was confirmed by the Schaeffer-Fulton malachite green-safranin differential staining method. Cell motility was evaluated by both wet-mount and hanging-drop preparations under phase contrast optics.

Biochemical Characterization and Species Identification

Isolates fulfilling morphological and staining criteria consistent with the genus Bacillus were subjected to a standardized 12-parameter biochemical characterization panel encompassing: catalase activity (3% H_2O_2 bubble production), oxidase activity (Kovács reagent), methyl red reaction (mixed-acid fermentation indicator), citrate utilization (Simmons citrate agar), urease production (Christensen urea agar), starch hydrolysis (iodine flooding after 48-hour incubation on starch agar), and fermentation of glucose, maltose, xylose, and mannitol using phenol red broth, complemented by assessment of motility and endospore production. All biochemical assays were performed in biological triplicate, and reaction outcomes were interpreted against the reference biochemical fingerprint of B. subtilis as tabulated in Bergey's Manual of Systematic Bacteriology, with additional cross-validation against published phenotypic identification criteria (Sitotaw et al., 2022; Lu et al., 2018).

Inoculum Preparation and Bacterial Cell Count

For deployment in biodegradation experiments, isolate B1 (confirmed B. subtilis) was propagated to late-exponential phase in 100 mL nutrient broth at 37°C with continuous orbital agitation at 150 rpm. Cells were harvested by centrifugation at 5,000 × g for 10 minutes, the spent supernatant was decanted, and the cell pellet was resuspended in sterile distilled water and adjusted to an optical density of 0.500 at 600 nm. This suspension density was empirically calibrated to a viable cell concentration of approximately 1.22×10^6 colony-forming units (CFU)/mL, as confirmed by the standard pour-plate enumeration method on nutrient agar following serial tenfold dilution.

Carbofuran Degradation Experiment

Biodegradation experiments were conducted in sterile 250 mL Erlenmeyer flasks, each containing 100 mL of carbofuran-supplemented MSM at one of three nominal substrate concentrations: 1.5, 3.0, or 4.5 µg/mL. Each flask received a 1 mL inoculum of the standardized B. subtilis B1 suspension to achieve a reproducible starting cell density. Flasks were incubated under continuous orbital agitation on temperature-controlled shakers set at three discrete temperatures (25, 35, and 45°C), each combined

factorially with three pH conditions (2.5, 6.5, and 4.5) adjusted by incremental addition of 0.1 M HCl or 0.1 M NaOH and monitored at the time of inoculation. Uninoculated abiotic control flasks were maintained in parallel under each treatment combination to quantify non-biological carbofuran losses attributable to hydrolysis and volatilization. All experimental treatments were executed in biological triplicate. At 24-hour intervals over five consecutive days, 2 mL aliquots were aseptically withdrawn, clarified by centrifugation to remove cellular biomass, and stored at 4°C pending chromatographic analysis.

RP-HPLC Analysis of Carbofuran Residues

Residual carbofuran in clarified culture supernatants was quantified by RP-HPLC on a Shimadzu LC-2030C chromatographic system equipped with a UV detector operating at 230 nm and an Inertsil ODS-3 C18 reversed-phase column (250 mm × 4.6 mm, 5 µm particle size). The optimized isocratic mobile phase consisted of HPLC-grade acetonitrile and ultrapure water (50:50, v/v), delivered at a constant flow rate of 1.0 mL/min at a column temperature of 30°C. The injection volume was 20 µL per run. External quantification was performed against a five-point calibration curve constructed from certified carbofuran reference standards at concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 µg/mL, encompassing the full range of experimental substrate concentrations. Percentage degradation was computed as the fractional reduction in residual carbofuran concentration relative to the initial concentration in each treatment flask, with arithmetic correction for abiotic control values to account for non-biological dissipation (Gangola et al., 2022).

Statistical Analysis

All biodegradation data were analyzed by two-way analysis of variance (ANOVA), incorporating carbofuran concentration and incubation temperature as fixed independent factors, followed by Duncan's Multiple Range Test (DMRT) for post hoc resolution of inter-group differences among treatment means. The threshold for statistical significance was established at $\alpha = 0.05$ throughout. Simple linear regression analysis was applied to characterize the temporal trajectory of degradation efficiency as a function of incubation duration. All statistical computations, including ANOVA decomposition, pairwise comparisons, and regression modelling, were performed using Minitab Statistical Software (Version 21.0; Minitab LLC, State College, PA, USA) (Das et al., 2022).

RESULTS

Isolation and Morphological Characterization of Bacillus Isolates

Enrichment culture of 15 composite soil samples yielded visible turbidity indicative of bacterial proliferation in 13 of 15 replicate flasks, confirming widespread distribution of carbofuran-tolerant

microbiota across the sampling sites. Plating of enriched cultures onto nutrient agar and systematic morphological screening identified two colony phenotypes consistent with the genus Bacillus, designated isolates B1 and B2. On nutrient agar after 48 hours at 37°C, isolate B1 produced large (5–8 mm), circular, flat, cream-coloured colonies exhibiting characteristic irregular to undulate margins and a dry, finely rugose surface texture. Isolate B2 formed smaller (2–3 mm), mucoid, pale-yellow colonies with entire margins. Gram staining of both isolates revealed Gram-positive, straight rod-shaped cells disposed singly or in short chains, with central to subterminal endospores and confirmed flagellar motility by wet-mount and hanging-drop preparations, collectively consistent with the genus-level phenotype of Bacillus.

Biochemical Characterization and Species Identification

The 12-parameter biochemical profiles of isolates B1 and B2 are presented in Table 1. Isolate B1 was strongly catalase-positive, oxidase-positive, citrate-utilization-positive, methyl red-negative, and urease-negative; it additionally demonstrated active starch hydrolysis and fermentative utilization of glucose, maltose, xylose, and mannitol, together with confirmed motility and endospore production under the assay conditions employed. This constellation of biochemical reactions was concordant in all 12 parameters with the reference diagnostic fingerprint of Bacillus subtilis as delineated in Bergey's Manual of Systematic Bacteriology and cross-referenced against established taxonomic criteria (Table 2). Isolate B2 exhibited discordant reactions in four of 12 parameters and failed to conform to any recognized Bacillus species profile; it was consequently excluded from all subsequent degradation experiments.

Table 1
Biochemical profiles of Bacillus isolates B1 and B2 recovered from carbofuran-enriched agricultural soils. (+) = positive reaction; (−) = negative reaction.

Biochemical Test	Isolate B1 (Large)	Isolate B2 (Small)
Catalase	+	−
Oxidase	+	+
Methyl Red (MR)	−	+
Citrate Utilization	+	−
Urease	−	+
Starch Hydrolysis	+	+
Glucose Fermentation	+	+
Maltose Fermentation	+	−
Xylose Fermentation	+	+
Mannitol Fermentation	+	−
Motility	+	+
Spore Formation	+	+

Table 2
Comparison of the biochemical profile of isolate B1 with the reference biochemical fingerprint of Bacillus subtilis. Perfect concordance across all 12 parameters confirmed species identification.

Biochemical Test	Isolate B1	Reference B. subtilis
Catalase	+	+
Oxidase	+	+
Methyl Red (MR)	–	–
Citrate Utilization	+	+
Urease	–	–
Starch Hydrolysis	+	+
Glucose Fermentation	+	+
Maltose Fermentation	+	+
Xylose Fermentation	+	+
Mannitol Fermentation	+	+
Motility	+	+
Spore Formation	+	+

Growth on Selective Media and Colony Enumeration

Growth on mannitol-egg yolk-polymyxin (MYP) agar after 48 hours at 37°C yielded colonies of B. subtilis B1 surrounded by a characteristic opaque lecithinase-positive precipitation halo, consistent with the biosurfactant-producing phenotype associated with this species. Viable cell enumeration by the spread-plate method on nutrient agar at the time of experimental inoculation yielded a reproducible count of 1.22×10^6 CFU/mL across replicate preparations.

HPLC Calibration and Quantitative Analysis

A five-point external calibration function for carbofuran quantification was established using certified reference standards spanning concentrations of 0.5 to 8.0 µg/mL. The calibration response was robustly linear across the entire working range ($R^2 > 0.998$), satisfying the analytical linearity criterion for validated quantitative HPLC methods and confirming fitness for purpose across the full range of experimental substrate concentrations. Under the optimized chromatographic conditions, carbofuran eluted with high reproducibility at a retention time of 15.0 minutes, providing adequate resolution from potential matrix interferents in culture supernatant samples.

Effect of Concentration, Temperature, and pH on Carbofuran Degradation

Figure 2 presents the percentage carbofuran degradation achieved across all nine treatment combinations over five consecutive incubation days, encompassing the complete factorial design of substrate concentrations, temperatures, and pH conditions. Biodegradation profiles were markedly heterogeneous across treatment combinations, with degradation efficiency exhibiting strong systematic dependence on the interaction of thermal regime and pH condition, superimposed on a secondary modulating effect of substrate concentration.

Under optimal conditions of pH 6.5 and 35°C, isolate B1 achieved near-complete or complete elimination of carbofuran (99.9–100%) at 1.5 µg/mL by day 2 of incubation, and at both 3.0 µg/mL and 4.5 µg/mL by days 4 and 4–5, respectively, representing the highest degradation efficiencies recorded across the entire experimental matrix. By marked contrast, degradation under strongly acidic conditions (pH 2.5) combined with sub-optimal temperature (25°C) was substantially attenuated, yielding mean five-day degradation values of 49.63%, 20.57%, and 32.83% at 1.5, 3.0, and 4.5 µg/mL, respectively values that in several treatment combinations were less than one-third of those recorded under optimal conditions. Degradation efficiency at pH 4.5 and 45°C was consistently intermediate across all concentration levels, indicating partial but not maximal expression of catabolic capacity. A monotonic inverse relationship between initial substrate concentration and percentage degradation was evident across virtually all temperature-pH combinations, with the lowest concentration treatment (1.5 µg/mL) consistently yielding the highest fractional degradation, consistent with substrate inhibition of microbial catabolic activity at elevated xenobiotic loads.

RP-HPLC Chromatographic Analysis

Statistical Analysis of Carbofuran Degradation

Table 3
Two-way ANOVA table for carbofuran degradation (%) by Bacillus subtilis B1. DF = degrees of freedom; Adj SS = adjusted sum of squares; Adj MS = adjusted mean square.

Source of Variation	DF	Adj SS	Adj MS	F-value	p-value
Concentration (µg/mL)	2	3,558	1,779.2	5.11	0.011
Temperature (°C)	2	21,351	10,675.3	30.68	< 0.001
Time (hours)	4	3,575	893.8	2.57	0.054
Error	36	12,527	348.0		
Total	44	41,011			

Two-way ANOVA partitioned total degradation variance into statistically significant contributions from both carbofuran concentration ($F = 5.11$, $p = 0.011$) and incubation temperature ($F = 30.68$, $p < 0.001$), confirming that both factors exerted independent, statistically meaningful influences on biodegradation efficiency (Table 3). Notably, temperature accounted for a substantially larger proportion of total explained variance than substrate concentration, underscoring the primacy of thermal modulation over substrate loading as a determinant of B. subtilis catabolic performance within the concentration range investigated. The main effect of incubation time approached but did not attain conventional statistical significance ($F = 2.57$, $p = 0.054$), reflecting a consistent progressive trend in cumulative degradation across the five-day observation period that was nevertheless insufficiently powered to clear the $\alpha = 0.05$ threshold given the temporal sampling density employed.

Duncan's multiple range test of mean degradation across substrate concentration levels demonstrated that the 1.5 µg/mL treatment yielded significantly greater degradation than 3.0 µg/mL, while the 4.5 µg/mL treatment occupied a statistically intermediate position, exhibiting no significant difference from either of the flanking concentrations. This non-monotonic pattern of statistical significance wherein the extreme concentrations are not mutually distinguishable yet both differ from an intermediate value is mechanistically consistent with Haldane-type substrate inhibition kinetics, in which excessive pollutant concentrations suppress catabolic enzyme activity through competitive or non-competitive inhibition, thereby partially counteracting the mass-action advantage conferred by elevated substrate availability.

Duncan's Multiple Range Test for temperature revealed that the 35°C regime produced mean degradation values significantly exceeding those recorded at both 25°C and 45°C, while 25°C and 45°C were not statistically distinguishable from each other. The unambiguous identification of 35°C as the thermal optimum is concordant with the established mesophilic physiological range of B. subtilis and corresponds to the temperature of maximal specific activity for the carbamate hydrolase and esterase isoforms implicated in carbofuran catabolism in this species.

Post hoc temporal pairwise comparisons demonstrated a statistically progressive accumulation of degradation across the five-day incubation period, with the greatest magnitude of incremental increase observed between 24 and 96 hours. The degradation efficiency recorded at 96 hours (Day 4) was statistically indistinguishable from that at 120 hours (Day 5), indicating that the biodegradation system had effectively reached a near-maximal steady state under optimal conditions by the fourth day of incubation, consistent with substrate depletion rather than enzyme inactivation as the limiting process.

DISCUSSION

The present investigation unequivocally established the capacity of a soil-derived B. subtilis isolate to metabolize carbofuran across a physiologically relevant matrix of substrate concentrations and physicochemical conditions, achieving near-complete pesticide elimination under a defined optimal regime. The selective enrichment strategy employed serial propagation in carbofuran-supplemented mineral salt medium with the insecticide as sole carbon source constitutes a well-validated and

reproducible methodology for enriching degradative competence from complex soil microbiomes and recovering numerically minor but functionally specialized populations that would remain below the detection threshold of direct plating on non-selective media (Randika and Bandara, 2022). The unambiguous phenotypic identification of isolate B1 as B. subtilis via complete 12-parameter biochemical concordance with the type-strain reference profile is consistent with the extensive documentation of this species as a metabolically versatile environmental microorganism capable of utilizing structurally diverse xenobiotic substrates as growth-supporting carbon sources (Omeiri et al., 2022).

The optimal degradation conditions identified in this investigation pH 6.5 and 35°C correspond precisely to the physiological optima for growth and enzyme synthesis in B. subtilis as a neutrophilic, mesophilic bacterium, and are mechanistically consistent with the kinetic properties of carbofuran-hydrolyzing enzymes, most plausibly carbamate hydrolases or broad-specificity esterases that catalyze nucleophilic attack on the ester carbonyl of carbofuran, liberating carbofuran phenol and methylcarbamic acid as primary hydrolytic products (Park et al., 2022). The well-established sensitivity of microbial enzymatic activity to pH and temperature through governing the ionization states of catalytic histidine and serine residues in hydrolase active sites, modulating substrate-enzyme binding geometry, and determining the thermodynamic stability of the protein tertiary fold provides a coherent mechanistic basis for the strong environmental dependence of biodegradation efficiency observed across treatment combinations (Ataikiru and Odesiri-Eruteyan, 2021). The sharp, dose-dependent decline in degradation efficiency under strongly acidic conditions (pH 2.5) is attributable to multiple concurring mechanisms: protonation of active-site catalytic residues abolishing hydrolytic activity, disruption of membrane potential and proton motive force essential for substrate import, and direct membrane structural damage at extreme pH. The degradation attenuation observed at 45°C likely reflects partial thermal denaturation of mesophilic catabolic enzyme complexes and an increase in membrane lipid bilayer fluidity that compromises the structural integrity of membrane-associated biodegradative machinery (Mishra et al., 2020).

The inverse relationship between initial carbofuran concentration and percentage degradation efficiency consistently manifest across all nine treatment combinations represents a canonical signature of Haldane-type substrate inhibition kinetics and has been reported with high reproducibility in published biodegradation literature across diverse pesticide-microorganism systems. At concentrations of 3.0 and 4.5 µg/mL, percentage degradation was markedly suppressed relative to 1.5 µg/mL under identical temperature and pH conditions, indicating that elevated substrate concentrations exceeded the catabolic capacity of the standardized inoculum (1.22×10^6 CFU/mL), imposed cytotoxic or metabolic stress through accumulation of reactive carbofuran transformation intermediates, and inhibited catabolic enzyme activity through competitive binding to non-productive enzyme-substrate complexes or allosteric modulation. An analogous concentration-dependent attenuation of degradation efficiency has been documented for Bacillus sp. DT1, which exhibited progressive reductions in carbofuran elimination rate with increasing substrate concentration in soil slurry systems (Duc and Huy, 2022). For translational application in field bioremediation contexts characterized by high residual carbofuran burdens, these data collectively indicate that effective biological treatment will require adoption of strategies to

overcome substrate inhibition, including augmented inoculum densities, pre-adaptation of the inoculant to progressively higher substrate concentrations, cell immobilization to protect against cytotoxic exposure, or staged substrate addition protocols.

The achievement of near-complete (99.9%) carbofuran elimination at the lowest tested substrate concentration (1.5 µg/mL) within four days of in vitro incubation constitutes a quantitatively significant outcome that compares favourably with biodegradation efficiencies reported for well-characterized carbofuran-degrading bacterial systems in the published literature, with the caveat that cross-study comparisons are substantially confounded by differences in inoculum density, substrate concentration range, medium composition, and analytical methods employed. Duc and Huy (2022) reported complete carbofuran elimination at 60 mg/L a substrate concentration approximately four orders of magnitude greater than the current study by immobilized Bacillus sp. DT1 preparations in complex soil slurry matrices, indicating that the biodegradation capacity characterized in the present investigation could likely be substantially enhanced under higher-density and immobilized-cell configurations appropriate for field deployment. Park et al. (2022) employed high-resolution non-targeted metabolomics to elucidate the complete catabolic cascade in Chryseobacterium sp. BSC2-3, identifying 3-hydroxycarbofuran as the dominant first-generation transformation product and demonstrating progressive metabolic channelling through the 2-aminobenzoate degradation pathway to yield tricarboxylic acid cycle intermediates. While the metabolite profile of carbofuran transformation by isolate B1 was not characterized in the present study, the kinetic signatures of degradation rapid early-phase removal followed by plateau dynamics are mechanistically consistent with the initiating enzymatic step of carbamate hydrolysis, as characterized for Bacillus-associated carbamate hydrolase isoenzymes (Omeiri et al., 2022).

The temporal dynamics of biodegradation characterized by a phase of accelerating elimination between 24 and 96 hours of incubation, succeeded by rate attenuation and near-plateau behavior by day 4–5 under optimal conditions are mechanistically consistent with the coupled kinetics of bacterial population growth and inducible catabolic enzyme expression. During the initial lag phase (0–24 hours), the inoculated B. subtilis population is proposed to undergo transcriptional and translational re-programming to induce the carbofuran-specific or broad-specificity hydrolase gene(s), with concomitant synthesis of membrane transport proteins facilitating intracellular substrate accumulation; measurable degradation rates during this period are accordingly minimal. As the population enters exponential growth, the progressive increase in both cell density and per-cell enzyme complement drives a corresponding acceleration of substrate elimination. The plateau dynamics observed by days 4–5 are most parsimoniously attributed to substrate depletion rather than to enzyme inactivation or population decline, as the approach to exhaustion of the limiting substrate constrains the hyperbolic Michaelis-Menten kinetics of the catabolic enzyme system (Ataikiru and Okorhi, 2022). This temporal pattern exhibits strong concordance with published accounts: Sopiah et al. (2022) documented peak carbofuran elimination efficiency between 48 and 72 hours by indigenous Indonesian bacterial communities, while Patowary et al. (2023) recorded maximum degradation of 89.2% by day 5 of incubation in Pseudomonas aeruginosa S07 liquid cultures.

The two-way ANOVA framework adopted in this investigation provided rigorous, model-based partitioning of biodegradation variance attributable to each principal physicochemical determinant. The pronounced dominance of temperature ($F = 30.68$, $p < 0.001$) over substrate concentration ($F = 5.11$, $p = 0.011$) as a source of variance quantitatively demonstrates that enzymatic modulation by the thermal regime exerts a more powerful regulatory influence on B. subtilis carbofuran catabolic capacity than substrate loading within the concentration range evaluated. This hierarchy of effects carries substantive implications for field-scale bioremediation strategy development: in situ temperature management approaches including targeted application of insulating organic mulches, biochar amendments that modulate soil thermal buffering capacity, or adjustment of inoculant application timing to coincide with seasonal or diurnal temperature windows approximating 35°C may confer greater gains in biodegradation performance than proportional reductions in applied carbofuran concentration or manipulation of substrate availability per se (Huang and Lu, 2021).

From the perspective of translational biotechnology, B. subtilis presents a compelling combination of attributes that collectively support its development as an industrial-scale bioremediation platform organism. Its formal GRAS certification under United States and European regulatory frameworks, extensive safety record as an agricultural biocontrol and growth-promotion inoculant, capacity for endospore-mediated dormancy conferring multi-year shelf stability in dried formulations without refrigerated storage, and highly characterized and tractable secondary metabolite biosynthetic gene clusters position it as an ideal chassis organism for conversion into registered biostimulant soil inoculant formulations suitable for direct on-farm application (Omeiri et al., 2022). Priority directions for follow-on investigation include: (i) molecular cloning and functional characterization of the carbofuran hydrolase structural gene(s) in isolate B1 to enable heterologous expression and enzyme engineering; (ii) comprehensive metabolite profiling of the carbofuran biotransformation cascade by LC-MS/MS to delineate the complete catabolic pathway and assess the ecological risk of transformation products; (iii) evaluation of in situ biodegradation performance in intact field soil microcosms incorporating authentic spatial heterogeneity in physicochemical parameters; and (iv) metagenomics-based assessment of the impact of B. subtilis B1 inoculation on resident soil microbial community structure and functional diversity (Duc and Huy, 2022; Huang and Lu, 2021).

CONCLUSION

The present investigation successfully isolated, phenotypically characterized, and evaluated the in vitro carbofuran biodegradation capacity of Bacillus subtilis B1 from contaminated agricultural soils of Lahore, Pakistan. Under optimal conditions of 35°C, pH 6.5, and 1.5 µg/mL initial substrate concentration, isolate B1 achieved near-complete carbofuran elimination (99.9%) within four to five days of incubation, representing a quantitatively significant bioremediation outcome under laboratory conditions. Two-way ANOVA demonstrated that both incubation temperature and initial substrate concentration exerted statistically significant independent effects on biodegradation efficiency, with temperature accounting for the dominant fraction of total variability. The concentration-dependent suppression of degradation efficiency at higher substrate loads is mechanistically consistent with

Haldane-type substrate inhibition kinetics, with direct implications for inoculum density optimization in high-residue contamination scenarios. RP-HPLC provided a selective, sensitive, and reproducible analytical framework for the quantitative monitoring of biodegradation kinetics under complex biological matrix conditions. These findings establish B. subtilis as a scientifically validated and practically tractable biological agent for the remediation of carbofuran-contaminated agricultural soils, and provide a quantitative empirical foundation for the rational design of cost-effective, environmentally sustainable bioaugmentation strategies under the agroclimatic and soil conditions prevailing in pesticide-intensive agricultural systems of Pakistan.

Declarations

Conflict of Interest:

All authors declare no potential conflict of interest.

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Author Contribution

A.S. conceived and designed the study, supervised the experiments, and wrote the main manuscript text. M.K. performed the bacterial isolation, enrichment culture, and biochemical characterization. A.M.K. conducted the biodegradation experiments and RP-HPLC analysis. A.S. (Aqsa Sajid) performed the statistical analysis and prepared the figures and tables. All authors reviewed and approved the final manuscript.Key:A.S. = Azeem Sharif (corresponding author)M.K. = Maira KhalilA.M.K. = Abdul Muqeet KhanA.S. (second) = Aqsa Sajid

Data Availability Statement:

Data will be available upon reasonable request.

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Figures

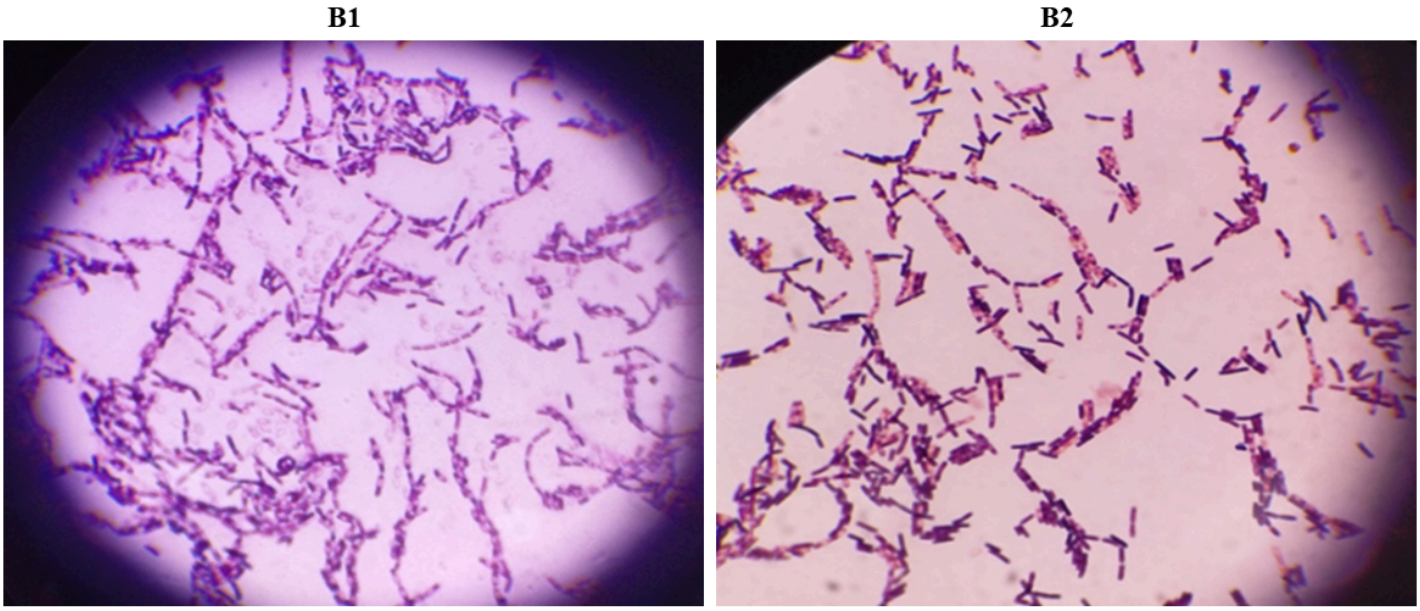


Figure 1

Photomicrographs of Gram-stained preparations of isolates B1 and B2 at 1000x magnification under oil immersion. Gram-positive, rod-shaped bacilli are visible with subterminal endospores.



Figure 4

Representative RP-HPLC chromatogram of the carbofuran standard at 8.0 µg/mL showing the characteristic retention peak at 15 minutes (UV 230 nm). Peak area integration values are annotated.

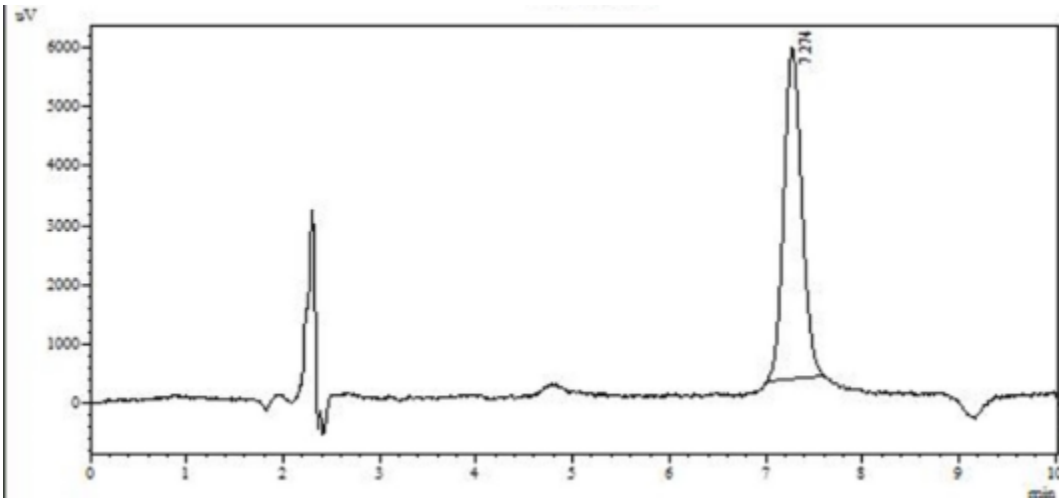


Figure 5

RP-HPLC chromatogram of the culture supernatant at 1.5 µg/mL carbofuran (UV 230 nm). Residual carbofuran peak at retention time 15 minutes reflects partial degradation by B. subtilis B1 at the indicated sampling time point.