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Recent advancements in microbe-pesticide interaction: A smart-soil bioremediation approach, 2nd edition

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Recent advancements in microbe-pesticide interaction: A smart-soil bioremediation approach, 2nd edition

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Table of contents

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Editorial: Recent advancements in microbe-pesticide interaction: a smart-soil bioremediation approach

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Editorial on the Research Topic
Recent advancements in microbe-pesticide interaction: a smart-soil bioremediation approach

Introduction

In recent years, there has been a growing interest in the development of eco-friendly solutions to control pests and promote soil health. One promising approach is the use of microbe-pesticide interactions to achieve smart-soil bioremediation (Bhatt et al., 2020). This approach involves the use of microorganisms that can degrade pesticides, detoxify the soil, and promote plant growth. Recent advancements in this field have shown great promise for sustainable agriculture (Wu et al., 2023). One of the most significant recent advancements in microbe-pesticide interaction is the discovery of new strains of microorganisms that can degrade a wide range of pesticides. These microorganisms can break down pesticides into harmless compounds, which helps to reduce their toxicity and prevent their accumulation in the soil. This not only benefits the health of the soil but also reduces the risk of pesticide contamination in food and water sources (Alexandrino et al., 2022).

Another key advancement in this field is the development of biopesticides, which are pesticides that are derived from natural sources, such as microorganisms, plants, or animals. Unlike conventional pesticides, biopesticides are non-toxic and do not harm the environment or non-target organisms. By using biopesticides in combination with microbe-pesticide interactions, it is possible to achieve effective pest control while promoting soil health. Moreover, recent studies have shown that microbe-pesticide interactions can promote plant growth and increase crop yield. Microorganisms can produce growth-promoting compounds that stimulate plant growth and enhance nutrient uptake. By promoting plant growth, microbe-pesticide interactions can help to increase the efficiency of crop production and reduce the need for chemical fertilizers (Glare and O'Callaghan, 2019).

Therefore, the recent advancements in microbe-pesticide interactions have opened new possibilities for sustainable agriculture. By using smart-soil bioremediation approaches that combine the use of microorganisms and biopesticides, it is possible to achieve effective pest control while promoting soil health and enhancing crop yield. These eco-friendly solutions can help to address some of the major challenges facing modern agriculture, such as pesticide resistance, soil degradation, and food security. As such, we must continue to

invest in research and development in this field to ensure a sustainable and healthy future for our planet.

In this Research Topic, we have accepted 16 papers authored by 118 authors. The themes of the papers covered microbe-pesticide interactions, bioremediation and the response of gut microbiota to pesticides. The major highlights of the Research Topics are described below.

Biodegradation of pesticides in environmental microorganisms

Qian et al. describes an investigation on the potential of using a combination of arbuscular mycorrhizal fungus (AMF) and *Hansschlegelia zhihuaiae* S113 to remediate soils contaminated with the herbicide bensulfuron-methyl. The researchers found that the combined use of AMF and *H. zhihuaiae* S113 was more effective in reducing bensulfuron-methyl residues in soil compared to the use of either organism alone. The study also showed that the combination treatment improved soil nutrient availability and microbial activity, which may have contributed to the enhanced biodegradation of bensulfuron-methyl. In another study, Kumar et al. suggested that the use of environment-restoring microbes isolated from the rhizosphere of horticultural crops under subtropics may provide a promising approach for the bioremediation of chlorpyrifos-contaminated soils. Sharma et al. identified the rhizospheric bacteria for the removal of the carbendazim in soil. They showed the inhibitory effect of carbendazim in isolated bacterial strain. They showed carbendazim (3,000 μg/mL) decreases the concentration of indole acetic acid, ACC deaminase and siderophore production in bacterial cells.

Li et al. suggest that the L1 microbial consortium has the potential to be used for the bioremediation of chlorimuron-ethyl-contaminated soils. However, further research is needed to optimize the conditions for the degradation process and to assess the long-term effectiveness and safety of this approach. The findings of this study may also have implications for the development of microbial consortia for the bioremediation of other herbicides and pollutants. The identification and characterization of the novel dmdA gene and its product may also have implications for the development of new herbicide-resistant crops and the biotechnological production of valuable compounds from dicamba. Zong et al. reported on the novel pyrethroid degrading enzyme SGNH esterase Est882, which can degrade diverse pyrethroids significantly.

Singh and Saxena discussed the recent findings on the pesticide-microbe interaction and showed the relationship among environmental factors at various trophic levels. He et al. discovered the cypermethrin-degrading binary consortium of the genus *Rhodococcus* and *Comamonas*. The developed consortium was effective for the elimination of pyrethroids from the environment. Wahla et al. reported that biochar-immobilized bacteria enhanced metribuzin degradation in the environment. Similarly, Pan et al. observed that immobilized bacteria can accelerate atrazine degradation in soil. Zhao et al. suggested development of the solid agents can facilitate the soil microbial degradation of pesticides.

Toxicity of pesticides in living systems

Baazeem et al. reported *Bacillus subtilis* and other bacterial strains identified in their study can be used as an alternative to chemical pesticides for the control of *Aphis punicae* and *Aphis illinoisensis*. However, further research is needed to evaluate the efficacy of these bacterial strains under field conditions and to assess their long-term impact on the environment. Siddique et al. highlight the importance of insect gut microbiota in the degradation of pesticides. The gut microbiota of insects can play a critical role in detoxifying pesticides and reducing the accumulation of pesticide residues in the environment. The gut microbiota can also modify the physicochemical properties of pesticides, making them more susceptible to degradation. The composition of gut microbiota in insects can be influenced by several factors, including diet, host genetics, and environmental conditions. Therefore, the gut microbiota of insects can adapt to the presence of pesticides, leading to the development of pesticide-resistant gut microbiota. Astaykina et al. reported the impact of three pesticides on the gut microbiota of a earthworm *Lumbricus terrestris*. Pathak et al. discussed the negative impacts of pesticides on the environment, and living systems. They suggested the adoption of more sustainable and eco-friendly management strategies using microbial technology.

Jaffer et al. described the the gut microbiota in insect physiology and the biodegradation of pesticides, and suggests that this knowledge could be leveraged for the development of more sustainable and eco-friendly approaches to pest management.

Future research

The articles published on this Research Topic advanced our understanding of pesticide-microbe interactions in soil and other environments. The Research Topic focuses on the degradation of pesticides using various bacterial and fungal strains. In addition, the Research Topic gives insights into the impact of pesticides on the gut microbiota. Further work is needed to meet the major challenges in the field of microbe-pesticide interaction. Advances in 'omics technologies will promote discoveries of novel methods and future applications capable of cleaning large-scale polluted sites in an eco-friendly manner.

Author contributions

The editorial draft was written by PB, SC, and CM. All authors contributed to the revision and improvements and approved the final version for submission. All the editors collaborated well for the successful completion of the Research Topic.

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Conflict of interest

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Combined Bioremediation of Bensulfuron-Methyl Contaminated Soils With Arbuscular Mycorrhizal Fungus and *Hansschlegelia zhihuaiae* S113

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Over the past decades, because of large-scale bensulfuron-methyl (BSM) application, environmental residues of BSM have massively increased, causing severe toxicity in rotation-sensitive crops. The removal of BSM from the environment has become essential. In this study, the combined bioremediation of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus intraradices* and BSM-degrading strain *Hansschlegelia zhihuaiae* S113 of BSM-polluted soil was investigated. BSM degradation by S113 in the maize rhizosphere could better promote AMF infection in the roots of maize, achieving an infection rate of 86.70% on the 36th day in the AMF + S113 + BSM group. Similarly, AMF enhanced the colonization and survival of S113 in maize rhizosphere, contributing 4.65×10^5 cells/g soil on the 15th day and 3.78×10^4 cells/g soil on the 20th day to a population of colonized-S113 (based possibly on the strong root system established by promoting plant-growth AMF). Both S113 and AMF coexisted in rhizosphere soil. The BSM-degrading strain S113 could completely remove BSM at 3 mg/kg from the maize rhizosphere soil within 12 days. AMF also promoted the growth of maize seedlings. When planted in BSM-contaminated soil, maize roots had a fresh weight of 2.59 ± 0.26 g in group S113 + AMF, 2.54 ± 0.20 g in group S113 + AMF + BSM, 2.02 ± 0.16 g in group S113 + BSM, and 2.61 ± 0.25 g in the AMF group, all of which exceeded weights of the control group on the 36th day except for the S113 + BSM group. Additionally, high-throughput sequencing results indicated that simultaneous inoculation with AMF and strain S113 of BSM-polluted maize root-soil almost left the indigenous bacterial community diversity and richness in maize rhizosphere soil unaltered. This represents a major advantage of bioremediation approaches resulting from the existing vital interactions among local microorganisms and plants in the soil. These findings may provide theoretical guidance for utilizing novel joint-bioremediation technologies, and constitute an important contribution to environmental pollution bioremediation while simultaneously ensuring crop safety and yield.

Keywords: ***Hansschlegelia zhihuaiae*** **S113, combined bioremediation, bensulfuron-methyl, arbuscular mycorrhizal fungi, 16s sequencing**

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INTRODUCTION

Because of their high efficiency, wide spectrum, and low toxicity, sulfonylurea herbicides are among the most widely used herbicides in the world for the control of field weeds. They inhibit catalytic activity of acetolactate synthase (ALS) in the biosynthesis pathway of branched-chain amino acids (such as valine, leucine, and isoleucine) in plants (Saeki and Toyota, 2004). Bensulfuron-methyl (BSM), a representative sulfonylurea herbicide, is usually employed for controlling broadleaf weeds in crop fields. It persists in soil at a half-life of 62 days (yellow-brown soil) or 129 days (yellow fluvo-aquic soil) and is poorly photodegraded (Delgado-Moreno et al., 2007). Unfortunately, their excessive and persistent usage has seriously damaged the natural environment, causing herbicide toxicity in the subsequent rotation of BSM-sensitive crops, changing of the indigenous microbial community structure or richness, and polluting water resources. Therefore, the systematic remediation of residual BSM in soil or water environments is essential to ensure crop safety and to further enhance agricultural yield.

Bioremediation techniques are highly efficient, eco-friendly, low cost, and are heralded as promising tools for the degradation of pollutants that remain in the environment (Pang et al., 2020; Zhang W. et al., 2020). Microbial degradation is a popular and accepted method for the removal of residual BSM from soil. Recently, several microorganisms, including fungi and bacteria, capable of degrading BSM have been reported, such as *Penicillium pinophilum* strain BP-H-02 (Peng et al., 2012), *Methylopila* sp. DKT (Duc and Oanh, 2020), *Bacillus megaterium* L1 (Lin et al., 2010), *Brevibacterium* sp. BH (Zhu et al., 2005) and *Bacillus subtilis* YB1 (Zhang Z. et al., 2020). The main focus of research has been the isolation of BSM-degrading strains, the identification of degradation pathways and products, and detoxification mechanisms of functional enzymes (Wang et al., 2019). In addition, the development of BSM-degrading bacterial inoculums or detoxified-enzyme products has also attracted research attention (Thouand et al., 2011). Nevertheless, the detoxification effect of pure cultures for BSM-contaminated environments (i) is restricted by the natural environmental conditions in the application process of polluted farmland soil, (ii) the survival of degrading strains cannot be maintained for an extended period, and (iii) the effects of a single population are restricted by the metabolism of toxic pollutants. Thus, a mixed community of microorganisms may constitute an alternative strategy for the degradation and removalS113 of BSM, and associations of fungi with bacteria are particularly promising. However, studies on the bioelimination of BSM by multiple microorganisms are rare.

Arbuscular mycorrhizal fungi (AMF), the most widely distributed type of symbionts in soils, and terrestrial plants have adopted an important and mutually beneficial symbiotic relationship. Previous research showed that the extension of the external mycelium of AMF can increase the nutrient absorption and biomass in plants and crops (Bowles et al., 2016; Brundrett and Tedersoo, 2018), including mineral nutrients and water (Leake et al., 2004; Parniske, 2008). The underlying causes are high-affinity inorganic phosphate (Pi) or nitrogen (N) transporters (Bonfante and Genre, 2010), as well as the increased productivity and quality of crops because of the control of pathogens (Baum et al., 2015; Li et al., 2019). Nakmee et al. (2016) also found that inoculation with AMF can produce the greatest biomass, grain dry weight, and total nitrogen uptake in sorghum shoots. Additionally, evidence indicates that AMF can also facilitate plants or crops' tolerance to potentially toxic elements (PTEs) and adverse environments, such as heavy metals, radioactive elements, or low temperature (Liu et al., 2014; Qiao et al., 2015). This is achieved by mediating the interaction between PTEs and plant roots (Yang G.W. et al., 2015) or alterations in H_2O_2 accumulation and ATPase activity. Hence, inoculation with AMF is an effective method for promoting the growth of sensitive crops grown in soils polluted with toxic substances. Furthermore, previous reports suggested that AMF can also enhance the degradation efficiency of a variety of pollutants in soil, including polychlorinated biphenyls and atrazine (Huang H. et al., 2007; Qin et al., 2014). Moreover, these effects also provide ideas and a foundation for combining inoculations of AMF with other biofertilizers or biodegradation agents.

Previous studies merely focused on the degradation of BSM by a pure strain or microbial communities. Similarly, one or limited number of AMF species were used to promote plant growth or the resistance to harsh environments. Nevertheless, a limited number of studies have assessed how herbicide-degrading strains and other functional bacteria or fungi (such as AMF) jointly remediate soils contaminated by organic pollutants. In the present study, the bacterial strain *Hansschlegelia zhihuaiae* S113, capable of degrading BSM into herbicidal-inactive acid by catalyzing de-esterification (Huang X. et al., 2007; Hang et al., 2012), was selected as herbicide-degrading strain. In addition, the AMF *Rhizophagus intraradices*, which markedly improves the transport and uptake of P and water under drought stresses (Li et al., 2014; Leyva-Morales et al., 2019), is added as a plant growth-promoting strain.

The objectives of the present study are to (i) assess the remediation effect of AMF and strain S113 on BSM-contaminated soil, and (ii) discuss the ecological effect of AMF and strain S113 on the remediation of BSM-contaminated soil. The combined bioremediation with AMF and an herbicide-degrading bacterial strain provides a promising remediation technique for the removal of residual herbicides from soils with the combined promotion of plant growth.

MATERIALS AND METHODS

Chemicals, Strains, and Media

BSM (analytical grade, purity > 99%) was purchased from J&K Scientific Company, Ltd (Beijing, China). Cleanert HXN SPE column was purchased from Agela Technologies Co., Ltd., Tianjin, China. HPLC-grade acetonitrile, dichloromethane, and methanol were purchased from Sigma Aldrich, United States.

All other reagents used in this study were of analytical grade or higher purity.

Hansschlegelia zhihuaiae S113 (Huang X. et al., 2007) was preserved in our laboratory. The AMF *Rhizophagus intraradices* was acquired from the Bank of Glomeromycota in China (BGC) of Beijing Academy of Agriculture and Forestry Sciences, China. Tryptone-yeast (TY) medium for strain S113 cultivation consisted of tryptone (5.0 g/L), yeast extract (3.0 g/L), and $CaCl_2$ (0.01 g/L). Mineral salts medium (MSM) contained (g/L): 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, 1.0 NH_4NO_3, and 0.2 $MgSO_4.7H_2O$. For solid media, 18.0 g of agar was added per liter.

Phosphate-buffered saline (PBS) buffer (pH 7.2) contained (g/L): 8.0 NaCl, 0.2 KCl, 1.42 Na_2HPO_4, and 0.27 KH_2PO_4. The BSM from rhizosphere soil was extracted with a mixture solution of PBS and acetonitrile (8:2, v:v).

Soil and Maize Seed Germination

The basic properties of soil used in this experiment are summarized in the following: pH 6.86, organic matter 12.8 mg/kg, available P 6.45 mg/kg, available K 105.48 mg/kg, nitrate-nitrogen 1.77 mg/kg, and ammonium nitrogen 6.24 mg/kg. Soil for the cultivation of maize was air-dried at room temperature and then sieved to a size of 2 mm. The maize seeds of Meiyu 5 (Jiangsu Mingtian Seed Co. Ltd., Nanjing, China) were surface sterilized with 10% H_2O_2 solution for 5 min, rinsed repeatedly using sterilized water until H_2O_2 was completely removed, and then soaked overnight in sterilized water. The water-swollen seeds were transferred to a glass culture dish covered with two layers of sterile, wet gauze at the bottom, and then left to germinate in the dark for three days. Maize seedlings with nearly equal lengths of root and stem were selected for further study (Zhang et al., 2018).

Preparation of Arbuscular Mycorrhizal Fungi Inoculant and S113 Suspension

Prior to propagation, pre-incubated soils were autoclave sterilized at 121°C for 2 h to remove indigenous AMF propagules and other microorganisms (Chang et al., 2018). AMF was firstly propagated on the host plant maize planted in a soil-sand mixture (sterilized soil: sand = 3:1) for 4 months. Then, this soil (containing spores, mycelia, and maize root segments) was collected for the preparation of AMF spore suspension, and their concentration was calculated using a hemocytometer. Each milliliter of inoculum contained approximately 9.43×10^4 spores. Each maize seedling root was inoculated with 200 μL spore suspension.

The preparation of strain S113 suspension was performed as described by Zhang et al. (2018) with minor adjustments. Briefly, strain S113 was grown in TY medium at 30°C on a rotary shaker (150 rpm). Cells were harvested in the late log growth phase by centrifugation at 4°C and 5000 *g* for 10 min, washed twice, and then resuspended in sterile water. The final concentration of optical cell density at 600 nm (OD_{600}) was adjusted to 1.5 for further investigation.

Remediation of Bensulfuron-Methyl-Contaminated Soil and Removal of Phytotoxicity to Maize by Arbuscular Mycorrhizal Fungi and Strain S113

Maize seedlings were prepared as described above, and then sown into soils with 0.1, 0.5, 1, 2, 3, and 5 mg/kg of BSM to test appropriate pollution levels, respectively. The growth of maize seedlings at different concentrations was compared after 10 days. To assess the most suitable inoculation amount of strain S113 used for repairing BSM-contaminated soil, a variable volume of S113 suspension (2, 5, 10, 15, and 25 mL) was inoculated into the roots of maize seedlings by root irrigation. Then, all pots were placed in an illumination incubator for 10 days under the following conditions: 14 h of light at 28°C and 10 h of dark at 20°C. ddH_2O of the same volume instead of S113 suspension served as control. All treatments were carried out in triplicate.

To explore cooperative remediation of BSM-polluted soil by AMF and strain S113, seven treatments and one control were set as follows: (A) control (without any addition), (B) strain S113, (C) BSM, (D) AMF (*Rhizophagus intraradices*), (E) S113 + BSM, (F) AMF + BSM, (G) S113 + AMF, and (H) S113 + AMF + BSM. Maize seedlings were randomly collected to measure the growth parameters, as well as to visualize the infection of AMF on maize roots at 12 d, 24 d, or 36 d. In addition, rhizosphere soil was also collected every 6 days to measure the residual concentration of BSM and to extract the total DNA of soil for studying the microbial community in maize rhizosphere soil. Each treatment was replicated three times. The degradation experiment refers to Huang X. et al. (2007).

Determination of Arbuscular Mycorrhizal Fungi Infection Rate

Total root systems of maize from each treatment were cut into 1 cm pieces and fixed in FAA solution (13 mL formaldehyde, 5 mL acetic acid, 200 mL 50% ethanol). Fixed roots were heated at 90°C for 1 h in 10% potassium hydroxide (KOH) solution. The roots were rinsed with water and acidified with 2% HCl for about 5 min, then stained at 90°C for 30 min using 0.05% trypan blue in a mixture solution of lactic acid, glycerol, and distilled water (1:1:1, v:v:v) (Phillips and Hayman, 1970). Finally, entire roots were soaked in decoloring solution (50 mL lactic acid, 100 mL glycerin, and 50 mL distilled water) overnight. Subsequently, 30 root segments were selected randomly and their AMF colonization was observed under an ordinary optical microscope at 200 × magnification. The mycorrhizal colonization rate was estimated using the method of Biermann and Linderman (1981).

Effects of Arbuscular Mycorrhizal Fungi on the Colonization of Strain S113

To facilitate colonization observation, strain S113-*gfp* (containing reporter genes *gfp*) was constructed as previously described (Zhang et al., 2018). Colonization on the root surface was detected by confocal laser scanning microscope (CLSM, Leica TCS SP3). The maize seedlings, prepared as described

in Section "Soil and maize seed germination," were irrigated by S113-*gfp* (OD_{600} = 1.5, 15 mL), S113-*gfp* (OD_{600} = 1.5, 15 mL) + AMF (200 μL), or sterile water of the same volume, then incubated in a growth chamber for 25 days. The seedlings were gently pulled out of pots at an interval of five days to observe the colonization of S113 on the roots. In addition, root and rhizosphere soil samples were collected for the quantitative determination of S113 colonization. The total DNA of both rhizosphere soil and maize roots were extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals™) and plant DNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China), respectively.

The S113 colonization in maize root or rhizosphere soil was detected by quantitative real-time PCR (qPCR). The qPCR reaction solution contained 10.0 μL of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazym), 0.4 μL of *sulE*-F/R (10 μM), 2.0 μL of template DNA (from maize root or rhizosphere soil), and 7.2 μL double distilled water. qPCR was performed in a QuantStudio 6 Flex system using the following procedure: 95°C for 3 min, followed by 40 cycles of 95°C for 5 s, and 60°C for 34 s. DNA standard curves were prepared as previously described (Chi et al., 2013) using the known gene *sulE,* encoding a de-esterification esterase that degraded BSM in the genome sequence of strain S113, because it is a single-copy gene and has low sequence similarities (44% identity) (Zhang et al., 2018). Briefly, the partial sequence of gene *sulE* (202 bp) amplified from S113 genomic DNA with the primers *sulE*-F (5′-CGCCAAGAACGTGAGGGGGAT-3′) and *sulE*-R (5′-CTGTGAGCGTAGCGAGTGACT-3′) was cloned into the vector pMD19-T (TaKaRa, Beijing, China). Plasmids (pMD19-T/*sulE*) were extracted from recombinants and used as standards for quantitative analyses. The concentration of recombinant plasmid DNA was determined by NanoDrop 2000/2000c (ThermoFisher Scientific, Wilmington, DE, United States). Ten-fold serial dilutions of known concentrations of the plasmid DNA were used as template for qPCR to generate an external standard curve.

Soil Bacterial Community Analyses

Extracts of total DNA of rhizosphere soil were obtained from eight group samples (see Section "Effects of AMF on the colonization of strain S113" for DNA extraction). The concentration of DNA in samples containing visible bands was assessed using NanoDrop 2000/2000c before sequencing. The quality of soil total DNA was tested via 1% agarose gel electrophoresis. The hypervariable V_4–V_5 fragment of the 16S rRNA gene was amplified by the universal primer pair 338F (5′-ACTCCTACGGGAGGCAGCA-3′)/806R (5′-ACTCCTACGGGAGGCAGCA-3′) with barcode. PCR reactions were carried out in 30 μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), using 0.2 μM of forward and reverse primers, and 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s. Finally, 72°C for 5 min. PCR products were assessed via 2% agarose gel electrophoresis and purified the bright main strip between 400 and 450 bp with GeneJET Gel Extraction Kit (Thermo Scientific). Purified PCR products were mixed for generating sequencing libraries using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's recommendations. The library quality was assessed with Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina HiSeq platform and 250 bp paired-end reads were generated, then merged using FLASH (Magoč and Salzberg, 2011). Briefly, reads were firstly filtered by QIIME quality filters. Then, sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). A representative sequence for each OTU was chosen to annotate taxonomic information using the RDP classifier (Wang et al., 2007).

Chemical Analysis

For extraction of BSM from rhizosphere soil, a 10 g rhizosphere soil sample was extracted with 20 mL mixed PBS and acetonitrile solution, then shocked at 150 rpm for 1 h, and centrifuged at 8000 *g* for 10 min. The extraction process was repeated three times for every sample and all supernatants were collected and acidified to a pH of 2.5. A Cleanert HXN cartridge was used to purify supernatants. The elution solution was air-dried, then resuspended in 1 mL methanol, and the residual BSM was measured by high-performance liquid chromatography (HPLC) (Dionex UltiMate 3000) equipped with a C_{18} reverse-phase chromatographic column (4.6 × 250 mm, 5 μm, Agilent Technologies, Palo Alto, CA, United States), using a UV detector at 230 nm. Water:acetonitrile:acetic acid (40:60:0.5, v:v:v) was used as the mobile phase at a flow rate of 1 mL/min for detection. The concentration was determined via comparison against values in the calibration curve.

Statistical Analysis

Data were analyzed by one-way ANOVA and Duncan's test to compare means. p values < 0.05 were considered to represent statistically significant differences. Statistical data were analyzed using GraphPad Prism v8.0.2.263 software (San Diego, CA, United States).

Data Availability

All of the sequencing data involved in this manuscript had been deposited in the NCBI database (BioProject ID:PRJNA793182), and could be download from the link: https://www.ncbi.nlm.nih.gov/sra?linkname=bioproject_sra_all&from_uid=793182.

RESULTS

Promotion of S113 on Arbuscular Mycorrhizal Fungi Infection to the Maize Root System

Photomicrographs of the root segments of maize infected by AMF are shown in **Figure 1A**. Typical structures of AMF and plant symbiosis, such as arbuscular hyphae, mycelium,

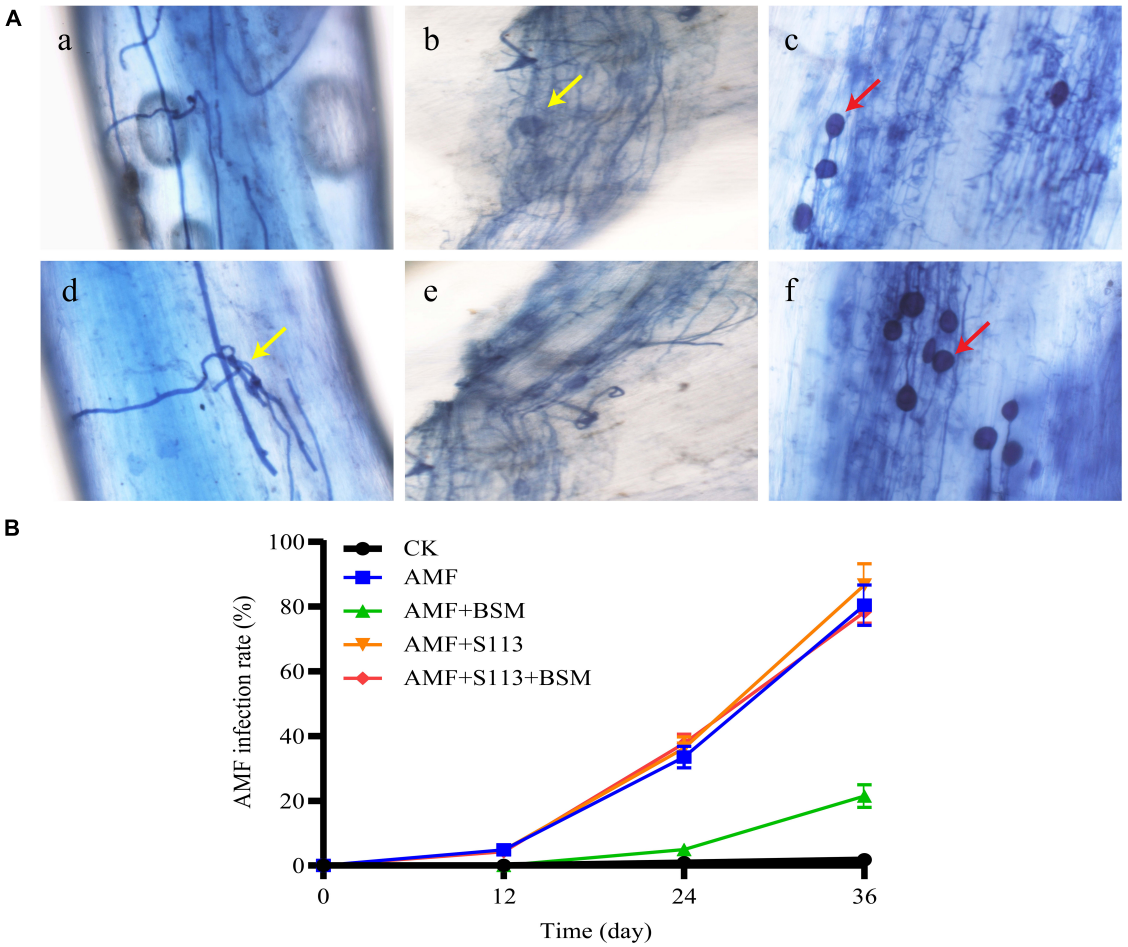

FIGURE 1 | Infection of the Arbuscular mycorrhizal fungi (AMF) to the maize root system. **(A)** *Rhizophagus intraradices* colonization in maize roots, as observed under an optical microscope (200 ×). (a) (d) Root segment with low infection rate; (b) (e) Root segment with moderate infection rate; (c) (f) Root segment with high infection rate. The red arrows indicate spores; the yellow arrows indicate hyphae or mycelium. **(B)** The infection rate of AMF to maize root system under different treatments.

and spores, were distinctly observed by an ordinary optics microscope. These structures indicate a friendly symbiotic relationship between AMF and maize roots.

The maize roots were AMF infected to a certain extent (**Figure 1B**). On the 12th day, the lower infection rates of 4.83%, 4.80%, and 4.30% were observed in the treatment groups D (AMF), G (S113 + AMF), and H (S113 + AMF + BSM), respectively. However, the AMF infection rates in groups D, G, and H on the 24th day reached 33.50%, 36.26%, and 37.76%, respectively, while the infection rate of group F (AMF + BSM) was only 4.93%. This may be the result of BSM causing serious toxicity to the maize root system, which in turn may have affected AMF infection. With the inoculation of BSM-treated soil with BSM-degrading strain S113, the AMF infection rate markedly increased to about 78.30% on the 36th day (**Figure 1B**). The possible reason for the significant increase of AMF infection rate after inoculation of S113 degrading bacteria is that strain S113 removes BSM in rhizosphere soil and reduces the formation of phytotoxicity. Only 21.50% of the infection rate was observed in the BSM-amended soil on the 36th day (**Figure 1B**), which still showed a significant difference from the control.

Increase of Strain S113 Colonization by Arbuscular Mycorrhizal Fungi

To assess the colonization of rhizosphere microorganisms on the root surface and rhizosphere of crops, a plasmid carrying the *gfp* gene was introduced into strain S113. The cells of strain S113 strongly colonized the surface of corn roots, which could be observed under a confocal laser scanning microscope (CLSM) (**Figure 2**). The most apparent fluorescence intensity in maize root could be seen after irrigating S113-*gfp* on the 5th day. However, the colonization effect of strain S113-*gfp* gradually weakened after the 10th day. This may be the result of a gradual decline in the growth activity of strain S113-*gfp* during maize cultivation.

According to the standard curve, strain S113 colonizing the maize root surface and rhizosphere was quantified by qPCR (**Figure 3**). The initial density of strain S113 colonizing

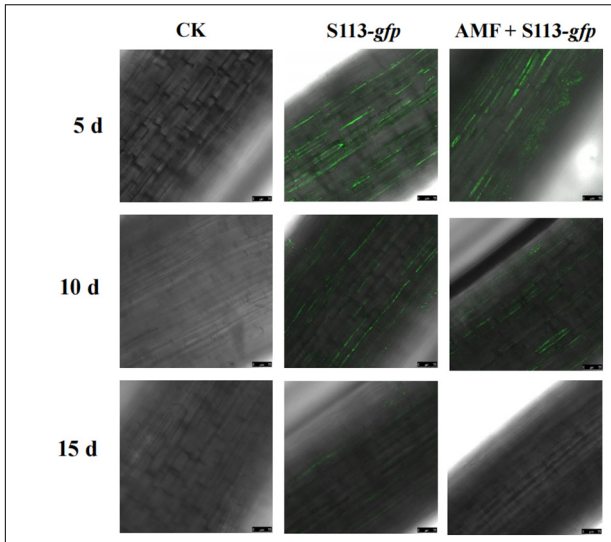

FIGURE 2 | Confocal laser scanning microscope images of S113 colonization on maize roots. The treatment was irrigated with S113 or S113 + Arbuscular mycorrhizal fungi (AMF). The control was treated with sterile water.

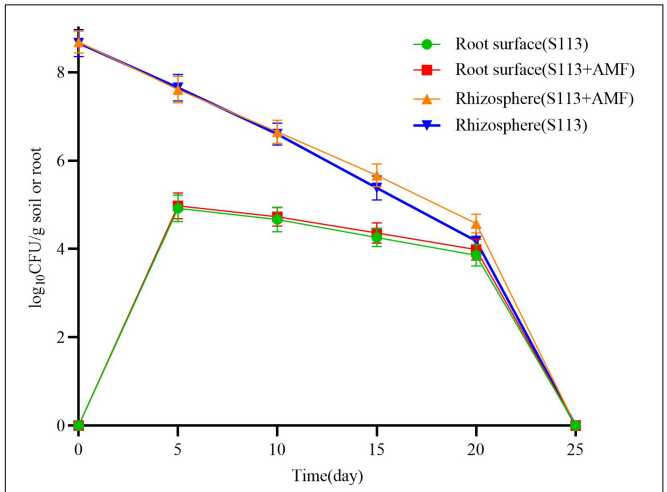

FIGURE 3 | Effects of Arbuscular mycorrhizal fungi (AMF) on the colonization of S113 in the rhizosphere and root surface. Dynamic of S113 population on roots surface and in the rhizosphere of maize under different treatments. The data are shown as $\log_{10}$CFU per gram soil or roots. The error bars indicate standard deviations for three samples.

rhizosphere soil reached 4.7×10^8 cells/g soil. The number of S113 in the rhizosphere gradually decreased with prolonged colonization time, and the population of strain S113 colonizing the corn root surface rapidly increased. On the 5th day, S113 reached maximum colonization (9.44×10^4 cells/g root) on the root surface. This result matches CLSM images of S113 colonization on maize roots (**Figure 2**). The amounts of strain S113 of 4.65×10^5 cells/g soil on the 15 days and 3.78×10^4 cells/g soil on the 20 days, colonized in the rhizosphere soil were higher compared with the treatment without AMF inoculation (2.39×10^5 cells/g soil on the 15 days and 1.52×10^4 cells/g soil on the 20 days). These results suggest that inoculation of AMF slightly promotes S113 colonization on maize root surface or rhizosphere soil.

Bioremediation of Bensulfuron-Methyl-Polluted Soil by the Combination of Arbuscular Mycorrhizal Fungi and S113

The appropriate inoculum of strain S113 and BSM contamination concentration were determined before assessing the elimination of the BSM phytotoxicity in maize by S113 and/or AMF. A variety of BSM concentrations caused a certain extent of phytotoxicity in maize seedlings (**Supplementary Figure 1A**). The leaves of maize seedlings were slightly shriveled, purple, and without moisture in 5 mg/kg BSM-contaminated soil, while maize seedlings did not wither after exposure to 3 mg/kg BSM. Therefore, a BSM concentration of 3 mg/kg was used to cultivate maize in subsequent pot experiments. Correspondingly, the strain S113 capable of biological repair could partially alleviate phytotoxicity to maize. Using > 15 mL suspension of strain S113 with $OD_{600} = 1.5$ irrigated to maize roots grown in 3 mg/kg BSM-contaminated soil achieved recovery of maize growth compared to control (**Supplementary Figure 1B**). Thus, a 15 mL suspension of strain S113 ($OD_{600} = 1.5$) was selected to inoculate the maize roots for all following remediation experiments.

The residual amount of BSM in rhizosphere soil is shown in **Figure 4**. The inoculation of strain S113 could notably reduce the concentration of BSM in rhizosphere soil on the 6th day (from 3 to about 0.5 mg/kg). Furthermore, the residual BSM could no longer be detected after 12 days. Nevertheless, the concentration of BSM in BSM + S113 treatment and BSM + S113 + AMF treatment did not show a significant difference during the entire remediation process of BSM contaminated soil. Removal of BSM in group F was still faster than the treatment without AMF (i.e., group C). The residual BSM concentrations were 1.90 mg/kg and 1.78 mg/kg soil in treatments C and F on the 24th day and 1.57 mg/kg and 1.35 mg/kg soil on the 36th day. This

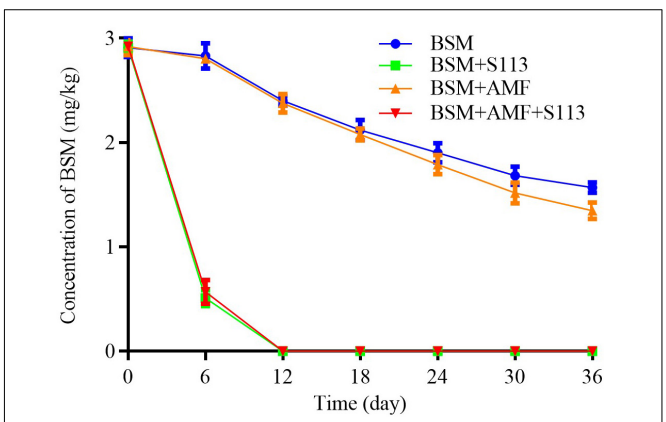

FIGURE 4 | The residual concentration of Bensulfuron-methyl (BSM) in Rhizosphere soil.

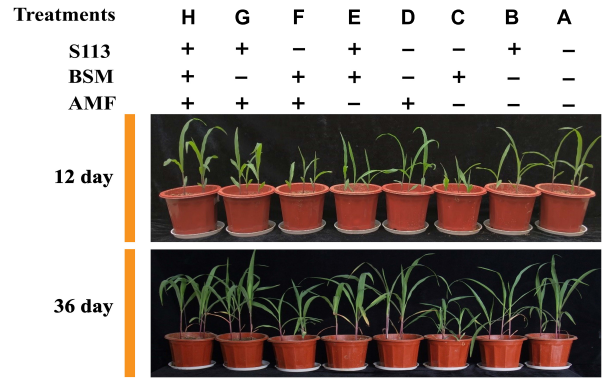

FIGURE 5 | The growth of maize under different treatments. (A) control (CK); (B) S113; (C) Bensulfuron-methyl (BSM); (D) Arbuscular mycorrhizal fungi (AMF); (E) S113 + BSM; (F) AMF + BSM; (G) S113 + AMF; (H) S113 + AMF + BSM. +: addition; −: without addition.

showed that AMF addition has a certain promoting effect on BSM degradation in soil.

Elimination of Bensulfuron-Methyl Phytotoxicity to the Maize by Combining Arbuscular Mycorrhizal Fungi and S113

The growth of maize under different treatments is shown in **Figures 5**, **6**. Strain S113 did not affect the growth of maize. The stem length, root length, fresh-stem weight, and fresh-root weight of maize in group C were 13.49 ± 1.28 cm, 10.92 ± 1.29 cm, 0.63 ± 0.05 g, and 0.37 ± 0.05 g on the 12th day, respectively, which were significantly lower than control. In contrast, addition of strain S113 (group E) could notably eliminate BSM phytotoxicity in maize seedlings. The length and fresh weight of stem and root gradually increased to 23.43 ± 2.20 cm, 26.14 ± 2.35 cm, 1.06 ± 0.05 g, and 0.92 ± 0.07 g on the 12th day, respectively, and were comparable to the control level. This indicated that BSM could strongly inhibit the growth of maize without S113 irrigation, and strain S113 could significantly alleviate the phytotoxicity of BSM to maize seedlings.

AMF could not directly degrade BSM from rhizosphere soil (there was no significant difference between BSM + AMF and BSM groups). However, AMF significantly enhanced the growth of maize regardless of whether it was grown in BSM-treated rhizosphere soil, especially S113 + AMF, S113 + AMF + BSM, and AMF. For example, the fresh weight of maize roots of groups S113 + AMF, S113 + AMF + BSM, and AMF was 2.59 ± 0.22 g, 2.54 ± 0.18 g, and 2.61 ± 0.19 g higher, respectively, compared to the CK group on the 36th day. The possible reason is that the lower infection rate still provided the possibility for information exchange between maize roots and AMF.

Recovery of the Soil Bacterial Community Structure Damaged by S113

Shannon diversity indices of different treatment groups were calculated using QIIME software to assess the diversity of the soil community structure. The Shannon diversity of group S113 + BSM presented a significant difference with only the addition of BSM according to the Duncan test on the 12th day (**Figure 7**). In other words, strain S113 markedly changed the diversity of the community structure of indigenous microorganisms at the beginning of inoculation. However, with the addition of AMF, the diversity of the microbial community structure in maize rhizosphere soil gradually recovered to the level of the native indigenous microorganisms on the 36th day (treatment S113 + BSM + AMF vs BSM) (**Figure 7**).

Different treatment conditions strongly impacted the abundance of the bacterial community. *Nocardioides*, *Arthrobacter*, *Sphingomonas*, *Gaiella*, *unidentified_Acidobacteria*, and *Candidatus_Udaeobacter* had consistently higher relative abundances in all samples over the entire remediation process of contaminated soil (**Figure 8**). The abundance of genus *Hansschlegelia* increased dramatically on days 12, and a decreasing trend was observed on the 36th day, indicating that strain S113 can survive in the soil for at least 12 d. Interestingly, a lower abundance was found for genus *Nocardioides* in S113 + BSM at any time.

DISCUSSION

Exploring sustainable and environmentally friendly remediation technologies for herbicide-contaminated soil has received considerable attention. Bioremediation is the most promising biotechnological approach to remove pollution from soil, including sulfonylureas herbicides (Egorova et al., 2017; Bhatt et al., 2020). To date, few microorganisms capable of degrading BSM have been isolated and identified. Prominent examples are *Brevibacterium* sp. (Luo et al., 2008), *Brevibacterium* sp. strain BH (Zhu et al., 2005), *Bacillus megaterium* strain L1 (Lin et al., 2010), *Penicillium pinophilum* strain BP-H-02 (Peng et al., 2012), *Bacillus subtilis* strain YB1 (Zhang Z. et al., 2020), and *Methylopila* sp. strain DKT (Duc and Oanh, 2020). In the present study, the highly efficient BSM-detoxifying strain *Hansschlegelia zhihuaiae* S113 was used for the remediation of BSM from soil, as relevant research showed that this strain achieved promising degradation effect (Huang X. et al., 2007; Hang et al., 2012). This provides valuable degrading-strain resources for the bioremediation of BSM-contaminated soil.

Associations between host plants and mycorrhizal fungi depend on a very close and useful relationship where plants provide nutrients and residency for mycorrhizal fungi, which in turn either directly or indirectly improve the nutrient status of their host plants and convey resistance to environmental stresses (Smith and Read, 2002). However, this symbiotic relationship between plants and AMF is not always beneficial for both. For example, AMF has been shows to also impose adverse effects on the growth of tobacco and sorghum plants (Watts-Williams et al., 2019). The findings of the present study demonstrate that AMF could neither degrade BSM nor improve the degradation effect of strain S113 to BSM in rhizosphere soil (**Figure 4**). However, it could, to varying degrees, promote the growth of

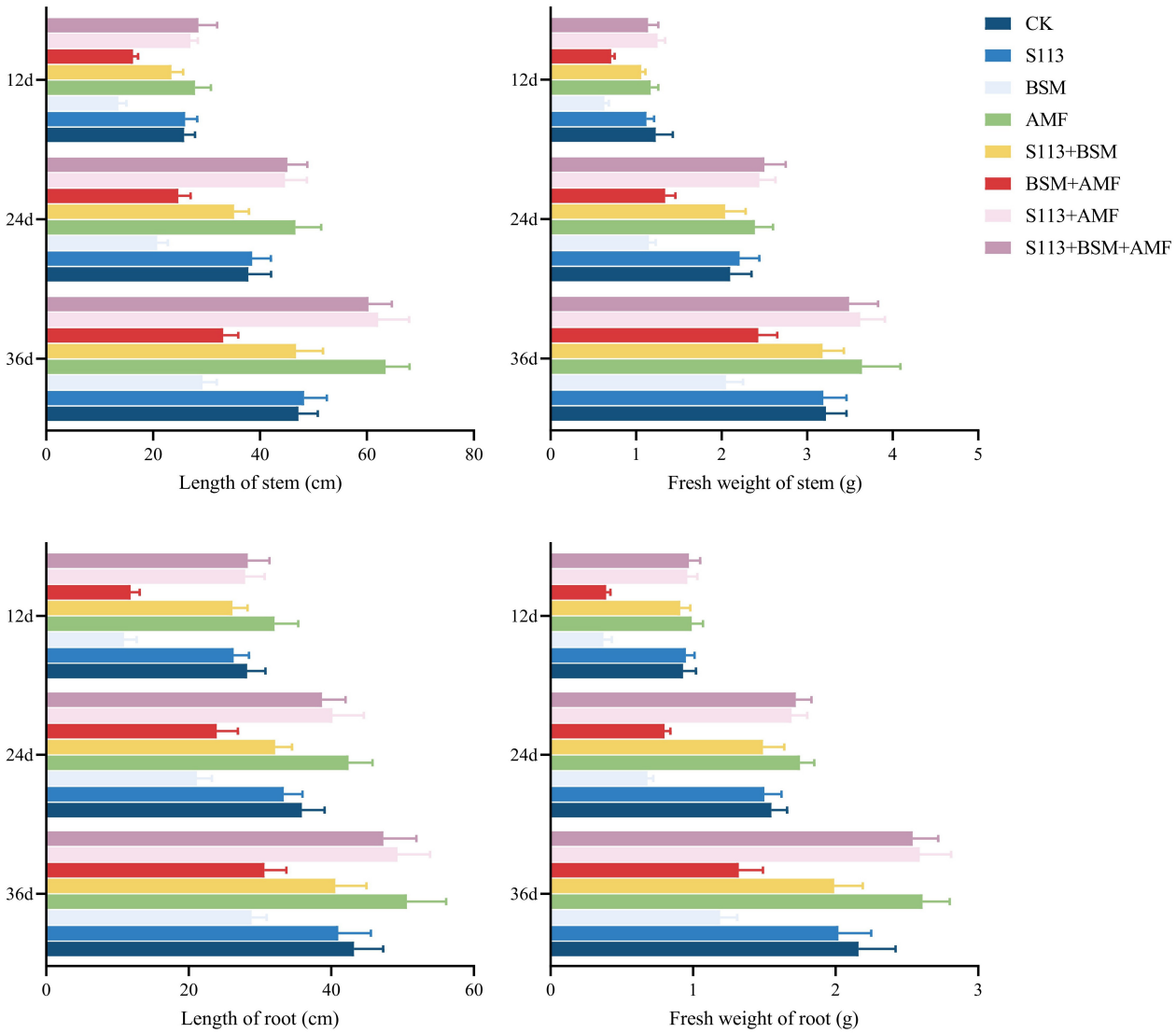

FIGURE 6 | The growth of maize under different treatments. CK: the seedlings without Bensulfuron-methyl (BSM) and S113 or Arbuscular mycorrhizal fungi (AMF); S113: seedlings were inoculated with S113 by root irrigation; BSM: soils were treated with bensulfuron-methyl, 3.0 mg/kg; AMF: seedlings were inoculated with AMF spore suspension (200 μL); S113 + BSM: seedlings were treated with BSM and inoculated with S113; AMF + BSM: seedlings were treated with BSM and inoculated with AMF spore suspension; S113 + AMF: seedlings were inoculated with S113 and AMF spore suspension; S113 + BSM + AMF: seedlings were inoculated with S113 and AMF spore suspension, and soils were treated with BSM.

maize whether it was planted in BSM-contaminated soil or not (BSM vs BSM + S113) (**Figures 5**, **6**), which is consistent with a previous report (Yang Y. et al., 2015). Most species of AMF belong to the sub-phylum Glomeromycotina of the phylum Myxomycetes (Spatafora et al., 2016), and already 25 genera have been described in this subphylum (Redecker et al., 2013). The possibility cannot be ruled out that other types of AMF are also capable of assisting the removal of BSM or other herbicides in plants' root or rhizosphere soil. For example, Huang H. et al. (2007) reported that AMF treatment enhances the degradation efficiency of the herbicide atrazine in soil. Hence, in future experiments, *Rhizophagus intraradices*, which was used in this study, could be replaced with other AMF and effects on the dissipation of BSM in soil by strain S113 can be assessed further.

The chemotaxis and colonization of rhizosphere microorganisms on plant root surfaces and rhizosphere are considered as a precondition for function, such as metabolizing pollutants that exist in the plant rhizosphere (Moore et al., 2006; Weyens et al., 2010). The leek endophyte *Sphingomonas* sp. HJY, which colonizes the roots, stems, and leaves of leek plants, efficiently degraded chlorpyrifos in leek and lets its growth condition gradually return to a healthy level (Feng et al., 2017). The biocontrol bacterium *Bacillus subtilis* CE1 was shown to be capable to inhibit the pathogen *Fusarium verticillioides* on the surface of corn roots. This reduces diseases

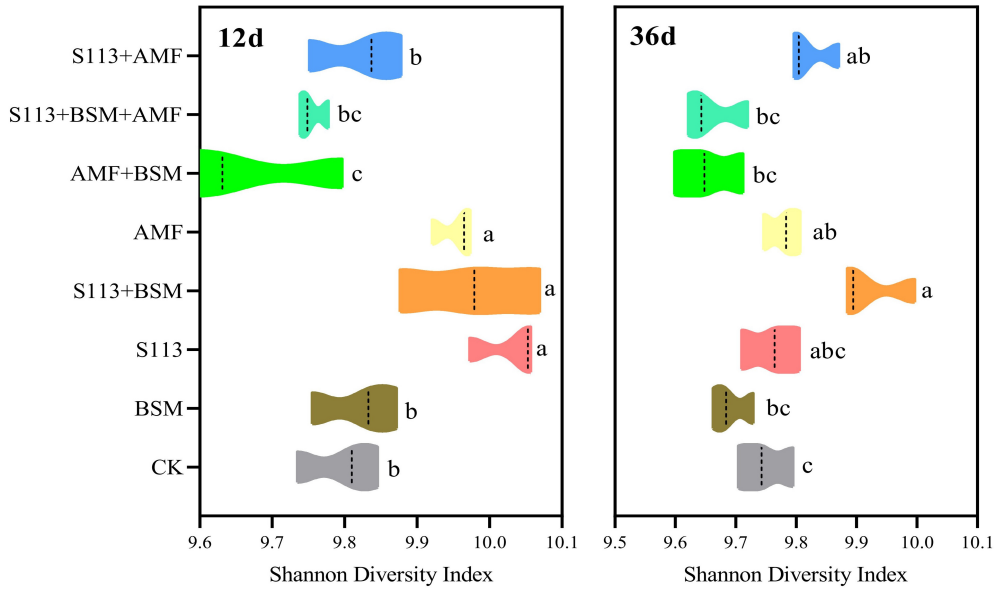

FIGURE 7 | Shannon diversity index for microbial communities of different treatments on the 12th day and 36th day. Note: CK: the seedlings without Bensulfuron-methyl (BSM) and S113 or Arbuscular mycorrhizal fungi (AMF); S113: seedlings were inoculated with S113 by root irrigation; BSM: soils were treated with bensulfuron-methyl, 3.0 mg/kg; AMF: seedlings were inoculated with AMF spore suspension (200 μL); S113 + BSM: seedlings were treated with BSM and inoculated with S113; AMF + BSM: seedlings were treated with BSM and inoculated with AMF spore suspension; S113 + AMF: seedlings were inoculated with S113 and AMF spore suspension; S113 + BSM + AMF: seedlings were inoculated with S113 and AMF spore suspension, and soils were treated with BSM. Values with different letters are significantly different at the $P < 0.05$ according to Duncan's test.

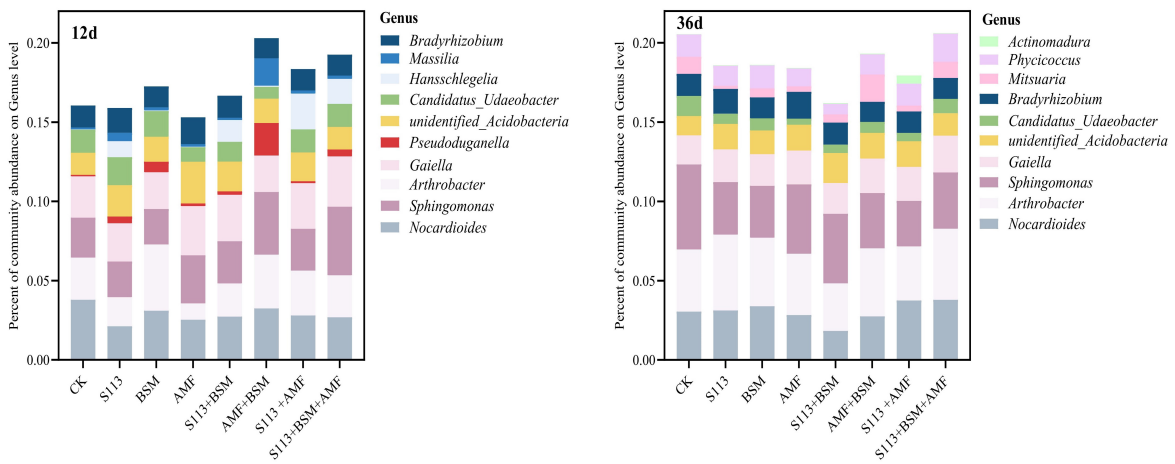

FIGURE 8 | Relative abundance histograms for different treatments in genus level. The top 10 of total species abundance are displayed. Each color represents a different genus level species. CK: the seedlings without BSM and S113 or AMF; S113: seedlings were inoculated with S113 by root irrigation; BSM: soils were treated with bensulfuron-methyl, 3.0 mg/kg; AMF: seedlings were inoculated with AMF spore suspension (200 μL); S113 + BSM: seedlings were treated with BSM and inoculated with S113; AMF + BSM: seedlings were treated with BSM and inoculated with AMF spore suspension; S113 + AMF: seedlings were inoculated with S113 and AMF spore suspension; S113 + BSM + AMF: seedlings were inoculated with S113 and AMF spore suspension, and soils were treated with BSM.

and promotes corn growth, which has largely been associated with its ability to colonize corn roots (Cavaglieri et al., 2005). *Klebsiella pneumonia* NG14 is also capable to colonize rice roots and participates in nitrogen fixation (Liu et al., 2011). Previous research showed that strain S113 could colonize maize roots by forming a biofilm to promote root exudates. The result of this research showed that on the 5th day, strain S113 reached its maximum colonization of the root surface (**Figures 2**, **3**) and could survive on the root surface or rhizosphere for at least 20 days in the presence of AMF (**Figure 3**). Moreover, the S113 population living on the maize root surface and rhizosphere increased slightly after AMF inoculation. This may explain why infection with AMF alternated the types and amounts of organic acids secreted by the roots of maize,

and further promoted S113 chemotaxis to maize roots (Feng et al., 2017; Shyam et al., 2017). It has been reported that AMF infecting the root surface of plants could efficiently regulate their own colonization and that of other strains by changing the composition of root exudates secreted by plants (Pinior et al., 1999). Another reasonable hypothesis is that AMF infection improved the growth condition of the maize root system, which further promoted the colonization and biofilm formation of strain S113 on the root surface. Overall, the AMF *Rhizophagus intraradices* helped the remediation of BSM-contaminated rhizosphere soil by S113, but also promoted the colonization of BSM-degrading strains and the growth of maize cultivated in BSM polluted soil. In other words, the plant-growth-promoting mycorrhizal fungi and BSM degrading bacteria AMF and S113 coexist and establish a successful interaction in the soil environment. Researching S113 colonization and survival in the presence of AMF in the rhizosphere soil or root surface might provide valuable information or new theoretical guidance for the bioremediation of herbicide-contaminated soil. Simultaneously, this can increase crop yield. However, the specific mechanism of how AMF affects the colonization of other rhizosphere microorganisms in the plant rhizosphere soil still merits further exploration.

In particular, the formation of AMF extra-root mycelia after colonization of the plant roots system can alter the structure and composition of the soil microbial community (Toljander et al., 2007; Luginbuehl and Oldroyd, 2017). In the present study, inoculation with AMF indeed increased the diversity and abundance of maize rhizosphere soil community (treatment AMF vs control). For instance, the α-diversity index of the AMF group was high ($p < 0.001$) during the entire experiment period. The relative richness of the genus *Sphingomonas* increased strongly after AMF addition on the 36th day (**Figures 7**, **8**). Although the formation of the mycorrhizal network with plants in the ecological environment is a key factor affecting the structure of the rhizosphere microbial community (Lekberg et al., 2007), the inoculation of other bacteria or bacterial consortia also affects the community structure. However, the ecological behavior of microbes that degrade chemical herbicides, including their interaction with soil indigenous microorganisms and their response to the herbicide substrates, remains less well defined. The results of high-throughput sequencing of 16S rRNA indicated that inoculation of maize root with BSM-degrading stain S113 had a variable effect on the local microbial diversity and relative abundance at the genus level (**Figure 8**). However, AMF-S113 combined remediation to the BSM contaminated soils would not influence the soil micro-ecosystem, which represents a major advantage of bioremediation approaches.

The joint remediation of BSM contaminated soil in this study was only carried out in a pot experiment. Thus, the effect of complex natural environmental factors on the combined bioremediation efficiency in the soil environment, as well as the survival rate of herbicide-degrading strains and plant-growth-promoting fungi in agricultural sites, remains to be studied in field applications. Moreover, the molecular mechanism of how strain S113 promotes AMF (*Rhizophagus intraradices*) infection in the maize rhizosphere, as well as the promotion restoration mechanism of the soil bacterial community by AMF, needs further investigation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: BioProject, PRJNA793182.

AUTHOR CONTRIBUTIONS

YQ designed the work, conducted the experiments, and wrote the manuscript. GZ and JZ statistically analyzed the data and participated in revising the manuscript. HZ conducted the experiments and analyzed the data. XH and TM guided the data analysis and revised the manuscript. All authors contributed to the study and approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.843525/full#supplementary-material

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Effects of Three Pesticides on the Earthworm *Lumbricus terrestris* Gut Microbiota

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Earthworms play a vital role in the terrestrial ecosystem functioning and maintenance of soil fertility. However, many pesticides, for example, imidacloprid, benomyl, and metribuzin that are world-widely used in agriculture, may be potentially dangerous to earthworms. At the same time, standard tests for pesticides acute and chronic toxicity do not reflect all aspects of their negative impact and might not be enough sensitive for effective assessment. In this paper, we studied the effects of non-lethal concentrations of imidacloprid, benomyl, and metribuzin on the gut bacterial community of *Lumbricus terrestris* using high-throughput sequencing approach. We found that pesticides reduced the total bacterial diversity in the earthworm's gut even at the recommended application rate. Under the applied pesticides, the structure of the gut prokaryotic community underwent changes in the relative abundance of the phyla Proteobacteria, Actinobacteria, Acidobacteria, Planctomyces, Verrucomicrobia, and Cyanobacteria, as well as the genera *Haliangium, Gaiella, Paenisporosarcina, Oryzihumus, Candidatus Udaeobacter,* and *Aquisphaera*. Moreover, the pesticides affected the abundance of *Verminephrobacter*—the earthworms' nephridia specific symbionts. In general, the negative impact of pesticides on bacterial biodiversity was significant even under pesticides content, which was much lower than their acute and chronic toxicity values for the earthworms. These results highlighted the fact that the earthworm's gut microbial community is highly sensitive to soil contamination with pesticides. Therefore, such examination should be considered in the pesticide risk assessment protocols.

Keywords: pesticides, next-generation sequencing, earthworm, gut microbiota, bacterial biodiversity

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INTRODUCTION

According to the Food and Agriculture Organization (FAO), more than 4 million tons of pesticides are used in the world annually;[1] when released into the environment, most of them might pollute air, soil, ground and surface waters, as well as pose a threat to non-target organisms (Van der Werf, 1996; Carriger et al., 2006; Duke, 2017). Pesticides, even at concentrations

[1]https://www.fao.org/faostat/en/#data/RP/visualize

not exceeding the Maximum Residue Levels (MRLs), lead to a decrease in biodiversity (Hole et al., 2005; Isenring, 2010; Beketov et al., 2013), in particular, soil microbial biodiversity (Lo, 2010; Puglisi, 2012; Fenner et al., 2013; Astaykina et al., 2020). Moreover, pesticides can change the activity of soil microorganisms. For instance, the fungicide chlorothalonil and insecticide chlorpyrifos have been inhibited dehydrogenase, catalase, urease and acid phosphatase activities (Jastrzębska, 2011; Baćmaga et al., 2018).

Earthworms as important representatives of soil invertebrates are often called 'ecosystem engineers' because their activity modifies physicochemical and biological properties of the habitat (Jones et al., 1994). Depending on the ecological-trophic group, earthworms can contribute to additional migration of pollutants in the soil (Kuzyakov and Blagodatskaya, 2015). For example, *Lumbricus terrestris* which belongs to the anecic earthworms (Bouché, 1977; Lavelle et al., 1989) can transport pesticides from the surface to the mineral horizons. *L. terrestris*, unlike the well-studied compost worm *Eisenia fetida,* is widespread in the arable horizons of most world soils. Furthermore, *L. terrestris* appeared to be a more sensitive species than *E. fetida* in the pesticide toxicity tests (Pelosi et al., 2013). Moreover, due to the structural features, such as the greater length of the intestinal tract, digestion in *L. terrestris* takes up to 6 h (Nechitaylo et al., 2010), consequently, when pesticides enter the digestive tract of *L. terrestris*, they are able to have long-term effects on their gut bacteria.

There are plenty of studies devoted to the analysis of the taxonomic composition of the earthworm's gut microbial community (Horn et al., 2003; Wüst et al., 2011; Sapkota et al., 2020), which usually differ from bacteria isolated from soils or composts (Byzov et al., 2009). The diversity and structure of bacteria in the earthworms' gut can vary depending on the ecological-trophic group of lumbricid (Egert et al., 2004). However, the study of the pesticide impact on the microbial biodiversity in the intestinal tracts of soil invertebrates remains practically out of sight of researchers, although this locus is generally recognized as a hotspot of microbial activity. For example, it was discovered that the insecticide fipronil may inhibit the growth of *Eudrilus eugeniae* gut bacteria plated on a sterile nutrient agar media (Salokhe and Deshapande, 2014). Besides, Kavitha et al. (2019) noted that organophosphate insecticide monocrotophos led to a reduction of the bacterial and fungal biodiversity and abundance in the earthworm *Lampito mauritii* gut. In addition, the herbicide glyphosate affected decrease of the microbial α-diversity in the earthworms *Alma millsoni*, *Eudrilus eugeniae* and *Libyodrilus violaceus* gut, while the relative abundance of *Enterobacter* spp. *DHL-02, Pseudomonas putida, Pantoea agglomerans* and *Pseudomonas taiwanensis* increased (Owagboriaye et al., 2021). Based on the results of molecular genetic analysis using high-throughput sequencing of the 16S RNA gene, it was found that the herbicide fomesafen might reduce bacterial diversity and shift the bacterial community structure of *Pheretima guillelmi* gut (Chang et al., 2021). Nonetheless, there are no studies which examine the effects of pesticides on the gut microbiota of *L. terrestris*, one of the most common earthworm species. Undoubtedly, this kind of research is important not only from the ecotoxicology perspective, but also for isolating bacteria which degrade pesticides (Sun et al., 2020).

Thus, based on the previous studies, we hypothesized that (1) pesticides might impact on the earthworm *L. terrestris* indirectly, through changing the gut microbial community taxonomic structure; (2) pesticides even at the recommended application rate may reduce the earthworm's gut microbial diversity; (3) the gut microbial community relatively quickly response to the pesticide application; (4) bacteria whose relative abundance increases under the impact of pesticides, can be recommended for further research in order to isolate strains that degrade pesticides. To test these hypotheses, we set up a laboratory incubation experiment to study the relationship between pesticide-contaminated soil and earthworm gut microbiota. The cutting-edge approach of total DNA metabarcoding allowed us to assess the taxonomic diversity of gut-associated bacteria without isolation and cultivation of microorganisms which guarantees the novelty of the obtained results.

MATERIALS AND METHODS

Soil and Earthworms Collection

For the laboratory experiment soil samples from the surface (0–10 cm) horizon of Umbric Albeluvisols (IUSS Working Group WRB, 2015) were taken in the Odintsovo district of the Moscow region (55°41′ N, 38°05′ E). The experimental site has been fallowed and has not been treated with pesticides and fertilizers over the past 5 years. Five separate soil samples were randomly selected (average distance between sampling points was 2 m) using a shovel. Large plant residues, live rhizomes, and roots were directly removed during collection. In the laboratory, the soil samples were dried at room temperature, sieved (<1 mm) and homogenized. The main properties of the soil are presented in **Supplementary Table S1**.

Mature specimens of the earthworm *L. terrestris* (Linnaeus, 1758) were collected on the same soil site. Earthworms were maintained at a constant temperature of +17 ± 1°C in the dark for 2 weeks before the start of the experiment in soil containing fresh litter of *Acer platanoides* as a food source.

Pesticides

For the experimental treatment we chose three pesticide formulations that were produced by «Avgust» Inc. (Russia): 700 g/kg metribuzin (herbicide), 200 g/L imidacloprid (insecticide), and 500 g/kg benomyl (fungicide). All actives were > 98.0% pure and met international standards Imidacloprid {4,5-dihydro-N-nitro-1-[(6-chloro-3-pyridyl)-methyl]-imidazolidin-2-ylene-amine} is a highly effective worldwide used neonicotinoid insecticide (Tišler et al., 2009), which is very stable in soil (DT_{50} > 120 days; PPDB: Pesticide Properties DataBase, 2016) and may have long-term effects on non-target species. Metribuzin [4-amino-6-tert-butyl-3-methylthio-1,2,4-triazin-5(4H)-one] is a triazinone herbicide, which has been widely used in agriculture for several

decades. Benomyl [N-[1-(butylcarbomoyl)-benzoimidazolyl-2]-0-methylcarbamate] is a systemic benzimidazole fungicide used to control fungal diseases in agriculture, forestry and veterinary. Benomyl, as well as its main metabolite carbendazim are pesticides stable for degradation in soil (DT_{50} = 61–120 days; PPDB: Pesticide Properties DataBase, 2016). The main properties of these pesticides are provided in **Supplementary Table 2S**. Imidacloprid, benomyl and metribuzin can be used together for the comprehensive protection of cereals, sugar beets and potatoes. Application of these pesticides includes both seed dressing and soil surface spraying before sowing the seeds so that virtually all the applied pesticide dose enters the soil and is not intercepted by the crop.

Experimental Design

The laboratory incubation experiment was carried out in four replicates at a constant temperature (+17 ± 1°C) in glass flasks covered with a perforated film to prevent water loss. The mass of moist soil (60% WHC) in each vessel was 500 g. The soil moisture was controlled by the weight method every 2 days.

The pesticides were applied according to the manufacturer's recommended application rates: 0.98 kg/ha metribuzin, 0.02 kg/ha imidacloprid, and 1.5 kg/ha benomyl. We also tested 2-fold and 10-fold application rates that simulated the worst-case scenarios. As was previously mentioned, imidacloprid and benomyl are stable pesticides, therefore, there are likely to accumulate in soil after many years of application on the same site. For a more thorough distribution of pesticides in the soil sample, initially we mixed the pesticide with 20 g of clear sand, and then the already treated sand was thoroughly mixed with the soil aliquot (500 g). In the control, the soil sample was mixed with untreated sand. In total, four variants of the experiment were carried out: a control and a mixture of three pesticides at the manufacturer's recommended application rates as well as the 2-fold and 10-fold application rates. In each vessel, we placed three specimens of *L. terrestris* (**Figure 1**). According to the OECD Guidelines for the Testing of Chemicals (OECD, 1984) the duration of the experiment was 14 days. To analyze the intestinal prokaryotic community, the worms were collected after 7 and 14 days, respectively.

Isolation and Cleaning of Earthworms' Guts

Before isolating the intestinal tract, the earthworms were washed and kept on wet filter paper for 24 h at a temperature of 6°C–7°C. The earthworms were anesthetized with hot water (100°C) for 3 s and then washed with 70% ethanol. To isolate guts, the worms were frozen on a freezer table (Peltier element) to −16°C and were dissected with a sterile scalpel immediately after defrosting, avoiding repeated freezing–thawing (Byzov et al., 2015). For a molecular genetic analysis, a section of the digestive tract was taken from the clitellum to the anus (**Figure 2**). The guts cut out in this way were placed in Eppendorf tubes containing 1 ml of sterile Milli-Q quality water and were centrifuged for 10 min at 10,000 *g*. The supernatant was removed and the obtained isolates of microorganisms were stored at −80°C until the subsequent isolation of total DNA.

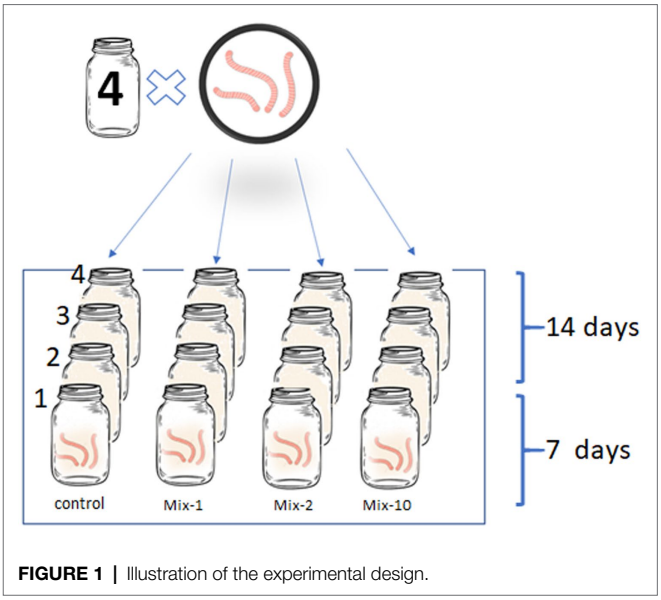

FIGURE 1 | Illustration of the experimental design.

Isolation of Total DNA and DNA Metabarcoding

The extraction of total DNA from a 0.5 mg sample of the earthworm's intestines was performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, United States) according to the manufacturer's protocol. The isolated DNA extracts were stored at −20°C.

The samples of the isolated DNA were 500-fold diluted. Amplification of the V4 variable region of the 16S rRNA gene was carried out in one round using forward and reverse primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with two-index multiplexing of the samples (Fadrosh et al., 2014). These primers are specific both to bacteria and to archaea. The PCR products were purified using the Cleanup Mini kit (Evrogen, Russia) for DNA isolation from reaction mixtures. The concentration of the obtained 16S rRNA libraries in the solution was measured on a Qubit fluorometer (Invitrogen, United states) using the Quant-iT dsDNA High-Sensitivity Assay Kit. The purified amplicons were mixed equimolarly depending on the concentration. The quality of the library prepared for sequencing was assessed by agarose gel electrophoresis. Further, sample preparation and sequencing of the pooled sample was carried out using the MiSeq Reagent Kit v2 (500 cycles) and an MiSeq sequencer (Illumina, United States).

Bioinformatics and Statistical Analyses

Bioinformatics analysis was performed as described in the work by Fadrosh et al. (2014). We used the DADA2 package for R, including error correction, inferring ribosomal sequencing variants (RSVs) separately for forward and reverse reads, elimination of chimeric RSVs and, finally, merging forward

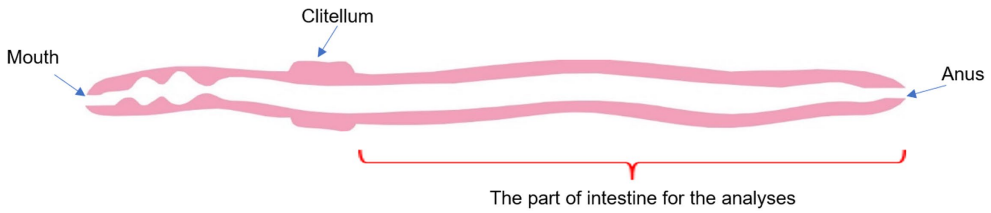

FIGURE 2 | Schematic diagram of the *Lumbricus terrestris* digestive tract.

and reverse RSVs. The obtained average RSV length was about 253 bp (with minimal variability) for bacterial 16S V4 fragments. In contrast to OTUs (operation taxonomic units), the analysis based on RSVs which are also referred to as amplicon sequence variants (ASVs) or exact sequence variants (ESVs) does not imply merging of closely related amplicon variants (<3% differences) into a single sequence (i.e., OTU) and therefore can identify single-nucleotide differences between species (Callahan et al., 2017).

For taxonomic annotation of the obtained RSV sequences, we also used the DADA2 package supplied with the Silva database (version 138) for bacterial communities. RSVs annotated as chloroplasts, mitochondria, Cercozoa, etc. were removed. The resulting read count data were normalized between the samples using the read count annotated at the domain (kingdom) level.

The rest of the analysis was also conducted in the R environment (version 3.6.3). To assess α-diversity, we calculated the Shannon, Chao1 and ACE indices using the fossil 0.4.0 and vegan 2.5–6 packages. When calculating these indices, the read count data were rarefied to match the sample with the minimum number of reads. For the beta diversity analysis, we used Bray-Curtis metric. The rest of the analyses and visualization were performed using the phyloseq 1.30.0, plotly 4.9.2.2, phytools 0.7–47, pheatmap 1.0.12, and ggplot2 3.3.3 packages. To visualize differences between the samples, we applied the metrical and non-metrical multidimensional scaling (MDS). The association analysis between time periods and taxon abundance was performed using the Spearman and Pearson correlations. For non-paired comparison of the two groups, we used the multiple *t*-tests as well as the Mann–Whitney and Pearson tests, and for paired comparisons, we used the Wilcoxon test. The FDR was calculated using the Benjamini–Hochberg adjustment for the obtained value of *p*s. All analyses were carried out in the R environment. For all types of the statistical analysis, the difference was considered significant at a significance level of value of $p < 0.05$.

RESULTS

Structure and Composition of Microbial Community of *Lumbricus terrestris* Gut With or Without Pesticide Impact

The study was based on 1,287,366 high-quality reads of 16S rRNA gene amplicons. In total, 208 bacterial genera from 45 classes that belong to 21 prokaryotic phyla were identified in the gut of *L. terrestris*.

Proteobacteria (50.3%), Actinobacteria (19.7%), and Firmicutes (17.6%) were found to be dominants of the microbial community. The other phyla were represented to a much lesser extent: Verrucomicrobia, Chloroflexi, Planctomycea up to 4%, Myxococcota, Crenarchaeota, and Acidobacteria up to 1% of all the obtained sequences (**Figure 3**). At the class level, gram-negative bacteria Gammaproteobacteria and gram-positive Bacilli and Actinobacteria dominated (>10% of all obtained sequences) in the prokaryotic community (**Figure 3**). At the genus level, *Klebsiella, Acinetobacter* and *Verminephrobacter* represented up to 31% of all assigned nucleotide sequences in the earthworm's gut (**Figure 3**).

Compared to the control, the structure of the prokaryotic community of the gut treated with pesticides changed as follows: (1) on day 7 of incubation, the relative abundance of the phylum Proteobacteria increased and the relative abundance of Actinobacteria decreased; (2) on day 14 of incubation, the relative abundance of the phyla Acidobacteria, Planctomyces, Verrucomicrobia, and Cyanobacteria increased ($p < 0.05$, **Supplementary Table 3S**). At the class level, on day 7 of incubation with pesticides, the relative abundance of gram-negative bacteria Gammaproteobacteria increased, while the relative abundance of Chloroflexia, Delta Proteobacteria and Anaerolineae decreased ($p < 0.05$, **Supplementary Table 4S**). After a 14-day incubation with pesticides, the relative abundance of the classes Ktedonobacteria, Planctomycetes, Acidobacteriia, Verrucomicrobiae and Cyanobacteriia increased ($p < 0.05$, **Supplementary Table 4S**). At the genus level, on day 7 of incubation with pesticides, the relative abundance of *Haliangium, Gaiella, Paenisporosarcina*, and *Oryzihumus* in *L. terrestris* gut decreased, while that of *Verminephrobacter* increased 3-fold ($p < 0.05$, **Supplementary Table 5S**). On day 14 of incubation of earthworms with pesticides, the relative abundance of *Candidatus Udaeobacter* and *Aquisphaera* in *L. terrestris* gut increased, while that of *Verminephrobacter* decreased 2-fold ($p < 0.05$, **Supplementary Table 5S**).

Pesticide-Sensitive Bacteria of *Lumbricus terrestris* Gut

The earthworm's gut associated bacteria that considerably changed in relative abundance under pesticide exposure are shown in **Figure 4**. In contrast to the control variants, as well as to the variants treated with pesticides at the recommended and 2-fold application rate, in the variants treated with pesticides at a

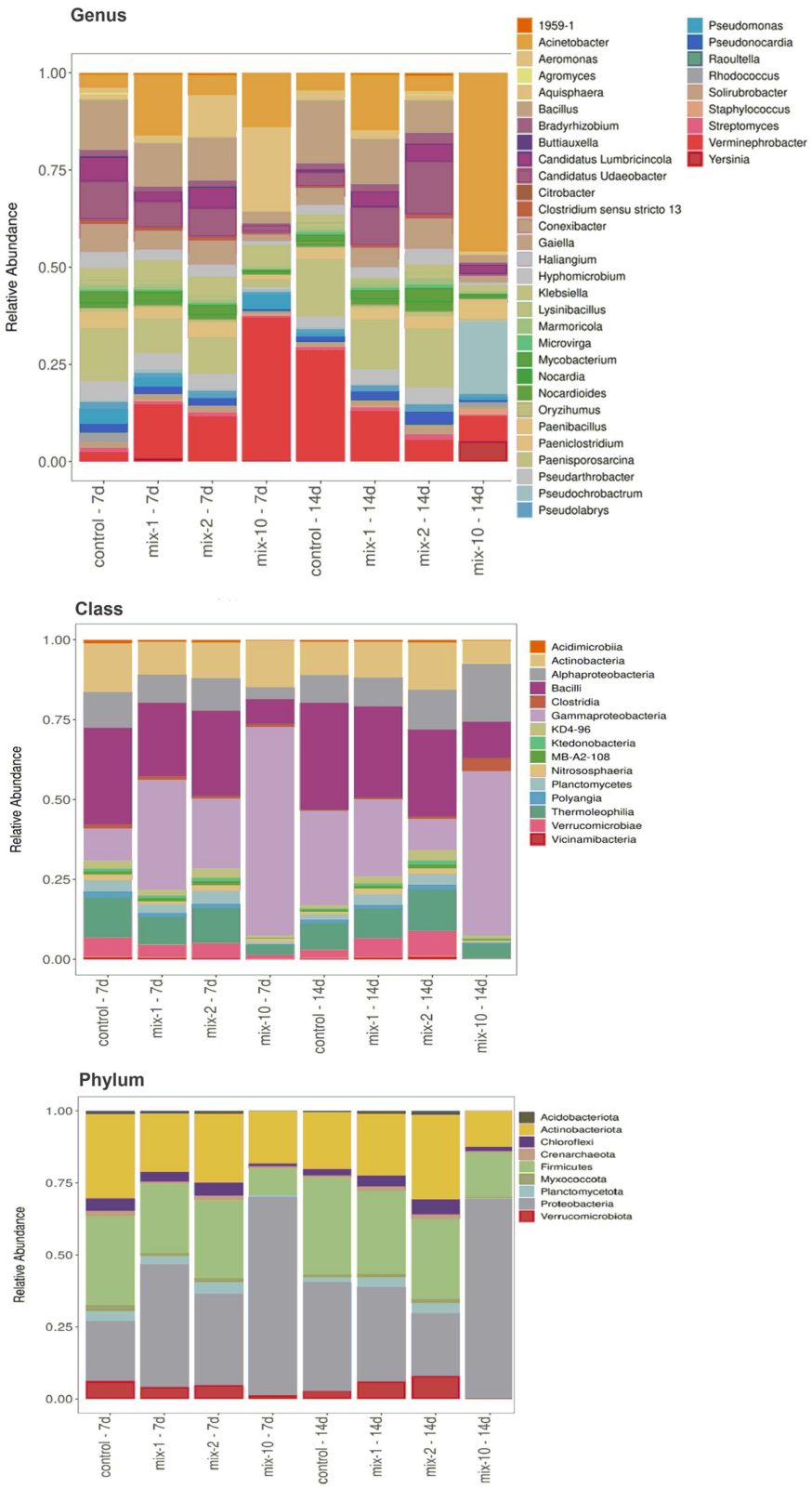

FIGURE 3 | The structure of the prokaryotic community of the intestinal tract of *L. terrestris* at the levels of phyla, classes and genera ($N = 2$). Legend: mix indicates pesticides in a mixture; 1/2/10 is the recommended, 2-fold and 10-fold pesticide application rates; 7 day/14 day is the incubation time. The relative abundance is shown for taxa with read counts >0.3%.

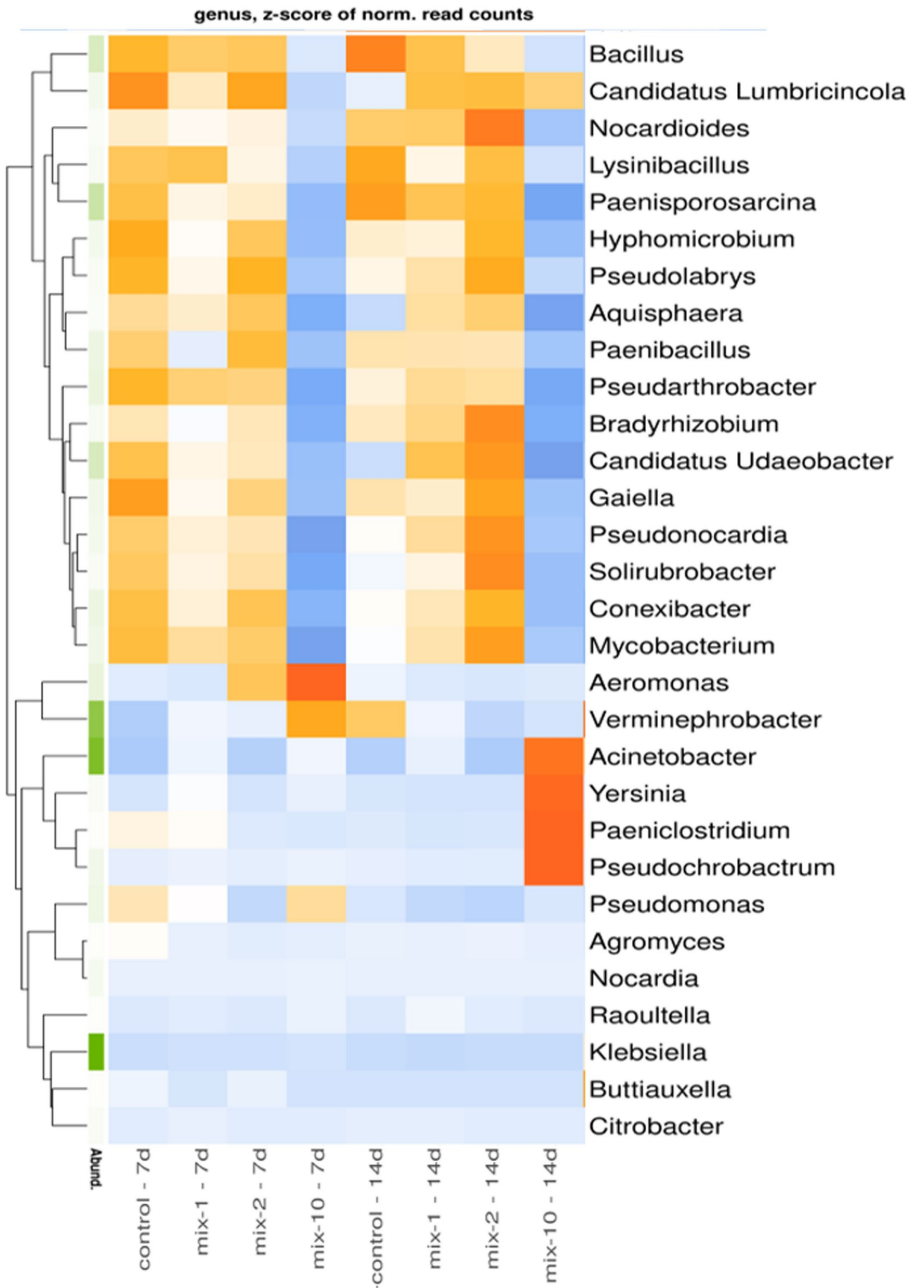

FIGURE 4 | Heatmap for relative abundance of top 30 microbial taxa in prokaryotic communities of the earthworm's guts at the genus level ($N = 2$). The data are presented as *z*-scores. Microbial genera with positive *z*-scores are marked in orange, genera with negative *z*-scores in blue. Legend: mix indicates pesticides in a mixture; 1/2/10 is the recommended, 2-fold and 10-fold pesticide application rates; 7 day/14 day is the incubation time.

10-fold application rate, the relative abundance of the genera *Bacillus*, *Candidatus Lumbricincola, Nocardioides, Lysinibacillus, Paenisporosarcina, Hyphomicrobium, Pseudolabrys, Aquisphaera, Paenibacillus, Pseudarthrobacter, Bradyrhizobium, Candidatus Udaeobacter, Gaiella, Pseudonocardia, Solirubrobacter, Conexibacter,* and *Mycobacterium* in *L. terrestris* gut decreased both after 7 and 14 days of the incubation experiment (**Figure 4**). Conversely, in the variants treated with pesticides at a 10-fold application rate, the relative abundance of *Acinetobacter, Yersinia, Paeniclostridium,* and *Pseudochrobactrum* in the earthworm's guts increased on day 14.

Gut Bacteria α-Diversity

Pesticides concentration affected the gut microbial α-diversity (**Figure 5**). For instance, the Shannon biodiversity index decreased from 3.6 in the control to 2.2 ($p < 0.05$) in the variants with a 10-fold application rate (**Figure 5**). The maximum values of the Chao1 and ACE biodiversity indexes were in the control

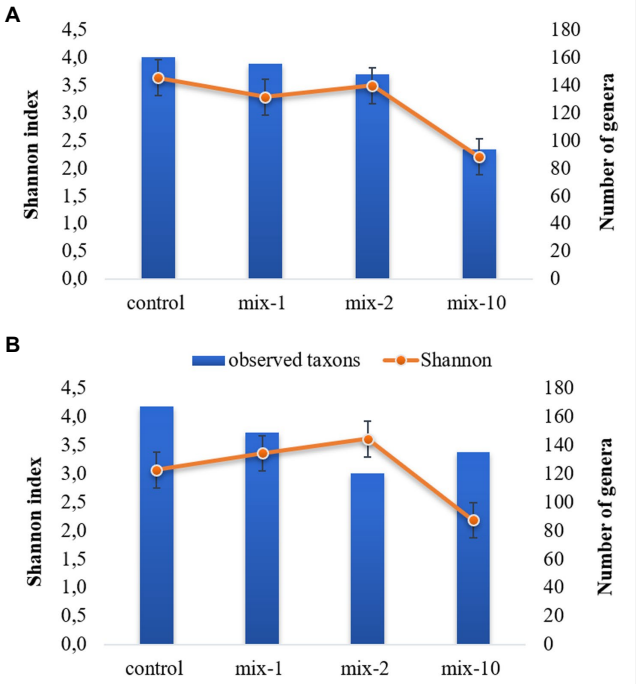

FIGURE 5 | Dynamics of Shannon index and number of genera on day 7 **(A)** and day 14 **(B)** of incubation (mean ± SE). 1/2/10 is the recommended, 2-fold and 10-fold pesticide application rates.

variants, but decreased ($p < 0.05$) in the variants with a 10-fold application rate of pesticides (the data are provided in **Supplementary Table 6S**).

The number of identified genera decreased from 161 to 94 on day 7 after the pesticides application. However, on day 14 of the experiment, the number of identified genera increased in the variants with a 10-fold application rate compared to the variants with a 2-fold application rate. At the same time, it was lower than in the control (**Figure 5**).

Gut Bacteria β-Diversity

Based on the Bray-Curtis metrics and PERMANOVA tests, all samples were divided into two distinct clusters: (1) control variants, samples with the recommended and 2-fold application rates; (2) samples with a 10-fold application rate of pesticides (**Figure 6**).

In general, the dissimilarities in the composition of the gut bacterial communities between the samples were high. Both after 7 and 14 days of the incubation experiment, the samples with the highest concentration of pesticides (a 10-fold application rate) tended to position together in the upper right corner of the plot, in contrast to the other variants.

DISCUSSION

Differences in Bacterial Communities of *Lumbricus terrestris* Gut and Soil

Our previous studies (Astaykina et al., 2020; Streletskii et al., 2022) were devoted to microbial biodiversity of Umbric Albiluvisols under pesticides treatment. We found that the pesticide exposure led to a reduction in the relative abundance of bacterial phyla Myxococcota, Bacteroidetes, Gemmatimonadetes, Proteobacteria. At the genus level pesticides increased the relative abundance of *Kitasatospora* and *Streptomyces* which could be explained by the involvement of these bacteria in the degradation of pesticides. Our current data has shown that Proteobacteria and Actinobacteria were dominant in the bacteria communities both in the earthworm's gut and in the surrounding soil. However, in contrast to the soil, Firmicutes was also the dominant phylum in the gut. This data corresponds with previous research, where Proteobacteria, Actinobacteria, Firmucutes, Bacteroidetes, and Verrucomicrobia have been shown to be the dominant phyla in *L. terrestris* gut (Knapp et al., 2009; Pass et al., 2015; Ma et al., 2017; Meier et al., 2021). The digestive tract of an earthworm is a microaerophilic and even anaerobic zone (Byzov et al., 2015); hence, phyla Firmicutes and Actinobacteria, which are facultative and/or obligate anaerobes, dominate in the prokaryotic community of the gut (Chang et al., 2021). On the other hand, Chloroflexi, the bacteria phylum which may be among dominants in *E. fetida* gut (Liu et al., 2018), was not a dominant in *L. terrestris* gut due to the differences in burrowing and feeding habits of worms.

Pesticides Alter the Structure of the Gut Bacteria Community

Pesticides effect on earthworms might be more complex than described by standard indicators such as LC_{50}, NOEC, body mass changes and behavioral disorders (Pelosi et al., 2014). For instance, some effects on the molecular and cellular levels (e.g., oxidative stress, DNA damage, teratogenesis) have been recently detected (Datta et al., 2016). The fungicide benomyl may cause the disruption of cell's microtubules (Hess and Nakai, 2000) and affect the development of *E. fetida* spermatozoa (Sorour and Larink, 2001). The insecticide imidacloprid can lead to a significant reduction in *E. fetida* fecundity, as well as to a damage of the epidermal and midgut cells of the earthworms (Wang et al., 2015). The fungicide benomyl, insecticide imidacloprid and herbicide metribuzin, separately or combined, have led to changes in the diversity and structure of the soil microbiota (Astaykina et al., 2020). However, there is no data regarding the effect of these pesticides on the earthworm gut microbiota. Our results showed that these pesticides increased the relative abundance of the phyla Proteobacteria, Acidobacteria, Planctomyces, Verrucomicrobia, and Cyanobacteria in the structure of their gut prokaryotic community, while Actinobacteria decreased. A reduction in this phylum indicated the decrease in the ability to produce enzymes necessary for the cellulose, hemicellulose and other natural polymers decomposition (Vieites et al., 2010).

Previously, Chang et al. (2021) also found that the herbicide fomesafen at the recommended application rate caused significant differences in the relative abundance of Actinobacteria, Firmicutes, and Proteobacteria. Furthermore, it was found that the increased abundance of Proteobacteria can be considered as a potential marker of imbalance in the gut microbiota of many earthworm species (Shin et al., 2015; Wang et al., 2022).

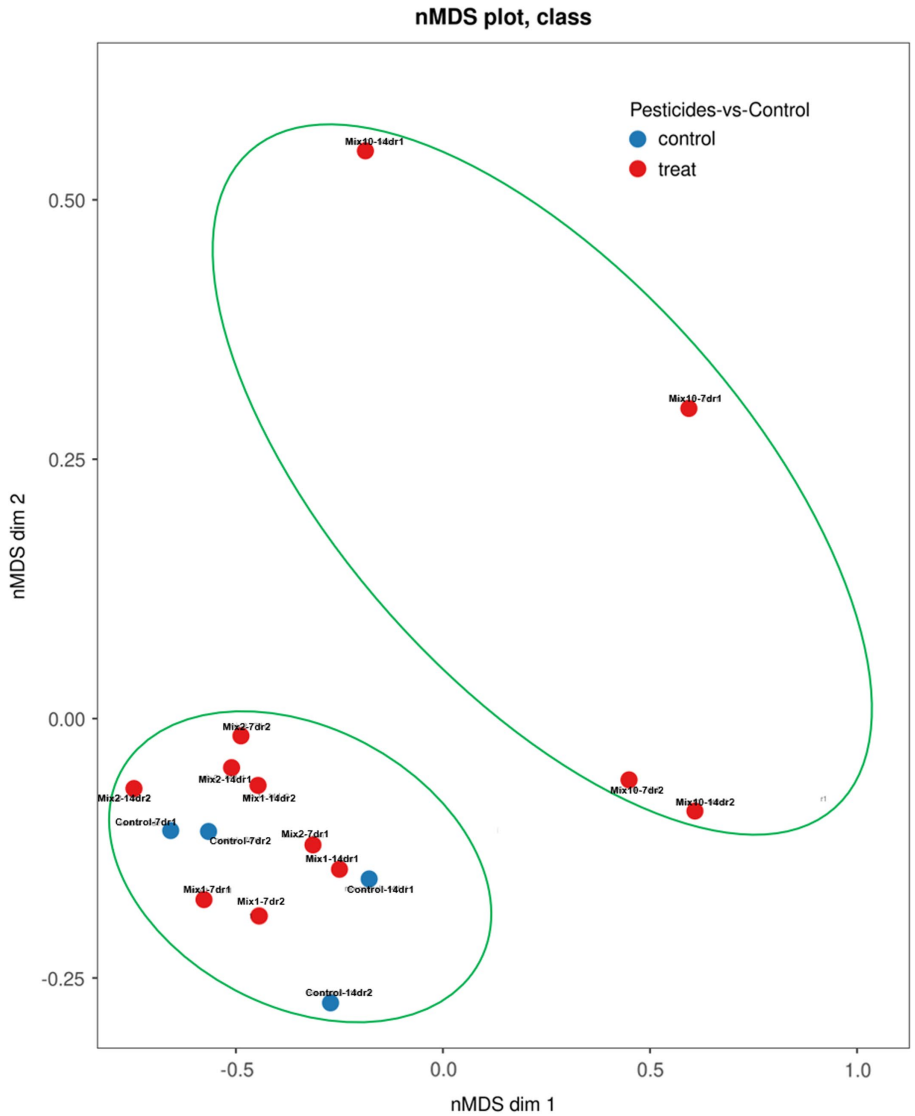

FIGURE 6 | Non-metric multidimensional scaling plot of the assembly patterns of prokaryotic communities of the earthworm's gut using the Bray–Curtis (BC) distance matrix at the genus level.

At the genus level, pesticides increased the relative abundance of gram-negative *Verminephrobacter* (the phylum Proteobacteria), while the relative abundance of *Haliangium* (myxobacteria from the phylum Proteobacteria), *Gaiella* and *Oryzihumus* (gram-positive Actinobacteria), *Paenisporosarcina* (the phylum Firmicutes) decreased after 7 days of pesticide exposure. Conversely, after 14 days of incubation the relative abundance of *Verminephrobacter* decreased, while the relative abundance of gram-negative *Candidatus Udaeobacter* (the phylum Verrucomicrobia) and *Aquisphaera* (the phylum Planctomyces) increased. The genus *Verminephrobacter* is the extracellular species-specific bacterial symbionts of Lumbricid earthworms inhabiting their nephridia, i.e., kidney-like osmoregulatory organs (Dulla et al., 2012; Lund et al., 2014). Nephridia are located next to the intestinal tract and may enter the sample during resection. Based on the results of molecular genetic analysis, bacteria of the genus *Verminephrobacter* were found to be more sensitive to the presence of pesticides in the soil than bacteria of the gut. Apparently, the pesticides application may increase the earthworms' excretory organs activity, consequently, the excretion rate of pesticide decomposition products may increase (Wells and Laverack, 1963). *Verminephrobacter* are likely to provide enzymes or other essential co-factors for these biochemical reactions (Lund et al., 2014). However, after 14 days of the experiment, the relative abundance of *Verminephrobacter* in the prokaryotic community of lumbricid significantly decreased. This can be explained by two factors: (1) the earthworms adapted to the presence of pesticides in the soil; (2) their metabolic capacity decreased after pesticide exposure, which can be considered as an indicator of pesticide toxicity.

The verrucomicrobial genus *Candidatus Udaeobacter* was also sensitive to pesticides in the soil. Our results showed that the pesticides application increased the relative abundance of this genus. According to the previously published research, *Candidatus Udaeobacter* are the most dominant uncultured soil bacteria that can oxidize the trace gas H_2 to generate energy and utilize nutrients due to antibiotic-driven lysis of other soil microbes (Willms et al., 2020). It is possible that pesticide treatment restructured the intestinal complex and amplified the antibiotic activity of *Candidatus Udaeobacter*. The functional role of these bacteria in the earthworm's gut remains to be determined.

The dominant gut bacterial genera which are sensitive to pesticide treatment play an important role in organic matter decomposition and nutrient cycling both in earthworms' guts and soils (Byzov et al., 2015). For instance, *Conexibacter* is involved in the nitrification as well as *Pseudarthrobacter* is denitrifiers (Su et al., 2019), and *Lysinibacillus* is nitrogen fixers. Therefore, a change in these bacteria abundance can affect the nitrogen cycle (Jien et al., 2021). Moreover, *Lysinibacillus sphaericus* can be used both in soil amendment in the replantation processes (Aguirre-Monroy et al., 2019) and as degraders of pesticides, in particular glyphosate (Pérez Rodríguez et al., 2019). Some *Nocardioides* species can degrade complex organic pollutants, so reducing their presence may affect the rate of natural remediation (Zhao et al., 2018).

Effects of Pesticides Application on the Earthworm Gut Bacterial Diversity

It was found that pesticides both at the recommended application rate and at the rate increased by 10 times decreased the values of α-biodiversity indices. It is similar with the effect of other xenobiotics, such as heavy metals, microplastics, antibiotics (triclosan), pesticides (fomesafen), which significantly reduced the bacterial biodiversity in the digestive tracts of soil invertebrates (Ma et al., 2017; Chen et al., 2020; Sun et al., 2020; Chang et al., 2021). Biodiversity reduction and changing of dominants in the bacterial community of the digestive tract led to a decrease in the biochemical activity of the intestinal contents and, therefore, affected the ability of the earthworm to assimilate the substrate. The pesticides may also alter the feeding behavior, leading to changes in the composition of intestinal microbiota (Zhu et al., 2018).

The β-diversity assessment showed that bacterial complexes of *L. terrestris* gut cluster according to the application rate of pesticides. The variants with a 10-fold pesticide application rate formed a separate cluster both after 7 and 14 days of incubation. Comparison of pesticide concentrations in the variants with a 10-fold application rate with the values of acute and chronic toxicity of these pesticides for earthworms showed that even when the recommended application rate is 10-fold increased, the final concentration of the pesticide is more than 100 times lower than the values of acute and chronic toxicity of the same pesticide (0.008 mg/kg metribuzin versus LC_{50} = 427 mg/kg and NOEC >52.3 mg/kg; 0.0002 mg/kg imidacloprid versus LC_{50} = 10.7 mg/kg and NOEC = 0.178 mg/kg; 0.01 mg/kg benomyl versus LC_{50} = 5.4 mg/kg and NOEC = 1.0 mg/kg; **Supplementary Table S2**). Thus, the microbial community of the intestinal tract is highly sensitive to soil pollutants (Chang et al., 2021), hence, this type of research should be included in the practice of assessing the risks of pesticide application for non-target organisms.

Pesticide-Degrading Bacteria

Numerous studies have demonstrated the crucial role of bacteria inhabiting the earthworm's guts in the transformation of organic pollutants in the environment (Sun et al., 2020). It is known that there are different ways to transform organic pollutants in the earthworm's gut such as the activity of transit bacteria, their free enzymes and intestinal symbionts (Byzov et al., 2015). It has been established that *Rhodococcus* and *Bacillus* bacteria from the earthworm's intestine can degrade pesticides (Kuipa et al., 2016). In this study, we showed that pesticide treatment increases the relative abundance of Proteobacteria phylum and at the genus level, increases the relative abundance of *Acinetobacter, Pseudochrobactrum* and bacterial symbionts *Verminephrobacter*.

Acinetobacter and *Pseudochrobactrum* are active microbiodegraders which can degrade many xenobiotics, including pesticides (Pawar and Mali, 2014; Kafilzadeh et al., 2015; Doolotkeldieva et al., 2018; Zhan et al., 2018). The discovered fact allows us to assume that bacteria of Proteobacteria phylum which possess high hydrolytic activity can be considered as pesticide-degrading bacteria. Therefore, further studies are required to assess the physiological and biochemical potential of Proteobacteria in the digestive tract of earthworms. For instance, it would be of great interest to design primers corresponding to carboxylesterases of bioscavengers involved in pesticide detoxification (Sanchez-Hernandez et al., 2009). It is also important to determine the role of *Verminephrobacter*, as a nephridium symbiont, in the mechanisms of pesticide detoxification.

CONCLUSION

In summary, based on the metagenomic analysis, pesticide-sensitive taxa, such as *Verminephrobacter, Acinetobacter, Candidatus Udaeobacter, Pseudochrobactrum* were identified in the *Lumbricus terrestris* gut. Depending on the duration of incubation, the reaction of the gut bacteria community on the presence of pesticides in the soil was different. Our results indicated that after 7 days of pesticide exposure the relative abundance of gram-negative *Verminephrobacter* increased, while the relative abundance of *Haliangium*, *Gaiella* and *Oryzihumus, Paenisporosarcina* decreased. On the contrary, after 14 days of incubation the relative abundance of *Verminephrobacter* decreased, while the relative abundance of gram-negative *Candidatus Udaeobacter* and *Aquisphaera* increased. It is possible that the gut microbiota adapted, and taxa that were initially subjected to toxic influence soon restored their abundance. We have discovered that pesticides can have a significant effect

on the composition of the earthworms gut bacterial community at concentrations that were many times less than their toxicity to earthworms. Therefore, standard methods for assessing risks of pesticides application do not have enough sensitivity and the NGS methods might be recommended for a better understanding of possible changes in the soil environment under pesticides application.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: NCBI BioProject—PRJNA797445.

AUTHOR CONTRIBUTIONS

AA, RS, and MM: conceptualization. RS and VG: methodology, resources, and funding acquisition. AA: software, formal analysis, data curation, writing—review and editing, visualization, and project administration. AA and GK: validation. AA and RS: investigation. MM and GK: writing—original draft preparation. MM and VG: supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.853535/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Role of Insect Gut Microbiota in Pesticide Degradation: A Review

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Insect pests cause significant agricultural and economic losses to crops worldwide due to their destructive activities. Pesticides are designed to be poisonous and are intentionally released into the environment to combat the menace caused by these noxious pests. To survive, these insects can resist toxic substances introduced by humans in the form of pesticides. According to recent findings, microbes that live in insect as symbionts have recently been found to protect their hosts against toxins. Symbioses that have been formed are between the pests and various microbes, a defensive mechanism against pathogens and pesticides. Insects' guts provide unique conditions for microbial colonization, and resident bacteria can deliver numerous benefits to their hosts. Insects vary significantly in their reliance on gut microbes for basic functions. Insect digestive tracts are very different in shape and chemical properties, which have a big impact on the structure and composition of the microbial community. Insect gut microbiota has been found to contribute to feeding, parasite and pathogen protection, immune response modulation, and pesticide breakdown. The current review will examine the roles of gut microbiota in pesticide detoxification and the mechanisms behind the development of resistance in insects to various pesticides. To better understand the detoxifying microbiota in agriculturally significant pest insects, we provided comprehensive information regarding the role of gut microbiota in the detoxification of pesticides.

Keywords: toxicology, microbial detoxification, insecticide degradation, resistant species, symbiotic bacteria

INTRODUCTION

Insects are the world's most diverse and abundant animals in terms of species diversity and body mass in all ecological habitats (Nagarajan et al., 2022). Their numerous interactions with beneficial microbes are essential for survival and diversity. Microbes that are living in the guts of insects play a vital role in the biology and behavior of their hosts, including assisting in the

digestion of recalcitrant food components, upgrading nutrient-poor diets, modulating the immune response, and protecting from predators, parasites, pathogens, and disease vectors. Other functions include facilitating plant specialization, governing mating preference and reproductive systems, and contributing to inter- and intraspecific communication (Sharon et al., 2010; Engel et al., 2012; Tokuda et al., 2018; Xia et al., 2018).

Many studies describing symbiotic connections between microbes and insects have been published (Funaro et al., 2011; Dang et al., 2017; Nicoletti and Becchimanzi, 2022). Most insects are thought to be in symbiotic partnerships with microbes, with estimates ranging from 15 to 20% of the total (Zhou et al., 2021). The role of microorganisms, particularly gut microbes, in insect function is important from various viewpoints, including agriculture, ecology, and medicine. Few insects are good laboratory models for studying microbe populations and their associations with hosts, especially immunology and metabolic associations (Hamilton and Perlman, 2013). Entomological studies of parasitic and mutualistic connections have focused on social insects like ants, which have evolved diverse interactions with other species at various levels, including individual and community interactions. These interactions can occur between bacteria and different insects and plants (Moreau, 2020).

Symbiotic bacteria can affect the efficacy of disease vectors or their developmental time, making them possible targets for disease control (Chouaia et al., 2012; Ricci et al., 2012). Microorganisms allied with pollinators and herbivores, and insects that feed on them are likely to impact the agricultural crops' health substantially. Insects and their gut microbial populations play vital roles in the nitrogen cycle and the decomposition of plant material in natural and human-impacted ecosystems (Fox-Dobbs et al., 2010; Engel and Moran, 2013). A symbiotic relationship with very adaptable bacteria may have opened new ecological niches and unbalanced food sources like plant sap or blood (Sudakaran et al., 2017). Mutualism between insects and microbes is unquestionably one of the primary drivers of insect evolution. It is one of the most important factors contributing to the remarkable success of this gigantic group of animals. Mutualism is described as an interaction between various species mutually advantageous to both parties (Armitage et al., 2022). Several fitness traits of insects are heavily influenced by associated microbiota (de Almeida et al., 2017). The association of insects with microbiota is very important for the evolution of ecological features and feeding habits in which insects exchange nutrients or specific functions, such as protection from adversaries or transit between parties (Kikuchi et al., 2012; Suárez-Moo et al., 2020). Symbiotic-associated bacteria allow insects to feed on hard-to-digest and nutritionally poor diets (Salem and Kaltenpoth, 2022). However, insects may be associated with various microbes that also play an important role in degrading pesticides.

Pesticides may have unintended harmful impacts on humans, non-target creatures, and the environment (surface, soils, and groundwater), as the products are designed to be poisonous and are intentionally discharged into the environment (Kamal et al., 2020). Pesticide hazard is a function of the pesticide's (eco) toxicological qualities and the pesticide's ability to harm humans, flora, and animals (Müller et al., 2014). In modern farming systems, pesticides have become an important part of the process. As a result of persistent pests' resurgence, the overreliance on pesticides for pest control may not end soon. Consequently, various biological and ecological factors mediate several available reports on insect pests' resistance against different pesticides (**Table 1**). As a result of the overdependence on synthetic pesticides, numerous concerns have been raised in lieu of their side effects, such as the development of resistance in the target insects, the pollution of the environment, and the effects on human health (Du et al., 2020). It has also been suggested that pesticide resistance may be influenced by gut microbiota, which adds another degree of complexity to the processes of resistance (Gressel, 2018). Bacteria have been demonstrated to directly break down organic pesticides such as chlorpyrifos, dimethoate, and ethoprophos (Nayak et al., 2018; Chen et al., 2020; Gunstone et al., 2021). Furthermore, agricultural pests regularly acquire these bacteria after ingesting them from various sources, including food and the environment (Kikuchi et al., 2012). The gut microbiome may also potentially aid in detoxification by modulating the immune system of the host (Xia et al., 2018). Gut bacteria that produce nutrients and other beneficial chemicals may help the host develop better and increased tolerance to food poisons, although direct experimental data remains sparse (Kohl and Dearing, 2016; Mason et al., 2019).

Increasing apprehensions about the dramatic upsurge in pesticide resistance in pests have prompted researchers to better understand the mechanisms through which insect gut microbiome may confer resistance. Insect gut microbial populations have been studied for their potential role in pesticide resistance—for example, in *Riptortus pedestris*, *Burkholderia* symbionts have been demonstrated to promote pesticide resistance, and fenitrothion-degrading *Burkholderia* strains can also be shifted horizontally to other insects (Kikuchi and Yumoto, 2013). Similarly, Cheng et al. (2017) found that trichlorfon-degrading *Citrobacter* sp. (CF-BD) isolated from the gut of *Bactrocera dorsalis* increased pesticide resistance in the cockroach gut. In addition, many non-septate fungi and bacteria, assumed to be mutualistic, were found in the small intestines of workers of cephalotinid ants. These bacteria live as a moderately dense flora that contains a diverse range of bacterial species, including gram-positive and gram-negative coccobacilli and anaerobes similar to *Bacterioides* and *Clostridia* species (Donelli et al., 2012).

We already know that the environment in the insect gut regulates or even determines the shape of the community microbiota diversity and its metabolic activities, which might cause physical consequences for insects (Tang et al., 2012; Xia et al., 2018). Variations in environmental situations have been shown to affect the microbiota interrelationships among insects and their microbiota and related gene expression (Possemiers et al., 2011; Stencel and Wloch-Salamon, 2018). Recently, emerging research have suggested associations between insect gut microbiome and pesticide resistance. Several studies ranging from community diversity surveys to molecular analyses have focused on the gut bacteria's interactions with the host immune systems (Kikuchi et al., 2012; Engel and Moran, 2013; Xia et al., 2013; Chmiel et al., 2019).

TABLE 1 | Some of the common pesticides that have been used against various resistant insect pests.

Pesticides	Common name of the targeted insect pests	Scientific name	References
Abamectin	American serpentine leafminer	*Liriomyza trifolii*	Ferguson, 2004
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
Acetamiprid	Melon and cotton aphid	*Aphis gossypii*	Bass et al., 2015
	Tobacco whitefly	*Bemisia tabaci*	Basit et al., 2013
	Asian citrus psyllid	*Diaphorina citri*	Naeem et al., 2016
	Colorado potato beetle	*Leptinotarsa decemlineata*	Bass et al., 2015
	Rice planthoppers	*Sogatella furcifera*	Zhang et al., 2017
	Codling moth	*Cydia pomonella*	Bass et al., 2015
	Cotton leafhopper	*Amrasca biguttula biguttula*	Chaudhari et al., 2015
	Western flower thrips	*Frankliniella occidentalis*	Bass et al., 2015
Azadirachtin	Tobacco whitefly	*Bemisia tabaci*	Dângelo et al., 2018
Benfuracarb	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
Bifenthrin	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
Buprofezin	Tobacco whitefly	*Bemisia tabaci*	Basit et al., 2013
	Rice planthoppers	*Sogatella furcifera*	Zhang et al., 2014
	The brown planthopper	*Nilaparvata lugens*	Wu S. F. et al., 2018
	Rice planthoppers	*Sogatella furcifera*	Jin et al., 2017
Carbamate	Cotton leafworm	*Spodoptera litura*	Saleem et al., 2008
Chlorantraniliprole	Beet armyworm	*Spodoptera exigua*	Lai and Su, 2011
	Tomato leafminer	*Tuta absoluta*	Roditakis et al., 2018
Chlorpyrifos	Rice planthoppers	*Sogatella furcifera*	He et al., 2015; Jin et al., 2017
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
Chlorpyriphos	Asian citrus psyllid	*Diaphorina citri*	Naeem et al., 2016; Chen et al., 2021
Clothianidin	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
	Colorado potato beetle	*Leptinotarsa decemlineata*	Bass et al., 2015
	Green peach aphid	*Myzus persicae*	Bass et al., 2015
	Rice planthoppers	*Sogatella furcifera*	Zhang et al., 2017
	The brown planthopper	*Nilaparvata lugens*	Khan et al., 2020
Cypermethrin	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012; Hafeez et al., 2020
Cyromazine	American serpentine leafminer	*Liriomyza trifolii*	Ferguson, 2004
Deltamethrin	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
	Tobacco whitefly	*Bemisia tabaci*	Longhurst et al., 2013
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012; Hafeez et al., 2019
	Red flour beetle	*Tribolium castaneum*	Zhu et al., 2016
Diamide	Diamondback moth	*Plutella xylostella*	Steinbach et al., 2015
	Tomato leafminer	*Tuta absoluta*	Roditakis et al., 2017
Diflubenuron	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
Dinotefuran	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
	Colorado potato beetle	*Leptinotarsa decemlineata*	Bass et al., 2015
	Rice planthoppers	*Sogatella furcifera*	Zhang et al., 2017
Emamectin benzoate	Housefly	*Musca domestica*	Khan et al., 2016
	Diamondback moth	*Plutella xylostella*	Patil et al., 2011
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Tomato leafminer	*Tuta absoluta*	Roditakis et al., 2018
Esfenvalerate	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
Ethiprole	The brown planthopper	*Nilaparvata lugens*	Garrood et al., 2016
Fenpropathrin	Asian citrus psyllid	*Diaphorina citri*	Tiwari et al., 2011
Fenvalerate	Beet armyworm	*Spodoptera exigua*	Musa Khan et al., 2021
Fipronil	Diamondback moth	*Plutella xylostella*	Wang et al., 2016a
	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
	Rice planthoppers	*Sogatella furcifera*	Tang et al., 2010; Jin et al., 2017
Flonicamid	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
Imidacloprid	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015; Kim et al., 2015
Imidacloprid	Asian citrus psyllid	*Diaphorina citri*	Bass et al., 2015
	Small brown planthopper	*Laodelphax striatellus*	Bass et al., 2015
	Housefly	*Musca domestica*	Bass et al., 2015
	Green peach aphid	*Myzus persicae*	Bass et al., 2015
	The brown planthopper	*Nilaparvata lugens*	Bass et al., 2015; Garrood et al., 2016; Wu S. F. et al., 2018
	Avocado thrips	*Scirtothrips perseae*	Byrne et al., 2005
	Rice planthoppers	*Sogatella furcifera*	Bass et al., 2015
	Cotton leafhopper	*Amrasca biguttula biguttula*	Chaudhari et al., 2015
	Tobacco whitefly	*Bemisia tabaci*	Longhurst et al., 2013

(Continued)

TABLE 1 | (Continued)

Pesticides	Common name of the targeted insect pests	Scientific name	References
	Asian citrus psyllid	*Diaphorina citri*	Tiwari et al., 2011; Naeem et al., 2016
	Western flower thrips	*Frankliniella occidentalis*	Bass et al., 2015
	Colorado potato beetle	*Leptinotarsa decemlineata*	Bass et al., 2015
	Rice planthoppers	*Sogatella furcifera*	Jin et al., 2017
	Greenhouse whitefly	*Trialeurodes vaporariorum*	Bass et al., 2015
	Tobacco whitefly	*Bemisia tabaci*	Hamada et al., 2019
	Chinese chive maggot	*Bradysia odoriphaga*	Chen et al., 2019
	Colorado potato beetle	*Leptinotarsa decemlineata*	Kalsi and Palli, 2017
	Asian citrus psyllid	*Diaphorina citri*	Kalsi and Palli, 2017
	The brown planthopper	*Nilaparvata lugens*	Hamada et al., 2020; Khan et al., 2020
	Grain aphid	*Sitobion avenae Fabricius*	Zhang et al., 2020a
	The western flower thrips	*Frankliniella occidentalis*	Wan et al., 2018
Imidaclothiz	Western flower thrips	*Frankliniella occidentalis*	Bass et al., 2015
Indoxacarb	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
	Tomato leafminer	*Tuta absoluta*	Roditakis et al., 2018
	Red imported fire ant	*Solenopsis invicta*	Siddiqui et al., 2022
Lambda-cyhalothrin	Tobacco whitefly	*Bemisia tabaci*	Dângelo et al., 2018
	Brown stink bug	*Euschistus heros*	Hegeto et al., 2015
	Fall armyworm	*Spodoptera frugiperda*	Hafeez et al., 2021
Lufenuron	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
Malathion	Asian citrus psyllid	*Diaphorina citri*	Tiwari et al., 2011
Methamidophos	Brown stink bug	*Euschistus heros*	Sosa-Gómez and da Silva, 2010
Methoxyfenozide	Housefly	*Musca domestica*	Shah et al., 2017
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
Neonicotinoids	Green peach aphid	*Myzus persicae*	Panini et al., 2014
Nitenpyram	Asian citrus psyllid	*Diaphorina citri*	Naeem et al., 2016
	Rice planthoppers	*Sogatella furcifera*	Zhang et al., 2017
	Tobacco whitefly	*Bemisia tabaci*	Basit et al., 2013
	The brown planthopper	*Nilaparvata lugens*	Khan et al., 2020
Organochlorinc	Cotton leafworm	*Spodoptera litura*	Saleem et al., 2008
Organophosphate	Cotton leafworm	*Spodoptera litura*	Saleem et al., 2008
Organophosphates	Currant–lettuce aphid	*Nasonovia ribisnigri*	Barber et al., 1999
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Greenhouse whitefly	*Trialeurodes vaporariorum*	Bass et al., 2015
Organophosphorus	Colorado potato beetle	*Leptinotarsa decemlineata*	Malekmohammadi and Galehdari, 2016
	Onion thrips	*Thrips tabaci*	Nazemi et al., 2016
Phenylpyrazole	The brown planthopper	*Nilaparvata lugens*	Garrood et al., 2017
Pirimicarb	Currant–lettuce aphid	*Nasonovia ribisnigri*	Barber et al., 1999
Profenofos	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
	Tobacco whitefly	*Bemisia tabaci*	Longhurst et al., 2013
Pymetrozine	Greenhouse whitefly	*Trialeurodes vaporariorum*	Bass et al., 2015
	Rice planthoppers	*Sogatella furcifera*	Jin et al., 2017
Pyrethroids	German cockroach	*Blattella germanica*	Wei et al., 2001
	Pollen beetle	*Meligethes aeneus*	Zimmer and Nauen, 2011
	The brown planthopper	*Nilaparvata lugens*	Sun et al., 2017
	Diamondback moth	*Plutella xylostella*	Sonoda et al., 2012
	Cabbage stem flea beetle	*Psylliodes chrysocephala*	Zimmer et al., 2014; Højland et al., 2015
	Grain aphid	*Sitobion avenae*	Foster et al., 2014
	Cotton leafworm	*Spodoptera litura*	Saleem et al., 2008
	Onion thrips	*Thrips tabaci*	Toda and Morishita, 2009; Nazemi et al., 2016
	Green peach aphid	*Myzus persicae*	Panini et al., 2014
	Greenhouse whitefly	*Trialeurodes vaporariorum*	Bass et al., 2015
	Currant–lettuce aphid	*Nasonovia ribisnigri*	Barber et al., 1999
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012
Spinetoram	Western flower thrips	*Frankliniella occidentalis*	Wang et al., 2016b
Spinosad	Oriental fruit fly	*Bactrocera dorsalis*	Sparks et al., 2012
	Olive fruit fly	*Bactrocera oleae*	Sparks et al., 2012
	Braconid wasp	*Cotesia plutellae*	Sparks et al., 2012
	Fruit fly	*Drosophila melanogaster*	Sparks et al., 2012
	Cotton bollworm	*Helicoverpa armigeria*	Sparks et al., 2012
	Tobacco budworm	*Heliothis virescens*	Sparks et al., 2012

(Continued)

TABLE 1 | (Continued)

Pesticides	Common name of the targeted insect pests	Scientific name	References
	Oblique-banded leafroller	*Lepidoptera Choristoneura rosaceana*	Sparks et al., 2012
	American serpentine leafminer	*Liriomyza trifolii*	Sparks et al., 2012
	American serpentine leafminer	*Liriomyza trifolii*	Ferguson, 2004
	Housefly	*Musca domestica*	Sparks et al., 2012
	Diamondback moth	*Plutella xylostella*	Sparks et al., 2012
	Beet armyworm	*Spodoptera exigua*	Ishtiaq et al., 2012; Sparks et al., 2012
	Cotton leafworm	*Spodoptera litura*	Ahmad et al., 2008
	The western flower thrips	*Frankliniella occidentalis*	Sparks et al., 2012
	Tomato leafminer	*Tuta absoluta*	Silva et al., 2016
	Western flower thrips	*Frankliniella occidentalis*	Wang et al., 2016b
Spiromesifen	Tobacco whitefly	*Bemisia tabaci*	Dângelo et al., 2018
Sulfoxaflor	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
Thiacloprid	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
	Tobacco whitefly	*Bemisia tabaci*	Basit et al., 2013
	Codling moth	*Cydia pomonella*	Bass et al., 2015; İşci and Ay, 2017
	Colorado potato beetle	*Leptinotarsa decemlineata*	Bass et al., 2015
	Pollen beetle	*Meligethes aeneus*	Zimmer and Nauen, 2011
Thiamethoxam	Cotton leafhopper	*Amrasca biguttula biguttula*	Chaudhari et al., 2015
	Melon and cotton aphid	*Aphis gossypii*	Koo et al., 2014; Bass et al., 2015
	Asian citrus psyllid	*Diaphorina citri*	Bass et al., 2015
	Brown stink bug	*Euschistus heros*	Hegeto et al., 2015
	Housefly	*Musca domestica*	Bass et al., 2015
	Asian citrus psyllid	*Diaphorina citri*	Tiwari et al., 2011; Naeem et al., 2016
	Western flower thrips	*Frankliniella occidentalis*	Bass et al., 2015
	The brown planthopper	*Nilaparvata lugens*	Wu S. F. et al., 2018; Khan et al., 2020
	Rice planthoppers	*Sogatella furcifera*	Jin et al., 2017

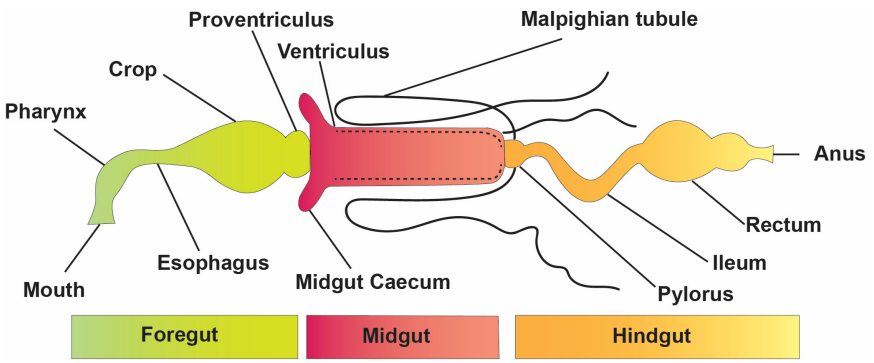

FIGURE 1 | Basic structure and divisions of the insect digestive system.

However, despite compelling reasons to further understand the roles played by insect gut microorganisms and a recent increase in research on microbes that live in insect guts, there has been little progress in expanding the available knowledge on the role of insect gut microbiota in the degradation of pesticides. Currently, pest resistance issues need to be addressed, so the current review will explore the functions and mechanism of pesticide resistance aided by gut microbiota and elaborate their role in pesticide degradation.

INSECT GUT STRUCTURE AND FUNCTIONS

The elementary structure of the intestinal system is alike among insects, even though they have a variety of alterations connected with adaptation to diverse feeding styles and environmental conditions (**Figure 1**). The digestive tract is divided into three basic regions: the foregut, the midgut, and the hindgut (Simpson, 2013). The foregut and hindgut originate from the embryonic epithelium and are protected from pathogens by an exoskeleton of chitin and integument glycoproteins. This exoskeleton is shed at each ecdysis, separating the gastrointestinal lumen from the epithelia. When divided into functionally different subgroups, the foregut is frequently distinguished by another diverticula or crop for impermanent food storage (Linser and Dinglasan, 2014). The hindgut includes distinct portions like fermentation compartments and a distinct rectum for retaining feces during earlier evacuation, among other things. In many insects, the midgut is the main location of absorption and digestion. It lacks an exoskeletal lining and develops from endodermal cells rather than the rest of the body (Engel and Moran, 2013).

A protective envelope known as the peritrophic matrix (or peritrophic membrane) is released by the midgut epithelial cells of many insects. This envelope, constantly being renewed as lost, is essential for the insect's survival. The midgut has two parts: the endo- and ectoperitrophic space. Microorganisms are generally kept in the endo-peritrophic area, which prevents them from coming into direct contact with the epithelium. Peritrophic matrixes are classified into two discrete categories, namely, type I and type II. Type I refers to the whole midgut and is occasionally active when particular foods are consumed, whereas type II is in the remote location of the anterior mid-gut (Engel and Moran, 2013).

The peritrophic matrix shields the epithelium against mechanical injury by food elements, toxins in food, invasive microbes, and absorbed food and digestive enzymes (Kuraishi et al., 2013; Dastranj et al., 2016). In other circumstances, the peritrophic medium wraps around the undigested food mass as it passes along the digestive tract. Tiny pores in the peritrophic matrix prevent most microbes from passing through while allowing enzymes and small molecules from digested food to get through (Terra and Ferreira, 2012; Engel and Moran, 2013). Several insect species, including most sap-feeding species (Hemiptera), various other species of family Formicidae, and order Coleoptera (Nardi and Bee, 2012), that rely solely on cell sap or honeydew do not form a peritrophic matrix (Engel and Moran, 2013).

The Malpighian tubules of insects are excretory structures that extend from the anterior hindgut into the body void and ingest wastes, such as uric acid supplied to the hindgut (**Figure 1**). As a result, the hindgut of insects comprises a distinct nutritional environment which is well documented for water resorption (Simpson, 2013); the hindgut might function as a location of nutrient assimilation, as verified for different insect pests, including termites (Ayitso and Onyango, 2016), crickets (Smith et al., 2017), cockroaches (Tinker and Ottesen, 2016), and heteropteran (Gutiérrez-Cabrera et al., 2016)—for instance, intercellular passages in the hindgut membrane of several cockroaches permit nutrients, such as amino acids and fatty acids made *via* the biota in the hindgut, to flow from the hindgut lumen to the insect hemolymph (O'Donnell and Donini, 2017). The basic form of an insect gut has undergone numerous alterations due to adaptations to specialized niches and eating patterns.

INSECT GUT MICROBIOME COMPOSITION

A wide range of parameters can influence gut microbiota composition, including insect growth, biochemical changes in different intestinal areas, and the insect's ability to obtain available resources (Bruno et al., 2019b). The hindgut of insects, which serves as an extension of the body cavity, is one of these structures that collect dietary waste. Therefore, it provides a great food environment to the gut microbiota, encouraging their proliferation and diversification (Engel and Moran, 2013; Bruno et al., 2019a).

The insect gut microbiome includes protozoa, fungus, archaea, and bacteria. Protists occupy almost 90% of the hindgut of subterranean termites—for example, lower and higher termites' guts include bacteria and archaea (Hongoh, 2010). Scientists revealed that the digestive regions of adult workers of honeybee (*Apis mellifera*) are dominated by a diverse group of nine bacterial species (five of which are *Snodgrassella alvi* and *Gilliamella apicola*, two species of *Lactobacillus*, and a species of *Bifidobacterium*) (Douglas, 2018). Additionally, the gut microbiota is rarely directly touched with intestinal epithelial cells due to their unique placement. Most of the time, bacteria that live in the gut are found in the lumen of the endoperitrophic space, a chitinous barrier that lines the middle of the gut (Erlandson et al., 2019). Yun et al. (2014) have comprehensively categorized and thoroughly defined the insect-associated gut bacteria of 305 samples belonging to 218 species in 21 taxonomic orders. The results indicated that Proteobacteria and Firmicutes were found to make up 62.1 and 20.7% of the total reads in the insect gut microbiota, respectively. Moreover, *Wolbachia* made up 14.1% of the total reads.

INTERACTION OF INSECT AND THEIR RELATED MICROBIOTA

Insect–microbiota interactions are quite diverse. Insects rely on symbiotic bacteria for a variety of essential activities. Symbiotic bacteria can be critical for host survival and growth (Consortium, 2012; Douglas, 2015; Berasategui et al., 2016). They can help break down food, provide energy, make vitamins, and even help shape the body's natural defenses (Cheng et al., 2019; **Figure 2**). Microbial symbionts have been proven to have many consequences on insect health and behavior (Sampson and Mazmanian, 2015). Certain insects have specialized organs that can only house a few symbiont species, while others have a far more diverse and variable flora in their guts and other internal organs. Numerous associations are developed with a sole or a few species of microbiota. They might require establishing specialized insect organs and cells (i.e., subsequent midgut crypts, mycangia, and microbiome) to house definite obligate symbionts (Zaidman-Rémy et al., 2018; Kuechler et al., 2019; Maire et al., 2019; Trappeniers et al., 2019). In these partnerships, the genetic integral of biochemical processes essential for the persistence of both interrelating groups is frequently observed (Hansen and Moran, 2011).

Some insect species are more involved in symbiotic associations with bacteria than others. Among the insects, three taxonomic groups are regularly involved. These groups include Blattaria, Coleoptera, Homoptera, and Hymenoptera. Additionally, certain bacteria seem to be particularly adept at symbiotic interactions. Numerous arthropods carry representatives of the *Wolbachia* genus (Aikawa et al., 2022), which is closely linked to pathogenic *Rickettsia* (Shan et al., 2021) and is categorized in Proteobacteria's subgroup. The subgroup contains symbiotic organisms closely related to significant human diseases, such as *Francisella tulariensis*, *Coxiella burnetii*, and several Enterobacteriaceae (Dang et al., 2017). Symbionts

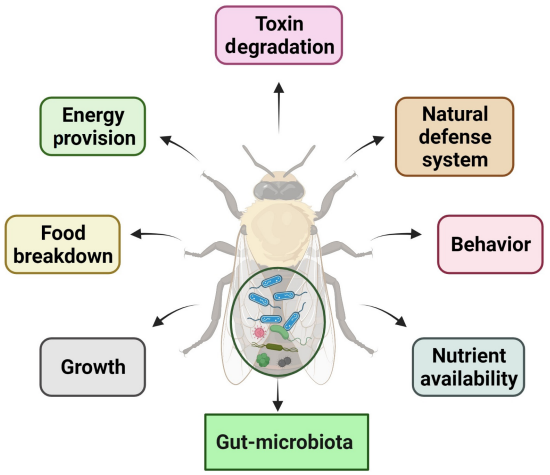

FIGURE 2 | Functions and impacts of gut microbiota on insect biology and physiology.

of mealybugs and the protist family Trypanosomatidae are members of the Proteobacteria β-subgroup (Boursaux-Eude and Gross, 2000). Cockroach mycetocyte symbionts (Blattaria) belong to the Flavobacterium–Bacteroides group (Guzman and Vilcinskas, 2020).

The maize weevil *Sitophilus zeamais*, for example, needs nutrients made by its endosymbiont *Sodalis pierantonius* to stay healthy. The symbionts' innate immune system is generally activated by the weevils' secretion of an antimicrobial peptide (AMP) in the microbiome, which prevents the weevils from generating a systemic antibacterial response against them (Wang et al., 2017; Maire et al., 2019; Trappeniers et al., 2019). When it comes to digesting plant tissues that are resistant to digestion, termites require more composite mutualism with lots of digestive-zone bacterial or protist species (Tokuda et al., 2018; Liu et al., 2019), and many of these microorganisms are termite-specific symbionts with a high degree of niche specialization (Bourguignon et al., 2018; Hervé et al., 2020). The microbiota of other various insects may be more varied and adaptable, as they do not rely on explicit critical symbionts (Coon et al., 2016; Scolari et al., 2019). The gut biota is critical for most insects' digestion, fertility, fecundity, and immunity (Heys et al., 2018; Salcedo-Porras et al., 2020), as growing axenic insects can be deadly (Flury et al., 2019). Insects need to get several symbionts that successfully make good and functional microbiota.

Primary symbionts are more common in insects having particularly nutrient-deficient foods (obligate hematophagy or phytophagy). In contrast, secondary symbionts are more common in polyphagous and omnivorous insects, which obtain a diverse microbiota from their surroundings (Salcedo-Porras et al., 2020). While most primary symbionts are internal (endosymbionts), secondary symbionts are external. There may be an association between the symbiont acquisition or transmission and the nature of the interactions between insects and symbionts. It is usual for female germline transmission to occur vertically through the female germline as with primary mutualists, for example, those present in aphids and weevils (Caspi-Fluger et al., 2012; Douglas, 2015; Hassan et al., 2020). It is common for environmental microorganisms to be transmitted across internal organs, some of which can form secondary symbioses without specialized organs.

Additionally, insect growth affects the time during which microbiota are acquired horizontally. Except for vertically transmitted microorganisms, most insects hatch practically germ-free and obtain their microbiome by cannibalism, trophallaxis, coprophagy, or ingesting their contaminated eggshells (Taylor et al., 2014; Salcedo-Porras et al., 2020). Holometabolous insects pupate in a nearly axenic state, and adults re-acquire some of their gut microbiota from the environment (Powell et al., 2014; Hammer and Moran, 2019; Rolff et al., 2019) after emerging from the pupal stage. The microbiota of adults in some species may differ greatly from the microbiome of the immature stages or may acquire a similar gut microbiota from the conspecifics or environment (Johnston et al., 2019; Majumder et al., 2020; Suárez-Moo et al., 2020). On the contrary, microorganisms attained after egg hatching can be preserved in hemimetabolous insects for an extended period (Rodríguez-Ruano et al., 2018; Hammer and Moran, 2019). Finally, social insects, whether hemimetabolous or holometabolous, can get microbiota from each other repeatedly, choosing and keeping a specific microbiota (Onchuru et al., 2018; Tokuda et al., 2018; Liu et al., 2019).

ACQUISITION RESISTANCE CHARACTERISTICS OF NATIVE GUT BACTERIA

The increased predominance of naturally existing inhibitory gut bacteria could be a viable alternative to para-transgenic techniques for reducing pathogen burden in natural populations of insects' vector. The configuration of the gut microbiome regulates vector capability by modulation of immunological reactions, competition for positions, or production of inhibitory compounds (Cirimotich et al., 2011; Boissière et al., 2012). The practical investigation of the gut microbiome to understand its contact with the parasite and host might lead to the development of innovative and more effective techniques to regulate vector-borne infections. As a result, future plant pest control efforts should consider this. Numerous microbial plant inflammations are conveyed *via* insect vectors, and the identification of these insects' intestinal bacteria has been conducted to create techniques to prevent pathogen spread (Raddadi et al., 2011; Engel and Moran, 2013). An excellent example is a disease (Pierce) of grapes produced *via* pathogenic *Xylella fastidiosa*. *Alcaligenes xylosoxidans* was isolated as a bacterial symbiont since the sharpshooter (Cicadellidae) spreads *X. fastidiosa*.

These bacteria live in insect's foregut, where they share space with *X. fastidiosa*, a bacterium that can be harmful to people. Because *A. xylosoxidans* are elated into the plants' xylem by insects feeding on sap, it is more likely to spread to other insects. These properties make *A. xylosoxidans* a promising option for use as a bio-control mediator against *X. fastidiosa*

establishment through modest position elimination or as a para-transgenic conveyer for providing anti-*Xylella* drugs among other applications (Miller, 2011).

IMPACTS OF GUT MICROBIOTA ON THE ACTIVITY OF PESTICIDES

The insect-associated microbial community is dynamic and responsive to various stressors (Zhang et al., 2022). The related microbiota, like the insect, is subject to natural selection pressure, and its composition can be influenced by variables such as dietary changes, food scarcity, and exposure to toxic substances (Adair and Douglas, 2017; Akami et al., 2022). The microbiota of hosts exposed to pesticides as a source of selection pressure may also assist the host in metabolizing these substances. It may act as a source of variation, resulting in the host's reduced susceptibility to pesticides (Akami et al., 2019a,b). Pesticide-degrading bacteria are prevalent throughout nature and have been identified in a variety of insect orders, including Lepidoptera (Ramya et al., 2016b; de Almeida et al., 2017), Hemiptera (Kikuchi et al., 2012), Diptera (Cheng et al., 2017), and Coleoptera (Akami et al., 2019b). There has been evidence that resistant strains of bacteria from the gut of *Plutella xylostella* Linnaeus (Xia et al., 2018) and *Spodoptera frugiperda* (de Almeida et al., 2017) have the capacity to breakdown many pesticides (Gomes et al., 2020). The selection of *S. frugiperda* strains based on pesticide-guided selection led to selecting pesticide-degrading bacteria absent in the microbiota of vulnerable, unselected larvae (de Almeida et al., 2017).

The microbial population of an insect's digestive tract comprises bacteria belonging to the phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacterioidetes, all of which can impact the biology of hosts (Paniagua Voirol et al., 2018; Gomes et al., 2020). Research on *Spodoptera littoralis* (Boisduval) found that the microbial community was mostly made up of Firmicutes, especially *Enterococcus* (Chen et al., 2016; Higuita Palacio et al., 2021). Firmicutes are found in the digestive tracts of many lepidopteran larvae, even though the digestive tracts of larvae are suggested to be not very suitable for bacteria to live. This includes *Spodoptera litura* Fabricius (Thakur et al., 2016), *Manduca sexta* Linnaeus (Holt, 2013), *Helicoverpa armigera* Hubner (Yuan et al., 2021), and many other lepidopteran species (Mereghetti et al., 2017; Gomes et al., 2020). Bacteria belonging to the genus *Enterococcus* are known to create a variety of bacteriocins, which are potent antibacterial chemicals that can influence the composition of the gut microbial communities (Van Arnam et al., 2018). The highest relative amount of Enterococcus was reported in *S. frugiperda* populations from the laboratory and from natural fields (Gomes et al., 2020).

According to various studies, the intestinal bacteria of insects have been shown to break down multiple pesticides and interfere with the effectiveness of pesticides used to control them (Ramya et al., 2016a; Cheng et al., 2017; de Almeida et al., 2017). The Proteobacteria families (Enterobacteria, Pseudomonada, and Burkholderia) could break down acephate, chlorpyrifos, trichlorfon, lambda-cyhalothrin, and Spinosad, respectively (Kikuchi et al., 2012; de Almeida et al., 2017; Itoh et al., 2018b; Gomes et al., 2020). Similarly, Actinobacteria and Firmicutes bacteria have also been shown to have a role in the process of removing toxins from the environment (de Almeida, 2013; Ramya et al., 2016b). The resistant strain of *S. frugiperda* harbor gut bacteria *Enterococcus* (Firmicutes) that were able to break down the pesticides (chlorpyrifos, lambda-cyhalothrin, deltamethrin, spinosad, and lufenuron) (Gomes et al., 2020). According to previous studies, there are several gut symbionts of different insects (orders Coleoptera, Diptera, Hemiptera, and Lepidoptera) that detoxify the pesticides (classes Benzoylurea, Carbamate, Methoprene, Neonicotinoid, Organochloride, and Organophosphate) by the different species of genera *Acetobacter*, *Actinobacteria*, *Aeromonas*, *Arsenphonus*, *Burkholderia*, *Citrobacter*, *Clostridium*, *Enterococcus*, *Exiguobacterium*, *Lachnospiracease*, *Lactobacillus*, *Lysinibacillus*, *Microbacterium*, *Pseudomonas*, *Staphylococcus*, *Symbiotaphrina*, and *Wolbachia* (**Table 2**).

Microorganisms' ability to utilize pesticides as a carbon source is contingent upon the coding of the biochemical systems required to cope with these substrates (Lourthuraj et al., 2022). Temperature and pH, nutrition availability, chemical concentration, and the size of the bacterial population all influence pesticide metabolization (Russell et al., 2011; Gomes et al., 2020). The pesticides' chemical composition and complexity play a role in how quickly and effectively bacteria use them as a food source (Hubbard et al., 2014). Microorganisms use a wide range of metabolic pathways to break down and change xenobiotics when they grow rapidly (Itoh et al., 2018b; Bhatt et al., 2019, 2021; Gangola et al., 2022)—for example, *Pseudomonas* spp. and *Ensifer adhaerens* metabolized the thiamethoxam pesticide. The principal metabolic pathway involves the transition of its N-nitroimino group (= $N\text{-}NO_2$) to N-nitrosimine/nitrosoguanidine (= N-NO, THX-II) and urea (= O; THX-III) metabolites (Hussain et al., 2016), which is shown in **Figure 3**. Another example is the symbionts species of genera *Arsenophonus* (Pang et al., 2018) and *Pseudomonas* (Pang et al., 2020b); *Ensifer* spp., *Stenotrophomonas* spp., *Variovorax* spp. (Hussain et al., 2016) have been reported to degrade imidacloprid. The mechanisms and associated metabolic pathways are shown in **Figure 4**, which indicates that nitro-reduction and oxidation are two of the main ways that bacteria break down imidacloprid (Lu et al., 2016; Fusetto et al., 2017). The gut microbiota produces enzymes that detoxify pesticides like pyrethroids, carbamates, diamides, and organochlorines, which have been identified (Russell et al., 2011; Khalid et al., 2016; Gomes et al., 2020; Lin et al., 2022).

Moreover, Plant secondary components, such as terpenes, alkaloids, glycosides, and phenolic compounds, are degraded by Proteobacteria in the presence of insects (Mereghetti et al., 2017; Gomes et al., 2020). Proteobacteria have the most diverse morphology and adaptability of all bacterial phylum, which offers them an advantage in various ecological niches (Shin et al., 2015). Proteobacteria may thus act as a source of available variety and a tool for host adaptation in nature when they interact with other organisms (Bradley and Pollard, 2017; Degli Esposti and Martinez Romero, 2017; Hauffe and Barelli, 2019).

TABLE 2 | List of insect gut microbiota involved in pesticide degradation.

Pesticides	Gut microbiota	Insect pests	References
Benzoylurea	*Enterococcus mundtii*	*Spodoptera frugiperda*	de Almeida et al., 2017
	Microbacterium arborescens		
	Staphylococcus sciuri subsp. *sciuri*		
Carbamate	*Pseudomonas melophthora*	*Rhagoletis pomonella*	Boush and Matsumura, 1967
Methoprene	*Clostridium* spp.	*Aedes* spp. and *Anopheles* spp.	Receveur et al., 2018; Giambò et al., 2021
	Lysinibacillus spp.		
	Staphylococcus spp.		
Neonicotinoid	*Acetobacter* spp.	*Drosophila melanogaster*	Chmiel et al., 2019
	Lactobacillus spp.		
	Lactobacillus plantarum		Giambò et al., 2021
	Arsenphonus spp.	*Nilaparvata lugens*	Pang et al., 2018
Organochloride	*Pseudomonas melophthora*	*Rhagoletis pomonella*	Boush and Matsumura, 1967
Organophosphate	*Microbacterium* sp.	*Anopheles stephensi*	Soltani et al., 2017
	Exiguobacterium sp.		
	Aeromonas spp.		
	Pseudomonas spp.		
	Citrobacter spp.	*Bactrocera dorsalis*	Cheng et al., 2017; Guo et al., 2017
	Actinobacteria spp.	*Bombyx mori*	Chen et al., 2020; Giambò et al., 2021
	Staphylococcus spp.		
	Enterococcus spp.		
	Lachnospiracease spp.		Li et al., 2020; Giambò et al., 2021
	Burkholderia spp.	*Cavelerius saccharivorus*	Kikuchi et al., 2012; Itoh et al., 2018a
	Wolbachia spp.	*Culex pipiens*	Berticat et al., 2002
	Symbiotaphrina kochii	*Lasioderma serricorne*	Shen and Dowd, 1991
	Enterobacter aburiae	*Plutella xylostella*	Ramya et al., 2016a
	Bacillus cereus		
	Pantoea agglomerans		
	Enterococcus spp.		Xia et al., 2018
	Pseudomonas melophthora	*Rhagoletis pomonella*	Boush and Matsumura, 1967
	Pseudomonas spp.	*Riptortus pedestris*	Kikuchi et al., 2012
	Flavobacterium spp.		
	Burkholderia spp.		Kikuchi et al., 2012; Itoh et al., 2018a
	Burkholderia spp.	*Cavelerius saccharivous*	Kikuchi et al., 2012
	Delftia lacustris	*Spodoptera frugiperda*	de Almeida et al., 2017
	Enterococcus casseliflavus		
	Enterococcus mundtii		
	Leclercia adecarboxylata		
	Microbacterium paraoxydans		
Oxadiazine	*Bacillis cereus*	*Plutella xylostella*	Ramya et al., 2016a
	Gammaproteobacteria spp.	*Blatella germanica*	Pietri et al., 2018
Pyrethroid	*Enterococcus casseliflavus*	*Spodoptera frugiperda*	de Almeida et al., 2017
	Enterococcus mundtii		
	Pseudomonas stutzeri		
	Arthrobacter nicotinovorans		
	Enterococcus casseliflavus		
Spinosyn	*Enterococcus casseliflavus*		
	Enterococcus mundtii		
	Pseudomonas psychrotolerans		

The gut microbiota has been linked to promoting the insecticidal action of *Bacillus thuringiensis*, the frequently used biological pesticide for herbivore pest management in agriculture (Mason et al., 2011; Eski et al., 2018). According to a study, when the gut microbial population was removed from gypsy moth larvae, the *B. thuringiensis* pesticide no longer worked as intended, whereas when some microbiota of the gut microbiome was added back in, the *B. thuringiensis*-facilitated mortality was reestablished (Polenogova et al., 2021). Several insect species have similar mechanisms for degrading imidacloprid

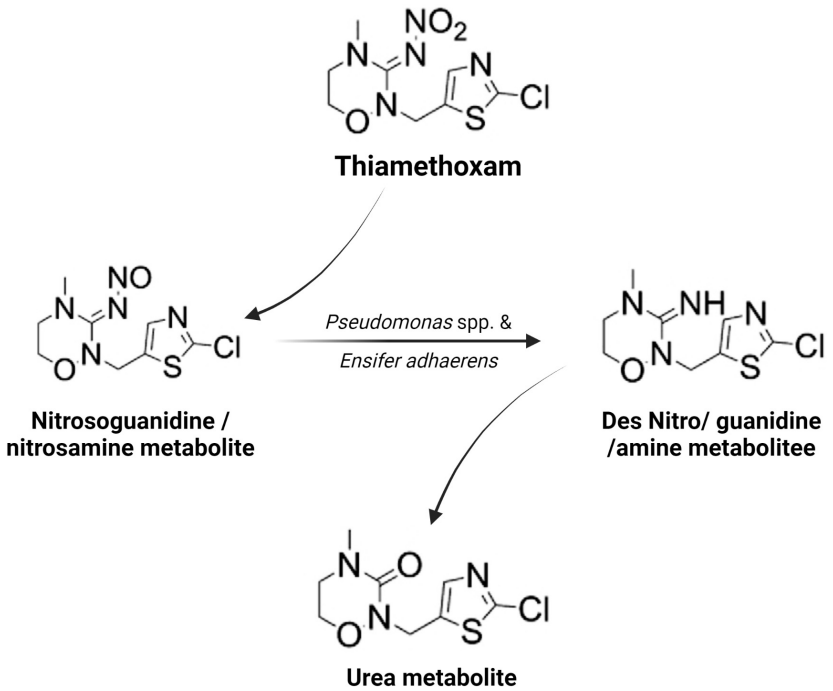

FIGURE 3 | Metabolic routes for bacterial degradation of the insecticide thiamethoxam (Hussain et al., 2016).

(Thurman et al., 2013). Additionally, the Cyp6g1 gene discovered in *Drosophila* is critical for imidacloprid breakdown in animals, regulating and promoting the generation of metabolites in the oxidation pathway (Fusetto et al., 2017). These findings indicate the importance of considering the gut microbiome of insects in the development of novel pest control methods.

SYMBIONT-MEDIATED PESTICIDE RESISTANCE

The rapid emergence of pesticide resistance in a wide range of organisms is a cause for concern, and it merits additional studies. Several mechanisms of pesticide resistance are ascribed to the physiology at the host level (Tabashnik and Carrière, 2010; van den Bosch and Welte, 2017), but few researchers have argued in recent years that some pesticides' resistance might be ascribed by symbiont detoxification. Detoxifying enzymes targeting harmful allelochemicals and pesticides have been found in fungal symbionts isolated from insects (Tabashnik and Carrière, 2010; Naik et al., 2018). Evidence showing that changes in mutualist-level physiology can cause pesticide resistance is very limited—for example, the *Burkholderia* mutualist in midgut crypts acquired *via* the environment in each generation instead of the "traditional" mode of vertical maternal spread, as is the case with humans (Garcia, 2015). The pesticide fenitrothion, a common organophosphorus chemical in agriculture, can be degraded by the symbiotic *Burkholderia* (Kikuchi et al., 2012).

Furthermore, the *R. pedestris* pest bug easily forms symbiotic relationships through fenitrothion, degrading *Burkholderia* mutualists, and have significantly more persistence rates on fenitrothion-treated plants than insects by non-demeaning *Burkholderia* mutualists (Kikuchi et al., 2012). Spraying of fenitrothion to the field enabled more bacteria to degrade fenitrothion in soil, which is thought to impact the dynamics of symbiotic-degrading *Burkholderia* spread *via* soil to stinkbugs (Tago et al., 2015). These discoveries imply that pesticide resistance may mature in the absence of pest insects in a field and then spread rapidly within sole insect pest generation (Kikuchi et al., 2012). In addition, *Burkholderia* symbionts of the established *R. pedestris* model give an excellent chance for research on microbial symbiotic aspects at the molecular level since they are cultivable and genetically manipulable (Kim and Lee, 2015). These studies help develop an ecological pesticide that uses gut symbionts to control insects. Such studies could be useful to learn about pesticide resistance mechanisms that have not been found yet.

MOLECULAR MECHANISMS BY WHICH ENZYMES MEDIATE PESTICIDE DETOXIFICATION

Detoxification enzymes occur naturally in various biological processes, functioning on the target sites to neutralize various toxins prevalent in the insect body (Lin et al., 2015; Bhandari et al., 2021; Siddiqui et al., 2022). According to some previous studies, the biochemical characterization of insect resistance to pesticides is connected to pesticide sensitivity at the target site and pesticide detoxification by metabolic enzymes

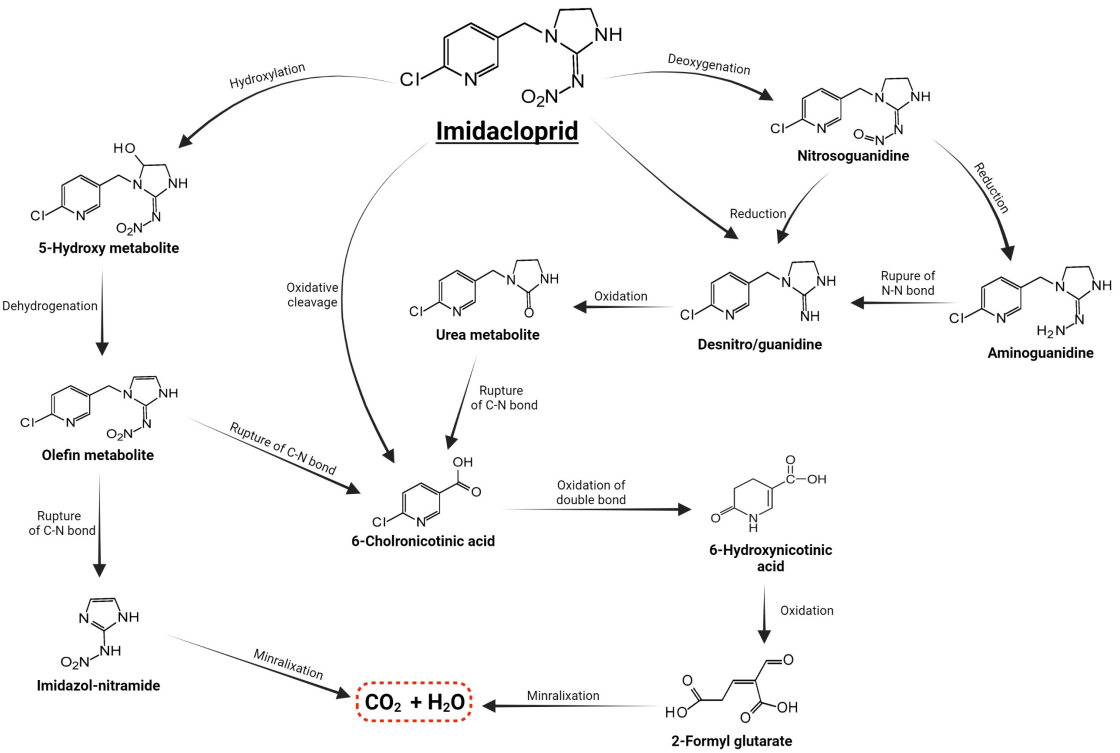

FIGURE 4 | Metabolic routes for bacterial degradation of the insecticide imidacloprid (Pang et al., 2020a).

(acetylcholinesterase, carboxylesterase, glutathione S-transferase, and cytochrome P450) (Wu et al., 2014; Ismail, 2020; Yang et al., 2021; Siddiqui et al., 2022). These enzymes are crucial in detoxifying xenobiotics (Hu et al., 2014), where their hosts can utilize these enzymes as biological indicators during pesticide detoxification (Khan et al., 2021)—for instance, Zhang et al. (2016) reported that detoxification enzymes (cytochrome P450 genes) were detected during detoxification of fipronil in the red imported fire ants (*Solenopsis invicta* Buren), where up to 36.4-fold rise in resistance was recorded following exposure of the ants to fipronil. Another related study has also linked cytochrome P450 enzymes with fluralaner detoxification in *S. invicta* (Xiong et al., 2020).

Additionally, these enzymes may raise the responder gene's copy number, mRNA levels, and coding sequence diversity by introducing point mutations (Pang et al., 2020a). These enzymes are involved in various processes, including biosynthesis and the metabolism of invading species, among others—for instance, P450 CYP6ER1 in *Nilaparvata lugens* and CYP6CM1 in *Bemisia tabaci* were used to characterize and assess imidacloprid metabolism. These findings revealed that amino acid changes in the binding site enhanced imidacloprid metabolism (Bao et al., 2016; Pang et al., 2016; Hamada et al., 2019). Puinean et al. (2010) discovered that the CYP6Y3 gene in *Myzus persicae* could confer resistance to neonicotinoids. Moreover, CYP353D1v2 was found to be overexpressed in several imidacloprid-resistant *Laodelphax striatellus* strains, and silencing this gene greatly reduced resistance (Elzaki et al., 2017). The effective suppression of CYP6CY14 transcription by RNAi in the overexpressed P450 gene of the CYP3 clade greatly improved the vulnerability of pesticide-resistant cotton aphids to thiamethoxam (Wu Y. et al., 2018).

In order to detoxify xenobiotics in the gut lumen, insects can employ various techniques. They can do so by creating an acidic environment and supplying a complex of enzymes (monooxygenases and esterases) that can cleave or alter the xenobiotic in preparation for excretion (de Almeida et al., 2017). It has been confirmed that microbial enzymatic activity in the gut lumen contributes to the breakdown of pesticides consumed by the host. The hydrolysis of these compounds provides resources for the microbiota to thrive (Mohammadi et al., 2021). The diversity and differences in prokaryote- and eukaryote-produced enzymes suggest that microbial enzymes could play a significant role in pesticide metabolization in contaminated insects (Russell et al., 2011; de Almeida et al., 2017).

PESTICIDE DEGRADATION BY SYMBIONTS IN INVASIVE SPECIES

Multiple resistance mechanisms have been functionally recognized as conveying pesticide resistance in several invasive insects, including penetration resistance *via* cuticle thickening or remodeling, metabolic resistance *via* the amplified activity of detoxification enzymes (e.g., esterases and cytochrome P450 monooxygenases), and knockdown resistance *via* kdr transmutations (Khan et al., 2021; Rigby et al., 2021). There are also possible behavioral and physiological resistance

mechanisms. These include point mutations that make esterases more active, GST, target place insensitivity, reformed AChE, GABA receptor insensitivity, and transformed nAChRs (Dang et al., 2017). The diamondback moth, *Plutella xylostella*, is an example of invasive species that act as a significant universal pest of various crops (Nakaishi et al., 2018). *P. xylostella* also generates an enzyme that avoids the generation of dietetic isothiocyanates that act as plant defense compounds emitted by the host plant and regulate feeding behavior of diamondback moths female (Hussain et al., 2019, 2020). Furthermore, *P. xylostella* has been discovered to be resistant to a wide range of chemical pesticides. Only three other pest species have established resistance to *Bacillus thuringiensis*-based pest control technologies, which is one of them (Furlong et al., 2013). The quick evolution of extremely resistant phenotypes in *P. xylostella* is partly ascribed to insect pests, including altered carbamate and organophosphate target locations, parathion metabolism by GST, and pyrethroid detoxification by P-450 monooxygenases (Ramya et al., 2016a). The indoxacarb-degrading microbiota (*B. cereus* bacteria) identified in the digestive tract of *P. xylostella* was found to degrade the pesticide by converting it into food (van den Bosch and Welte, 2017). Another pesticide, acephate, was quickly degraded by gut bacteria obtained from diamondback moth intestines. Together with earlier research on the gut microbiota of stinkbugs showing pesticide resistance (Kikuchi et al., 2012), these findings suggest that the gut biota might have a greater part in pesticide resistance than formerly assumed.

ROLE OF GUT MICROBIOTA IN TOLERANCE AND RESISTANCE

Insect's digestive systems are equipped with a multilevel defensive system, likely a primary driver in structuring gut microbiome communities. Different aspects of such a defensive system provide the host's ability to tolerate and reject harmful bacteria in the gut through various processes. While tolerance reduces the detrimental effects of a bacterial burden on the host's health, resistance reduces the bacterial burden so as not to harm the host (Moreno-García et al., 2014). Most immunological research have concentrated on resistance mechanisms, and there is little knowledge about the processes that mediate tolerance. However, host–microorganism associations in the insect gut are frequently commensalism or mutualism.

Compared to insects with sparsely populated digestive tracts, those with vast bacterial communities are more likely to be tolerant and less likely to be resistant to bacteria in their guts. As a result, the gut immunity mechanisms of diverse insects may be tailored to the definite desires of their hosts. As mentioned previously, the midguts of most insects produce a peritrophic medium composed of a network of chitin microfibrils implanted in a protein–carbohydrate medium (Muthukrishnan et al., 2012). The peritrophic medium is semi-permeable, allowing nutrients, digestive enzymes, and defense chemicals to flow while protecting the epithelial cell layer from a direct microbe or toxin exposure. The cuticle layer bordering the epithelial cell layer in the foregut and hindgut may have comparable protective roles.

These physiological barriers among the lumen and epithelium are decent instances of tolerance mechanisms since they minimize the influence of bacteria on the host rather than reduce the bacterial load in the gut. Certain parts of the insect gut can have a low or high pH or contain enzymes that target bacterial cell wall components, such as peptidoglycan or lysozymes (PGN) hydrolases (Liu et al., 2014; Moreno-García et al., 2014). Such systems can cause deliberate resistance by reducing the number of bacterial communities in specific parts of the gut, but they may be useful in bacterial cell breakdown to enhance nutrition.

Bacterial endosymbionts have been extensively examined in the context of biological invasions to detect or quantify their impact in increasing the invasion process of imported species (Klock et al., 2015; Taerum et al., 2016; Cheng et al., 2019). There is still a lack of understanding of the mechanisms that drive the responses of native species to invasive species' selective pressures. A deeper knowledge of the structure and function of bacterial mutualists, on the other hand, may disclose possible mechanisms for inhabitant hosts to adapt to exotic species, as variations in bacterial mutualists have been revealed to correlate with variations in food sources in both invertebrates and vertebrates (Rokhsefat et al., 2016; Shapira, 2016). Furthermore, identifying and describing the bacterial mutualists of inhabitant species may supply vital hints about handling exotic species in the future.

Plant defenses and pesticides can potentially interact with and supplement host immune systems (Mason, 2020). Secondary metabolites play a critical role in protecting plants from arthropod herbivores. Secondary chemicals play an important role in insect resistance and vulnerability (Erb and Kliebenstein, 2020). Plant secondary metabolites with antinutritive, deterring, antibacterial, and poisonous properties frequently affect the growth and productivity of phytophagous insects feeding on various host plants (Puri et al., 2022). In nature, the level of plant-defensive compounds varies by species and is determined by the plant's genotype, growing circumstances, and phenology. Plant allelochemicals impose a very strong selection pressure on herbivorous insects and the microbiota in their guts, which is particularly important for their survival (Douglas, 2015; Chen et al., 2022), for example, a study discovered that symbionts such as *Phenylobacterium*, *Ochrobactrum*, *Erwinia*, *Amycolatopsis*, and *Sediminibacterium* spp. may play critical roles in the metabolism of tea saponins, according to the findings. Two of them, *Acinetobacter calcoaceticus* and *Acinetobacter oleivorans*, were very important in the degradation of tea saponins (Zhang et al., 2020b).

The gut microorganisms' digesting abilities can also assist in the removal or inactivation of toxic compounds in food (Schmidt and Engel, 2021). Detoxification symbioses have been observed in a wide range of hosts, even though certain insects have these functions encoded in their genomes (Itoh et al., 2018b). They are particularly important for herbivorous insects since plants produce a diverse spectrum of phytotoxins that are toxic to them (Itoh et al., 2018b). Adaptation to the highly toxic terpenoids present in the bark of pine trees has been achieved by cooperation between the mountain pine beetle (*Dendroctonus ponerosae*) and the pine weevil (*Hylbius abietis*) and their gut microbiota.

Gammaproteobacteria, in particular, play an important role in the degradation of diterpenes (Berasategui et al., 2017; Schmidt and Engel, 2021).

An example of a social insect belonging to the genera *Apis* and *Bombus* harbors gut microbiota that plays important roles in their health, with a possible impact on pathogen protection and nutrient acquisition (Engel et al., 2016; Zheng et al., 2016). Glycolysis pathways and phosphotransferase systems have been found in the genomes of *Gilliamella apicola*, indicating that this bacterium functions as a saccharolytic fermenter that aids in the digestion of the host's carbohydrate-rich meal (Tilottama et al., 2021). The pollen grain of *G. apicola* was subjected to a metagenomic investigation, and the results revealed the presence of genes encoding pectin-degrading enzymes. These enzymes play a vital role in breaking down the stiff polysaccharide walls of pollen grains and release constituent monosaccharides (Zheng et al., 2016).

The coffee borer beetle (*Hypothenemus hampei*) engages in a detoxifying symbiosis to facilitate nutritional adaption to coffee beans, which contain high quantities of the poisonous alkaloid caffeine (Mejía-Alvarado et al., 2021). It was discovered that the beetle's gut microbiota is dominated by *Pseudomonas* species, which are seen in beetles from several coffee-producing countries (Ceja-Navarro et al., 2015). Beetle pseudomonad spores were able to develop on caffeine alone, and they were able to restore the breakdown of caffeine in beetles that had been previously treated with antibiotics (Schmidt and Engel, 2021). In addition to promoting nutritional adaptability, it has been observed that several pest species carry gut symbionts that are capable of degrading pesticides (de Almeida et al., 2017; Itoh et al., 2018b). The *Burkholderia* gut symbiont, *R. pedestris*, may degrade the pesticide fenitrothion and increase the survival of *R. pedestris* in soil infected with the pesticide (Itoh et al., 2018a). The wasp *Nasonia vitripennis*, for example, was found to have a greater survival rate in the presence of its gut microbiota in the exposure of atrazine (Wang et al., 2020). This research highlights the gut microbiota's ability to boost the adaptive capabilities of its insect host, which has crucial implications for pest and pollinator insect control.

Another defense mechanism is the inherent immune system of insect species, which comprises numerous immunological responses (Chambers et al., 2012; Engel and Moran, 2013) and summarizes the general principles of innate immunity in insects. A key inducible response permitting resident immunity at the gut epithelial cell layer has been identified, mostly through experiments with *D. melanogaster*. These are the creation of amino acids (AMPs) (**Figure 5**) and the combination of reactive oxygen species (ROS). The generated reactions may altogether be considered traditional resistance mechanisms; nevertheless, they contain undesirable response circles and modulatory mechanisms, conveying host tolerance toward the commensal gut microbiota. The Toll and IMD signaling channels are two of the most important signaling mechanisms causing AMP synthesis in *D. melanogaster*'s systemic immune response (Hanson and Lemaitre, 2020). The reaction in the gut is distinct in that only the IMD pathway is activated, resulting in the induction of resident AMP reactions in response to pathogen stimulation (Nehme et al., 2007; Lee et al., 2013). Initiation happens when different types of bacterial PGN bind to receptors on the outside or inside of the body's epithelium that belong to the peptidoglycan recognition protein (PGRP) family (Engel and Moran, 2013). Signaling downstream *via* the IMD pathway activates the transcriptome factor Relish, which stimulates the production of multiple AMPs and other immunity-associated genes (**Figure 5**). Introduction to pathogens also results in ROS formation in the gut of *D. melanogaster* through the membrane-related dual oxidase (DUOX) system (Alaraby et al., 2018). The PGN-independent

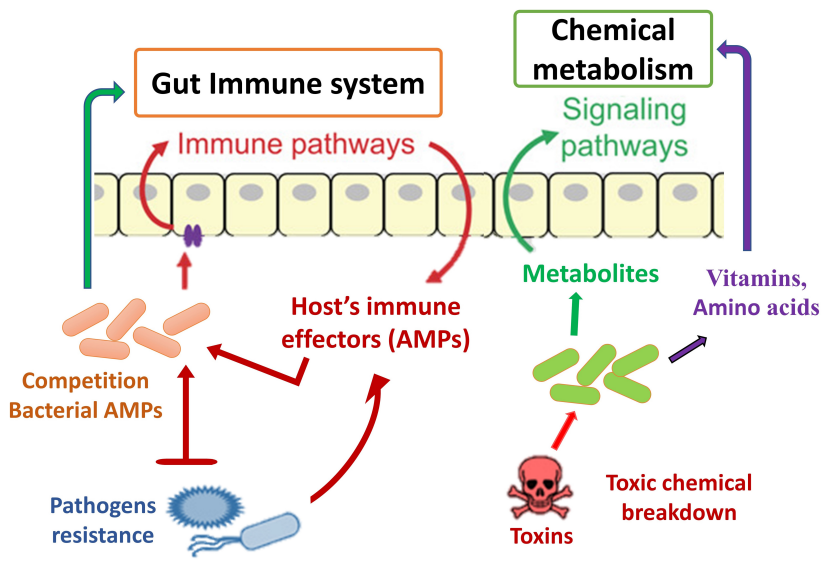

FIGURE 5 | Schematic diagram of the role of gut microbiota in immune and resistance mechanism.

and PGN-dependent signaling pathways are involved in this process (Charroux and Royet, 2012). In addition to the bacteria, the host's epithelial cells are also subjected to oxidative stress when ROS are produced.

Immune catalases are activated in *D. melanogaster* to remove excess ROS (Buchon et al., 2014). Tolerance is improved due to this catalase synthesis, which reduces the immune response-induced self-harm (Simpson et al., 2015). How these enzymes protect the host cells without affecting the pathogens' ability to produce ROS remains a mystery. One possible reason is that the catalase activity is constrained to a specific region of the epithelial surface—for instance, the vicinity of the epithelial surface. Immunological reactions to the related gut microbiome community have been confirmed in *D. melanogaster* by employing the DUOX system activity and IMD pathway at varying degrees. In the IMD pathway, the homeobox transcript factor caudal binds to the promoter regions of AMP genes in the gut and stops them from being made. The gut flora alters, and the epithelial cell layer breaks down in caudal-defective flies because of a constant generation of AMP (Ryu et al., 2008; Broderick and Lemaitre, 2012). As a result, it appears that caudal avoids over-encouragement of the immunity system by mutualistic gut biota. Additional immunological regulatory operations in *D. melanogaster* are regulated by amidases produced by the midgut cells of the epithelium and cleave pro-inflammatory PGN into passive systems (Bischoff et al., 2006; Zaidman-Rémy et al., 2006; Engel and Moran, 2013).

There are many ways in which obligatory insect-associated bacteria contribute to their host insect's overall health and well-being; however, the primary contribution of these bacteria is connected only to their ability to provide nutrients. Secondary bacterial symbionts boost the host's immunological response to entomophagy (Vorburger et al., 2010) and entomopathogens (Jaenike et al., 2010), impact host plant selection (Frago et al., 2012), defend against heat stress (Pons et al., 2022), and aid in the detoxification of compounds produced for herbivore defense (Hammer and Bowers, 2015). Microbes also plays a role in detoxifying xenobiotics by catabolizing organic compounds used in applied pest management, as demonstrated by degradation (Pietri et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

Microbes are known to degrade a wide variety of allelochemicals and pesticides, providing numerous opportunities for insects to develop detoxifying symbiotic relationships. The gut microbiota plays various roles in the host's physiology, including immunological modulation and toxin degradations. Arguably, the microbiota evolves more rapidly than their host insects, resulting in rapid pest adaptation to pesticides through the employment of mutualistic microbes. Additionally, insects can swiftly obtain novel metabolic activities and colonize new ecological niches through symbiotic interactions with microbiota that previously have fully developed well-tuned metabolic pathways. As results of the ever-dynamic climatic conditions and human populations, it is imperative that additional/novel insect pest management strategies are implemented to synergize the existing ones. Exploring symbiotic microorganisms as a means of managing their associated hosts could be one way to meet this need. Currently, sterile insect technology, introduction of natural enemies such as parasitoids or predators, application of entomopathogenic fungi or bacteria, etc., are some of the most commonly used integrated pest management techniques. Additional research into (detoxifying) symbiosis may result in environmentally acceptable and long-term ways of controlling large pest insect populations. Insect pest status, for example, may be heavily influenced by microbiota genotype, allowing for the identification and selection of genotypes most suited for addressing specific pest management priorities, ideally through low-tech means. In the same vein, detoxifying microbiota that can be isolated could be used in bioremediation or to treat pesticide poisoning. To better understand detoxifying microbiota in agriculturally significant pest insects, we provided comprehensive information regarding the role of gut microbiota in the detoxification of pesticides. Further investigation may be helpful to produce an effective integrated pest management program.

AUTHOR CONTRIBUTIONS

JS, MK, BB, and MH wrote the initial draft. YX financially supported and supervised the manuscript. JS and YX conceptualized and developed the document. MQ and MTR provided critical feedback and reviewed the manuscript. MAR, SA, and MS revised the manuscript. All authors have read and agreed to the final version of the manuscript.

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Characterizing the Microbial Consortium L1 Capable of Efficiently Degrading Chlorimuron-Ethyl *via* Metagenome Combining 16S rDNA Sequencing

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Excessive application of the herbicide chlorimuron-ethyl (CE) severely harms subsequent crops and poses severe risks to environmental health. Therefore, methods for efficiently decreasing and eliminating CE residues are urgently needed. Microbial consortia show potential for bioremediation due to their strong metabolic complementarity and synthesis. In this study, a microbial consortium entitled L1 was enriched from soil contaminated with CE by a "top-down" synthetic biology strategy. The consortium could degrade 98.04% of 100 mg L^{-1} CE within 6 days. We characterized it from the samples at four time points during the degradation process and a sample without degradation activity *via* metagenome and 16S rDNA sequencing. The results revealed 39 genera in consortium L1, among which *Methyloversatilis* (34.31%), *Starkeya* (28.60%), and *Pseudoxanthomonas* (7.01%) showed relatively high abundances. Temporal succession and the loss of degradability did not alter the diversity and community composition of L1 but changed the community structure. Taxon-functional contribution analysis predicted that glutathione transferase [EC 2.5.1.18], urease [EC 3.5.1.5], and allophanate hydrolase [EC 3.5.1.54] are relevant for the degradation of CE and that *Methyloversatilis*, *Pseudoxanthomonas*, *Methylopila*, *Hyphomicrobium*, *Stenotrophomonas*, and *Sphingomonas* were the main degrading genera. The degradation pathway of CE by L1 may involve cleavage of the CE carbamide bridge to produce 2-amino-4-chloro-6-methoxypyrimidine and ethyl o-sulfonamide benzoate. The results of network analysis indicated close interactions, cross-feeding, and co-metabolic relationships between strains in the consortium, and most of the above six degrading genera were keystone taxa in the network. Additionally, the degradation of CE by L1 required not only "functional bacteria" with degradation capacity but also "auxiliary bacteria" without degradation capacity but that indirectly facilitate/inhibit the degradation process; however, the abundance of "auxiliary bacteria" should be controlled in an

appropriate range. These findings improve the understanding of the synergistic effects of degrading bacterial consortia, which will provide insight for isolating degrading bacterial resources and constructing artificial efficient bacterial consortia. Furthermore, our results provide a new route for pollution control and biodegradation of sulfonylurea herbicides.

Keywords: biodegradation, chlorimuron-ethyl, consortium, metagenomics, network

INTRODUCTION

The herbicide chlorimuron-ethyl (CE) is extensively used in soybean fields to control annual grass weeds, sedges, and broadleaf weeds (Reddy et al., 1995). However, CE has a half-life of ≈7–70 days in soils and remains present for 2–3 years; CE is phytotoxic toward current and subsequent crops and leads to reductions in the yield and quality of crops (Sharma et al., 2012; Zang et al., 2020a). Moreover, CE residues in the soil can alter the structure of soil microbial communities, reduce soil enzyme activities (Zawoznik and Tomaro, 2005; Zhang et al., 2011), and enter the aquatic environment directly or indirectly, causing water pollution and promoting the growth of harming aquatic organisms (Battaglin et al., 2000; Fenoll et al., 2013). To overcome these issues, new methods and suitable technologies are urgently needed to eliminate CE and its intermediate metabolite residues from the environment. Microbial degradation has great advantages in the restoration of the environment with herbicide residues due to economic, eco-friendly, safety, and no secondary pollution (Singh and Singh, 2016; Jing et al., 2020), and many CE-degrading strains have been isolated, including fungi and bacteria (Zang et al., 2020b). These strains degrade CE under laboratory conditions but show some limitations such as low efficiency, unstable effects, incomplete degradation, and easy repellence by indigenous microorganisms in the remediation of *in situ* contaminated soil. Complex organic pollutants in nature cannot be degraded by a single microbial strain but rather by the syntrophism and metabolism of consortia (Jeon and Madsen, 2013, Vaidya et al., 2018).

Microbial consortia are composite microbial assemblies with stable structure and function and can be cocultivated and metabolized in a specific environment by two or more microorganisms through domestication (VerBerkmoes et al., 2009). Based on the synergistic interaction of different microorganisms in the population, bacterial consortia are more effective than single microbial or enzyme systems for environmental bioremediation (Wanapaisan et al., 2018) due to their better adaptability and tolerance to variable and complicated environments (Xu et al., 2020; Wang et al., 2021). Furthermore, microorganisms in consortia can exchange substances and communicate with each other through complex and efficient metabolic regulation networks and signaling molecules (Zafra et al., 2017; Bai et al., 2022) to coordinate the overall function of the strain and achieve higher degradation efficiency compared with that of a single organism (Bhatia et al., 2018). Therefore, bacterial consortia are good models for studying the interactions between bacterial populations during bioremediation (Desai et al., 2010) and analyzing the network relationships among bacterial communities, metabolic exchange, and signal transmission. Moreover, the consortia are a resource library for cultivated functional bacteria and a powerful tool for evaluating the potential of viable but non-culturable bacteria and for determining the function of many unknown genes (Wintermute and Silver, 2010). Therefore, studies of the microbial ecology of the bacterial consortia are essential for understanding their roles and niches in the degradation process and for optimizing their performance (Eze et al., 2021).

Many bacterial consortia have been reported to degrade herbicides such as linuron (Dejonghe et al., 2003; Zhang et al., 2018), diuron (Ellegaard-Jensen et al., 2014), atrazine (Xu et al., 2019), metribuzin (Wahla et al., 2019), bispyribac sodium (Ahmad et al., 2018), and CE (Li et al., 2017). These consortia showed significantly higher degradation efficiency than a single microorganism, indicating that synergy among the strains can improve metabolic efficiency. The natural microbial consortium for herbicide degradation has not been widely examined, and research on artificial microbial consortia has only focused on their degradation efficiency. Studies of the community diversity, structure, and functional interactions of natural microbial consortiums can reveal the co-metabolism relationship and regulation mechanism during degradation and provide guidelines for constructing a high-efficiency artificial microbial consortium using synthetic biology methods.

In this study, we enriched a natural microbial consortia L1 (MC-L1) from CE-contaminated environments using the "top-down" strategy (Liang et al., 2022). To characterize this consortium, we analyzed the bacterial diversity, structure, function, pathway, and interactions of MC-L1 during the degradation process using 16S rRNA high-throughput sequencing and metagenomic sequencing. We predicted the possible pathways and keystone taxa related to CE degradation. Our results will greatly improve the understanding of degrading bacterial consortia and provide a foundation for applying bacterial consortia to herbicide residue elimination and environmental health remediation.

MATERIALS AND METHODS

Chemicals and Cultural Media

Chlorimuron-ethyl (purity ≥98.0%) was purchased from Acmec biochemical Co., Shanghai, China. All other chemicals, analytical grade or better, were purchased from Sinopharm Chemical Reagent Co., Ltd., China.

The composition of the inorganic salt medium was as follows (g/L): 2.0 g $NaNO_3$, 2.0 g KH_2PO_4, 0.125 g $MgSO_4{\cdot}7H_2O$, 0.5 g

NaCl, 0.02 g $FeSO_4 \cdot 7H_2O$. The pH of the medium was adjusted to 7.0.

Sample Collection and Degradation Capacity Determination

The soil sample was collected from a pesticide factory in Nanjing, Jiangsu, China and then was applied to enrich the CE degrading bacterial consortium. The experiment used standard successive enrichment culture procedures using 100 mg L^{-1} CE as the sole carbon and energy source. The enriched consortium has been maintained by weekly transfers in the inorganic salt medium. After 10 subcultures, a bacterial consortium named L1 with a stable degradation rate was obtained. To further determine the degradation capacity, MC-L1 was transferred into 100 ml of inorganic salt medium (pH = 7.0) containing 100 mg L^{-1} of CE and 2 ml L^{-1} of methanol with 5% (V/V) inoculum and incubated at 28°C and 150 rpm for 8 days. Optical density (OD_{600}) and herbicide concentration were measured every 24 h to determine the bacterial cell density and degradation rate of CE.

The degradation rate was measured by high-performance liquid chromatography (HPLC). In brief, 10 ml of culture medium and 10 ml of dichloromethane were mixed in a 150 ml separatory funnel, and the lower organic phase was combined after three rounds of extraction by vigorous shaking. The organic phase was dried with N_2, suspended in 10 ml acetonitrile, and filtered through a 0.22 μm nylon membrane. Then, a 20-μl sample was injected into the HPLC equipped with a Zorbax SB-C18 ODS Spherex column (4.6 × 250 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA) at 25°C; the mobile phase was acetonitrile (A): 0.05% acetic acid (B) at a flow rate of 1 ml/min, linear gradient 0–1 min 2% A; 1–10 min 2–70% A; 10–13 min 70–100% A; 13–13.5 min 100–2% A; and 13.5–15 min 2–0% A (Wang et al., 2016). CE was detected at 254 nm. The correlation coefficient (R^2) for the standard curve was 0.9996.

To use sequencing methods to study the interactions and changes of MC-L1 during the biodegradation process, the bacterial pellet samples were collected by centrifuging at 80,000 rpm for 15 min according to the degradation curve and growth curve. In addition, we also collected an incapacitated consortium (marked NO) belonging to the same generation and then also performed a sequencing analysis.

DNA Extraction

Total DNA was extracted using the E.Z.N.A.™ Soil DNA Kit (Omega Biotek, Inc., Norcross, GA, USA), and its concentration and purity were evaluated using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). After quality control, the extracted DNA samples were stored at −80°C in preparation for sequencing (Chen et al., 2018).

16S rRNA Gene Sequencing and Analysis

The PCR amplifications were performed using primers 338F/806R targeting the V3–V4 region of the bacterial 16S rRNA gene (Su et al., 2018). The amplicons were merged on an Illumina MiSeq PE300 platform (Illumina Inc., San Diego, USA) following the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Raw sequences were filtered for quality using Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) (Caporaso et al., 2012). After removing those low-quality sequences (quality scores < 20, length < 50 bp), operational taxonomic units (OTUs) were assigned from the reads using UPARSE (version 7.1 http://drive5.com/uparse/) at a 97% sequence similarity threshold (Edgar, 2013). The taxonomic identity of all phylotypes was then determined by the SILVA ribosomal RNA gene database project (Quast et al., 2013).

Metagenomic Sequencing and Annotation

Metagenomic sequencing based on the NovaSeq 6000 platform (Illumina, USA) was completed by Majorbio, Inc. (Shanghai, China). Metagenomic assembly, contigs binning, gene prediction, and abundance analysis were performed according to a previous study (Zhang et al., 2020b). The gene catalog was translated to putative amino acid sequences, which were all extracted from the NCBI NR database, evolutionary genealogy of genes: Non-supervised Orthologous Groups (EggNOG, http://eggnog.embl.de/, version 4.5.1) and the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/, version 94.2) (Ogata et al., 1999) databases with Diamond (http://www.diamondsearch.org/index.php, version 0.8.35) (*e*-value ≤ $1e^{-5}$). The original sequencing data have been uploaded to the NCBI database (accession number: PRJNA788073 for 16S rRNA high-throughput sequence, PRJNA788363 for metagenomic sequence).

Correlation Network Analyses

Network analysis was performed to reveal the complex associations within microbial communities and identify potential keystone taxa for degradation (Zheng et al., 2021). Networks of different time points and incapacitated consortia were implemented on the I-Sanger platform using Networkx and visualized with Cytoscape 3.8.2 (Banerjee et al., 2016). Co-occurrence relationships were considered as stable if the Pearson's correlation coefficient was >0.5 and $P < 0.05$. All operational taxonomic units (OTUs) were analyzed; the color level of each node is displayed as a level. Nodes showing a high degree were considered keystone taxa (Berry and Widder, 2014).

To visualize the associations between species and function, we established a function-taxon correlation network to identify the functional clusters of bacterial taxa in MC-L1. The top 50 genera and top 50 functions in pathway level 3 were selected for the network constructions. Pearson's correlation coefficients used in the network analysis were more than 0.5 and the cutoff of *P*-values was 0.05. Finally, we also used Cytoscape 3.8.2 (Banerjee et al., 2016) for visualization. The taxon nodes tending to have high degrees were identified as degradation keystone taxa.

Statistical Analyses

Degradation curve and growth analyses were performed using the GraphPad Prism 8 software (GraphPad, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) and Pearson's correlation analysis between CE residues and the OD_{600} were performed using the SPSS 25 software (SPSS, Inc., Chicago, IL, USA). Alpha diversity indices (within samples) were calculated using Mothur version 1.30.1 (Schloss and Westcott, 2011).

Beta diversity (between samples) was visualized using principal coordinate analysis (PCoA) of the Bray Curtis distance metric. Permutational multivariate ANOVA (PERMANOVA) based on the Bray-Curtis similarity (Bray and Curtis, 1957) was also performed to identify variations among different groups with 999 permutations. Hierarchical clustering analysis of the dataset was performed using the Bray-Curtis distance at the genus level. Venn diagrams and composition diagrams of the sample microbiota were drawn in the Vegan package (version 2.4.3) using R (The R Project for Statistical Computing, Vienna, Austria). Differentially abundant bacterial genera among groups were determined using linear discriminant analysis (LDA) effect size (LEfSe), applying the all-against-all strategy with a threshold of 3 on the logarithmic LDA score for discriminative features (Segata et al., 2011). The Circos graph, multigroup comparison, and relative contribution analysis were performed using the Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

RESULTS AND DISCUSSION

Enrichment and CE Degradation of L1 Consortium

After inoculation, CE was rapidly degraded without any lag period (**Figure 1**). MC-L1 almost completely degraded CE within 8 days (99.62%), with the degradation rate reaching 98.04% at 6 days (**Figure 1**). The degradation rate and tolerance to high contamination stress of MC-L1 were significantly better than the consortium reported previously (Li et al., 2017). This may be because the natural microbial consortium has evolved to have more complete co-metabolic networks, closer interactions, and stronger environmental adaptations (Kato et al., 2008). As shown in **Figure 1**, the OD_{600} increased rapidly from days 3 to 6 and then decreased slowly due to the depletion of carbon and energy materials after CE degradation. An extremely strong negative correlation was observed between the OD_{600} and CE residues ($R = -0.95$, $P < 0.01$), indicating that biomass is important for the efficient degradation of MC-L1. Therefore, corresponding to the pre-degradation, degradation, and post-degradation periods, consortium L1 was selected on days 1, 4, 5, and 7 for 16S rDNA and metagenomic sequencing.

Overview of 16S rRNA Sequencing and Metagenomic Sequencing

After sequence quality control, 1,032,755 effective sequence reads with an average length of 419.24 bp were obtained from all samples using 16S rRNA high-throughput sequencing. These sequences were assigned to 78 OTUs with 97% similarity. The number of observed OTUs, library coverage, species richness, and diversity indices is shown in **Supplementary Table S1**.

Metagenomic sequencing generated 1.15 million contigs comprising 1.66 billion contigs bases (**Supplementary Table S2**). To identify the potential biological pathways of the genes, we used genes related to xenobiotic biodegradation and metabolism (XBM) in the KEGG to construct and re-annotated a new gene set. All analyses of the metagenomic data were based on this new gene set, in which 5,281 genes were mapped into five branches with 89 pathways. The "xenobiotics biodegradation and metabolism" pathway belonged to level 3 and involved 21 different pathways, among which the most numerous pathways were related to the metabolism of benzene ring structures including "benzoate degradation," "styrene degradation," "xylene degradation," "toluene degradation," and "naphthalene degradation". Some pathways were associated with the degradation of chlorinated substances, such as chlorocyclohexane and chlorobenzene degradation and chloroalkane and chloroalkene degradation. These results suggest that many pathways and genes in MC-L1 are related to the degradation of a wide range of pollutants.

Richness and Diversity of CE-Degrading Consortium L1

In terms of alpha-diversity, the Chao and Simpson indices of MC-L1 did not significantly differ among groups (**Figures 2A,B**), whereas the Simpson indices significantly differed between the consortia on days 4 and 7 based on Student's *t*-test ($P = 0.04303$). The diversity of MC-L1 was relatively stable; therefore, the degradation capacity was not determined by the overall diversity but rather by the abundance of specific taxa (Banerjee et al., 2016). PCoA analysis was used to illustrate differences in β-diversity (**Figure 2C**). Bacterial community structures were divided into five groups at the genus level. Furthermore, PERMANOVA analysis indicated a significant effect of time on the bacterial structure ($R^2 = 0.7844$, $P = 0.001$). The hierarchical clustering tree showed significant phylogenetic differences among all groups (**Figure 2D**). Samples from the lower degradation rate at the beginning (day 1) and ending stages (day 7) of the degradation process clustered into one group based on the phylogenetic composition, whereas consortia at the rapid degradation stage between days 4 and 5 had a different microbiota. These results demonstrate that the time factor did not alter the overall diversity of species in the microbial community but changed the abundance of key species.

Composition and Structure of the Microbial Consortium in Different Groups

The Venn diagram showed unique and shared bacterial OTUs (**Figure 3A**) and genera (**Figure 3B**) in all five groups. The consortium on day 5 contained one unique OTU belonging to *Hyphomicrobium*, but no unique taxa were found at the genus level. Venn diagrams at the OTU and genus levels supported that there was no significant difference in the community composition between the consortium at different time points and incapacitated consortium. A previous study showed that using a single carbon source to enrich bacterial consortia results in the formation of a dense cross-feeding network, leading to collective interactions that simplify the interaction of the microbiome (Goldford et al., 2018). MC-L1 also formed relatively stable and close interactions under long-term CE stress as a single carbon source; therefore, the community composition did not change significantly.

The Circos figure illustrates that five phyla were identified in MC-L1 (**Figure 3C**), with Proteobacteria as the most dominant

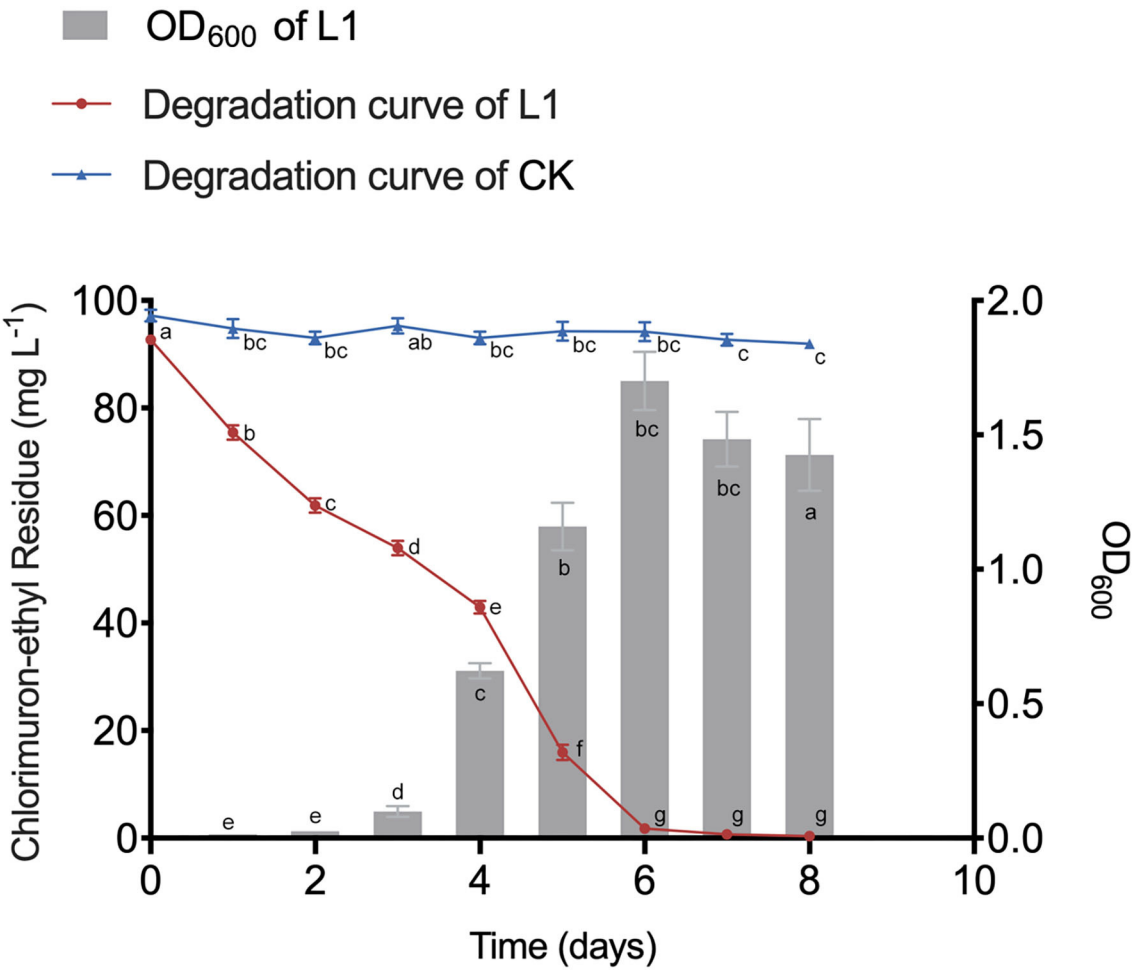

FIGURE 1 | Growth dynamics of microbial consortia L1 (MC-L1) and its degradation curve to chlorimuron-ethyl.

phylum (96.52%), particularly on day 5 (Kruskal–Wallis (K–W) H test, $P < 0.05$). The results were consistent with the report that Proteobacteria was the most abundant phylum in the resources of herbicide-degrading bacteria (Singh and Singh, 2016). At the genus level (**Figure 3D**), the bacterial communities were dominated by *Methyloversatilis* (34.31%, on days 1, 4, and 7) and *Starkeya* (28.60%, on day 5 and NO), followed by *Pseudoxanthomonas* (7.01%), *Hydrogenophaga* (6.83%), *Legionella* (3.47%), and *Chitinophaga* (3.12%). During degradation, *Starkeya* and *Hydrogenophaga* (K–W test, $P < 0.05$) first showed an increasing trend followed by a decreasing trend, and their abundance in the NO group was relatively high. The genus *Pseudoxanthomonas* showed an increasing trend, but its abundance in the NO group was the lowest (K–W test, $P < 0.05$), and the genus *Legionella* was set to increase (K–W test, $P < 0.05$). Significant temporal variations of bacterial community structure at the genus level were observed among different groups. Although none of these dominant genera have been reported to degrade CE, their consortium showed high degradation potential, possibly *via* cooperative catabolism, where one strain transforms the herbicide into products that are used by another strain (Ellegaard-Jensen et al., 2014).

Furthermore, we evaluated the presence of different bacterial genera by LEfSe analysis ($P < 0.05$, LDA score >3) and identified 45 taxonomic clades with different abundances and LDA scores higher than 3.0 (**Figures 3E,F**). We found that 6, 1, 3, 13, and 22 taxa were significantly enriched in each group, with the NO sample showing the largest number of significantly enriched microbes, such as Bacteroidota (from phylum to genus), Actinobacteria (from phylum to genus), and Pseudomonas (from order to genus). Taken together, NO greatly differed in community structure from the other consortium samples. Thus, the loss of degradation capacity can occur even in relatively stable consortia; additionally, although the overall community composition did not change, overgrowth and/or growth failure of certain members can result in structural changes that may affect co-metabolic processes (Kato et al., 2008).

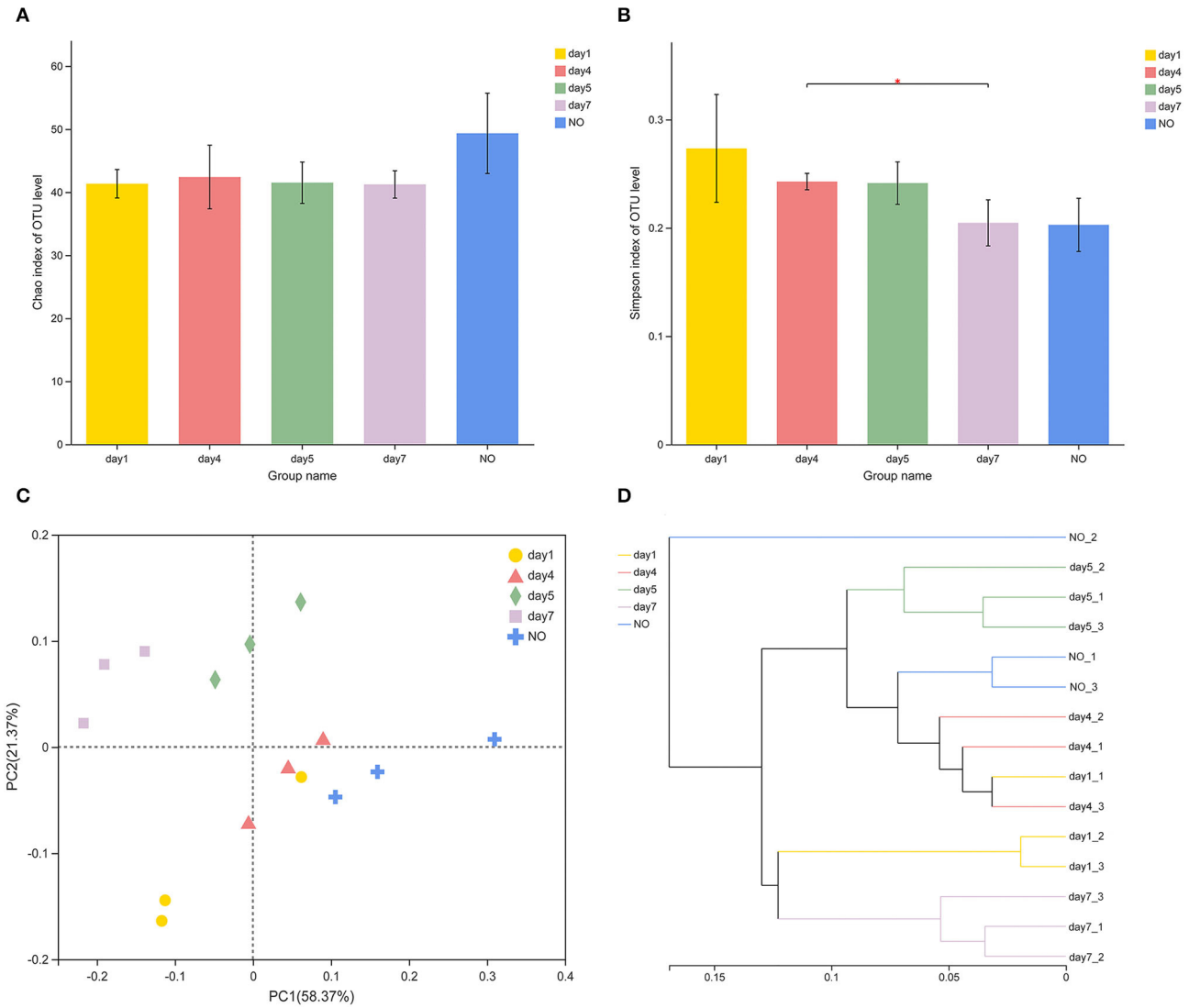

FIGURE 2 | Variations in diversity and community structure of different consortium samples. **(A,B)** Variations of Chao1 and Simpson indexes *$P < 0.05$ (Student's *t*-test); **(C)** principal coordinate analysis based on Bray–Curtis distances; **(D)** hierarchical cluster analysis using the Ward's method at the genus level.

Microbiome Function Annotation Based on Metagenomic Sequencing

The relative abundance of metagenomic next-generation sequencing (mNGS) and 16S rRNA sequencing showed that MC-L1 was clearly dominated by Rhizobiales (**Supplementary Figure S1A**). Other orders present in the consortium were Rhodocyclales, Burkholderiales, and Xanthomonadales. In addition, some orders such as Sphingomonadales and Legionellales showed a lower relative abundance in mNGS than in 16S rRNA sequencing. The main difference between the mNGS and 16S rRNA sequencing relative order abundance was found in Rhodocyclales, which was relatively abundant according to the mNGS results (37.90%) but absent from the 16S rRNA sequencing results. This result was explained by differences in sequencing principles and taxonomic identification databases (SILVA database/NCBI NR database). In addition, the genetic similarity of some species was high, which may lead to errors in phylogenetic analyses, such as *Sphingomonas* and *Sphingobium* (Zhao et al., 2017). Overall, the compositions of the four main orders in mNGS (93.89%) and 16S rRNA sequencing (89.06%) were similar, indicating consistency between the two sequencing results.

The results of PCoA analysis of KEGG at level 3 of "XBM" showed significant differences between the NO group and other groups (**Supplementary Figure S1B**). PERMANOVA confirmed these significant differences ($R^2 = 0.79897$, $P = 0.002$ in KEGG), enabling the analysis and prediction of functional bacteria with degradation ability. Based on this, the functional analysis should focus not only on the differences between the degradation consortium

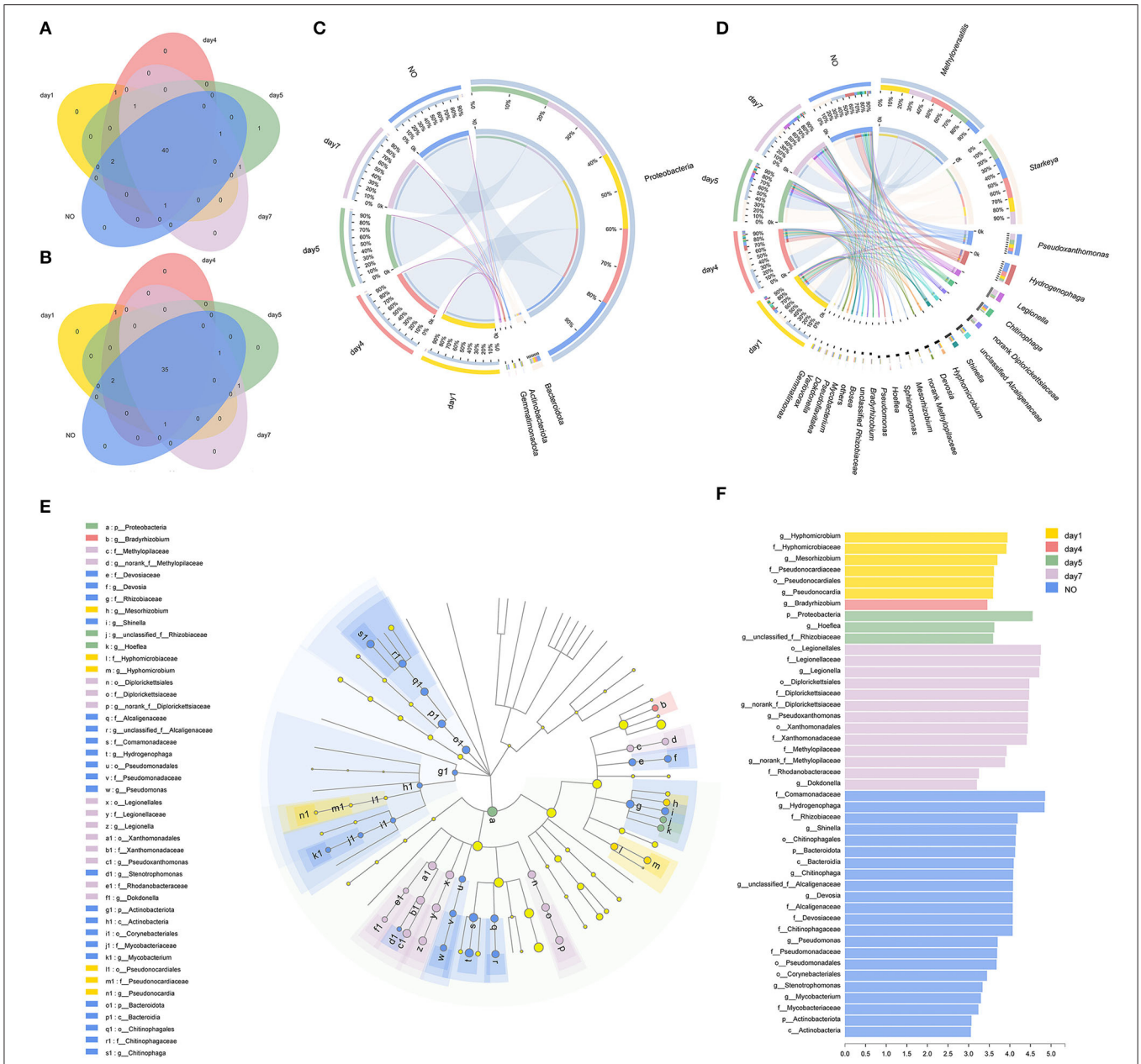

FIGURE 3 | The evolution of bacterial community compositions among different time points and the incapacitated consortium. **(A,B)** Venn diagrams showed the shared and unique numbers of bacterial operational taxonomic units (OTUs) (at 97% identity) and genera among the five groups; **(C,D)** relationships between samples and phyla/genera are shown in Circos figures. Phylogenetic groups accounting for ≤1% of all classified sequences are summarized in the artificial group "others"; **(E,F)** phylogenetic cladogram of biomarker bacteria and indicator bacteria with linear discriminant analysis (LDA) scores of ≥3 in bacterial communities.

at different time points but also on samples without degradation functions.

As shown in **Figure 4**, a multigroup comparison based on the K–W test revealed significant differences among the five groups for levels 1, 2, and 3. In both the KEGG categories of metabolism (**Figure 4A**) and "XBM" (**Figure 4B**), functional genes tend to first increase and then decrease during degradation. Particularly, samples on day 5 showed the most significant enrichment, whereas NO exhibited the lowest enrichment (K–W test, $P < 0.05$).

At level 3 in the "XBM" category, we analyzed the top 15 KEGG pathways (**Figure 4C**), among which 11 pathways exhibited significant differences. Among the 15 pathways, six were selected for further analysis, namely, ko00362 (benzoate degradation), ko00361 (chlorocyclohexane and chlorobenzene degradation), ko00625 (chloroalkane and chloroalkene degradation), ko00622 (xylene degradation), ko00623 (toluene

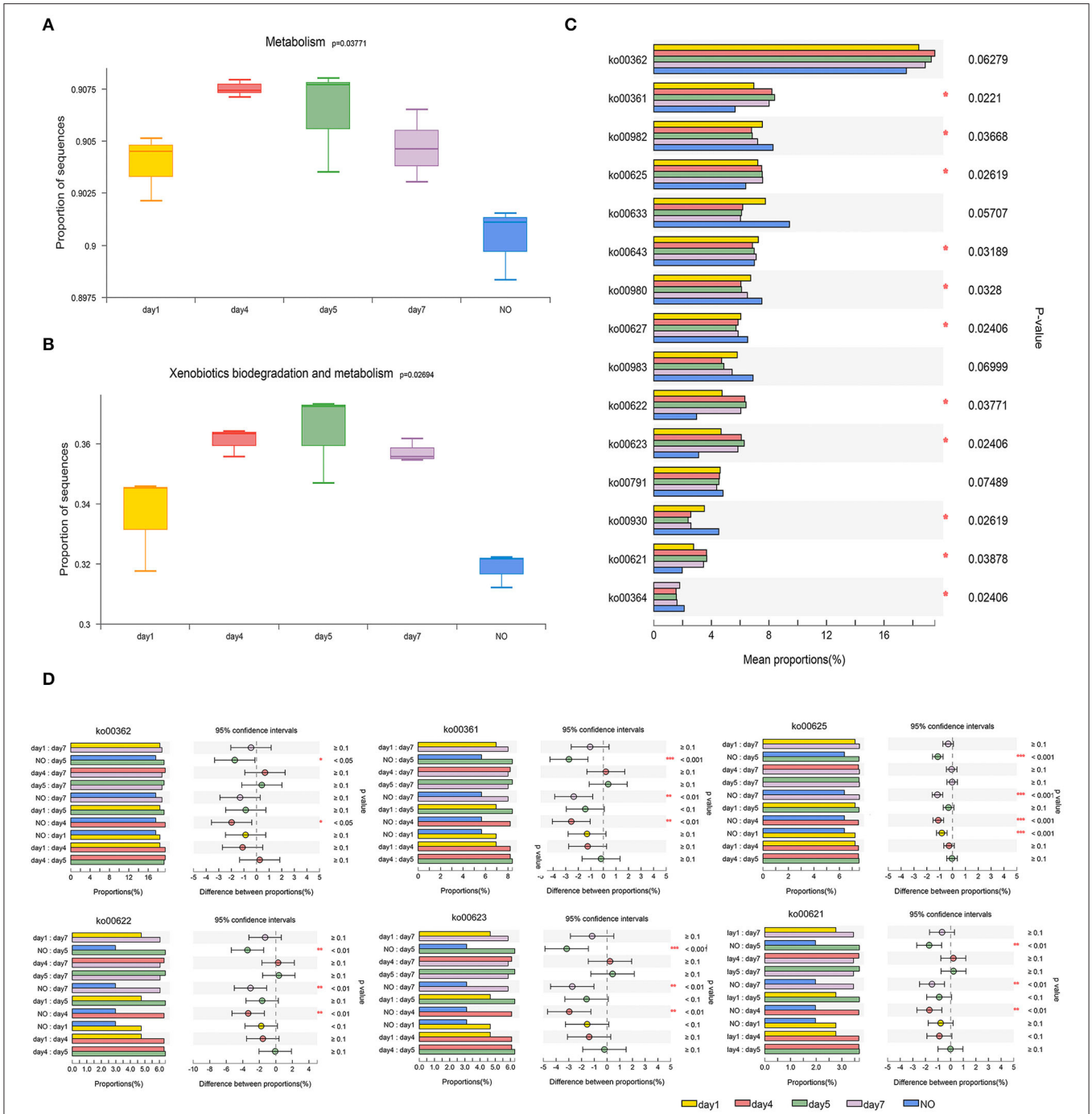

FIGURE 4 | The comparison of bacterial community functions analyzed at level 1, level 2, and level 3 between different groups. **(A)** and **(B)** Comparison of the Kyoto Encyclopedia of Genes and Genomes (KEGG) functions at level 1 related to "Metabolism" and level 2 related to "Xenobiotics Biodegradation and Metabolism"; **(C)** The Kruskal–Wallis (K–W) test of the top 15 abundance KEGG pathways at level 3 in the category of "Xenobiotics Biodegradation and Metabolism"; **(D)** Tukey-Kramer test using *post-hoc* analysis of six KEGG pathways. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

degradation), and ko00621 (dioxin degradation) because the functional genes of these six pathways in the NO group were significantly lower than those in samples at other time points. The abundance of functional genes was highly consistent with the trend of the degradation curve (**Figure 4D**).

The six pathways mentioned above involve degradation of the benzene ring structure and chloride, which agrees with previous studies on the degradation of CE *via* urea bridge cleavage, de-esterification, oxidation, cyclization, and cleavage of the N–C bond of the sulfonylurea bridge and pyrimidine ring (Li et al.,

2016). In addition, "toluene degradation," "chlorocyclohexane degradation, " and "chlorobenzene degradation" were reported to respond to CE in *Rhodococcus erythropolis* D310-1 (Cheng et al., 2018). The six pathways were tested using the Tukey-Kramer *post-hoc* analysis. Although ko00362 was not significant in the multigroup comparison, we found significant differences in ko00362 between NO and the samples on days 4 and 5. Four of the six pathways, ko00361, ko00622, ko00623, and ko00621, showed remarkable differences between NO and the samples on days 4, 5, and 7, respectively. In ko00625, NO was extremely significantly different from all other groups ($P < 0.001$). The decrease in functional genes involved in these metabolic pathways in NO may lead to loss of the degradation ability of MC-L1.

Linking the Potential Degrading Taxonomic and Functional Properties

To determine the relationship between potential degrading taxonomic and functional properties, we performed a relative contribution analysis at level 3 in the category of "XBM" and the six pathways (**Figure 5A**). *Methyloversatilis* was the main contributor among samples at different time points, but its contribution to NO was the lowest among the five groups (**Figure 5A**). The contribution of *Methyloversatilis* first increased and then decreased during degradation. This trend was not only reflected at these two levels but also was consistent with our results on the metabolic pathways and community structure of 16S rRNA sequencing. The relative contribution of *Starkeya* was highest in NO, particularly in ko00625, which is closely related to CE degradation. Thus, loss of the degradation ability of NO may be related to changes in the community structure, that is, a decrease in *Methyloversatilis* and an increase in *Starkeya*. Similarly, the contribution of taxa belonging to *Bosea* declined. The contribution of this genus was the highest on the first day and then decreased but was significantly higher in NO than in other samples, particularly in ko00361, ko00625, and ko00623. Interestingly, *Bosea* was nearly undetectable in ko00622 and ko00621, demonstrating that compared with other dominant strains, some pathways were lacking (**Figure 5A**). Additionally, some taxa showed higher contributions to NO than in other samples, such as *Afipia*, *Agrobacterium*, *Shinella*, and *Pseudacidovorax*, indicating that structural imbalances of these dominant genera can lead to a reduction in degrading bacteria and eventual loss of their degradation ability.

According to the K–W test (**Figure 5B**), the genera that may play important roles in CE degradation by MC-L1 were predicted. The following seven genera showed a high contribution to the degradation process but were low in NO: *Methyloversatilis*, *Comamonas,* *Pseudoxanthomonas*, *Methylopila*, *Sphingomonas*, *Hyphomicrobium*, and *Stenotrophomonas*. Analysis of the functional contribution of these genera at the enzyme level (top 50 enzymes) showed that *Methyloversatilis* contained the largest number of enzymes (32), whereas *Comamonas* contained the smallest number (2) (**Figure 6A**). *Methyloversatilis* likely has a wide range of metabolic functions and acts as the main functional strain during degradation. An analysis of the reactions catalyzed by the annotated enzymes, i.e., glutathione transferase [EC 2.5.1.18], urease [EC 3.5.1.5], and allophanate hydrolase [EC 3.5.1.54], was predicted to be relevant to CE degradation. All three enzymes can catalyze the cleavage of the CE carbamide bridge to produce 2-amino-4-chloro-6-methoxypyrimidine and ethyl o-sulfonamide benzoate, which is consistent with the previously reported degradation pathway of CE (Ma et al., 2009; Sharma et al., 2012; Zhang et al., 2020c). In terms of their functional contributions, glutathione transferase [EC 2.5.1.18] was annotated as the highest relative contributor in the six genera, except for *Comamonas*. Knockout experiments of *Klebsiella jilinsis* 2N3 confirmed the degradation capacity of glutathione transferase for CE (Zhang et al., 2020c), and comparative genome analysis of *Sphingomonas* showed that this bacterium produces amidohydrolase, which may catalyze the hydrolysis of the amide bond of CE (Cheng et al., 2019). This enzyme may play a dominant role in the degradation of CE by MC-L1, and *Methyloversatilis*, *Pseudoxanthomonas*, *Methylopila*, *Sphingomonas*, *Hyphomicrobium*, and *Stenotrophomonas*, which contain this enzyme, are the main degrading genera. In addition, allophanate hydrolase [EC 3.5.1.54] with a low relative contribution was annotated in *Methylopila* and *Hyphomicrobium*, and urease [EC 3.5.1.5] with a high relative contribution was annotated in *Methyloversatilis*, *Methylopila*, and *Hyphomicrobium*. The coexistence of all three enzymes in *Methylopila* and *Hyphomicrobium* suggested that these two genera had high degradation potentials. Although these three degradation-related enzymes have not been annotated in *Comamonas*, the high presence of alcohol dehydrogenase [EC1.1.1.1] suggests that this genus plays an important role in converting downstream degradation products for the energy supply. Additionally, previous studies showed that *Methyloversatilis* promotes the cleavage of ester bonds, which may be related to the production of 2-[[(4-chloro-6-methoxy-2-pyrimidinyl) carbamoyl] sulfamoyl] benzoic acid and 2-carboxy phenylsulfamide during the degradation of CE (Cai et al., 2011).

Although there are few reports of CE degradation by these six genera, substrate analyses suggested that they have a high potential for organic compound degradation. For example, *Methylopila* sp. can degrade a variety of sulfonylurea herbicides, such as metsulfuron-methyl and bensulfuron-methyl (Huang et al., 2007), and *Hyphomicrobium* sp. can degrade dichloromethane, methyl chloride, methamidophos, dimethyl sulfide, methanol, and polycyclic aromatic hydrocarbons (Yoshikawa et al., 2017; Hayoun et al., 2020; Jin et al., 2022). *Pseudoxanthomonas spadix* BD-a59 can metabolize all six BTEX compounds (Lee et al., 2012), and *Stenotrophomonas* sp. can metabolize aromatic compounds and highly chlorinated polychlorinated biphenyl congeners (Gao et al., 2013; Horváthová et al., 2018; Li et al., 2021). Additionally, *Stenotrophomonas maltophilia* D310-3 degraded 89% of 50.21 mg L^{-1} CE in 6 days (Zang et al., 2016). The genome of *Methyloversatilis universalis* FAM 5^T contained genes not only for the uptake and utilization of hydrogen and nitrogen compounds for energy metabolism but also for the utilization of cyanate, glycerol, long-chain amines, aromatic compounds,

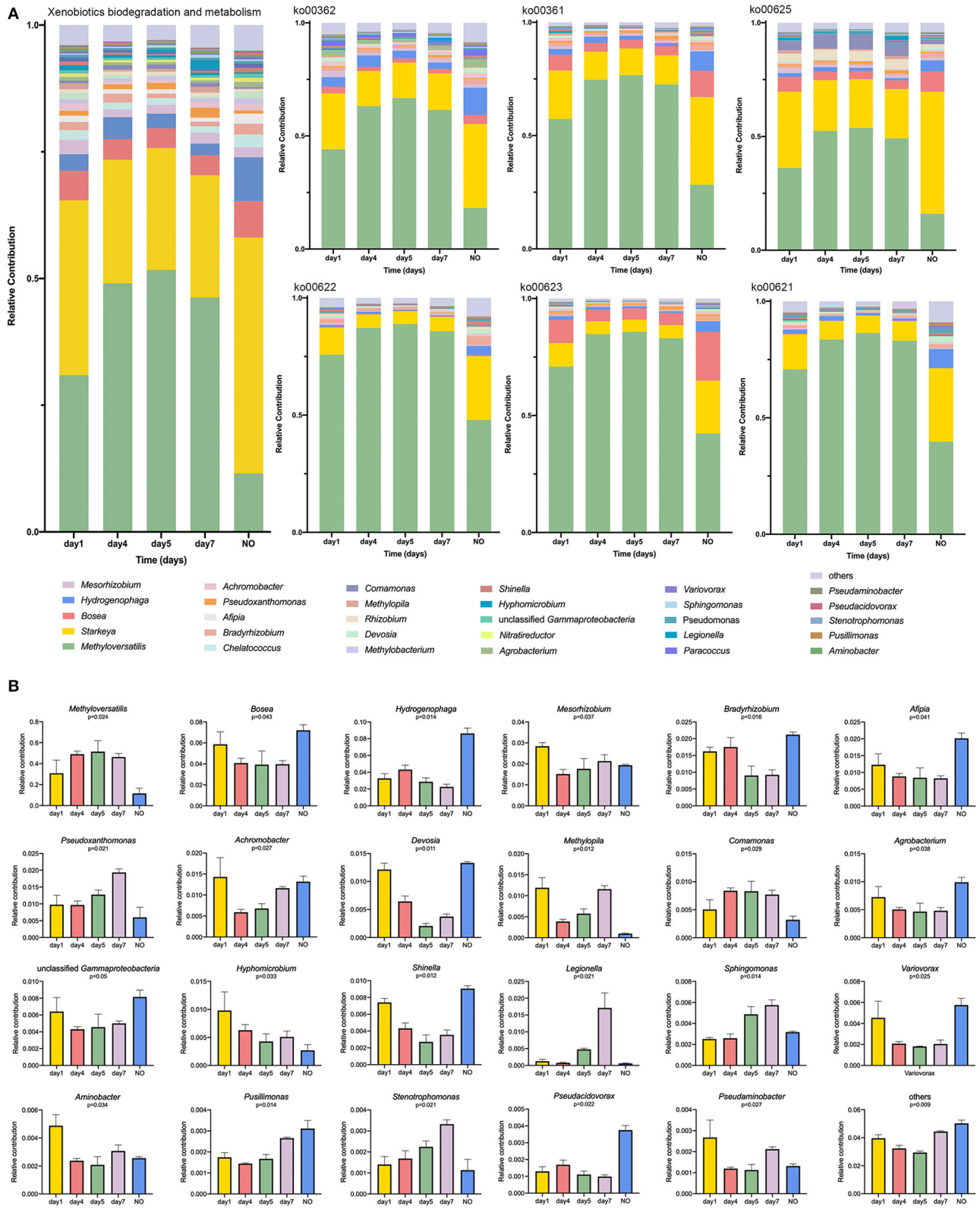

FIGURE 5 | (A) Relative contribution of different taxa at genus level to identified degradation-enriched functional attributes in different groups; **(B)** The K–W *H* test at level 3 in the category of "Xenobiotics Biodegradation and Metabolism."

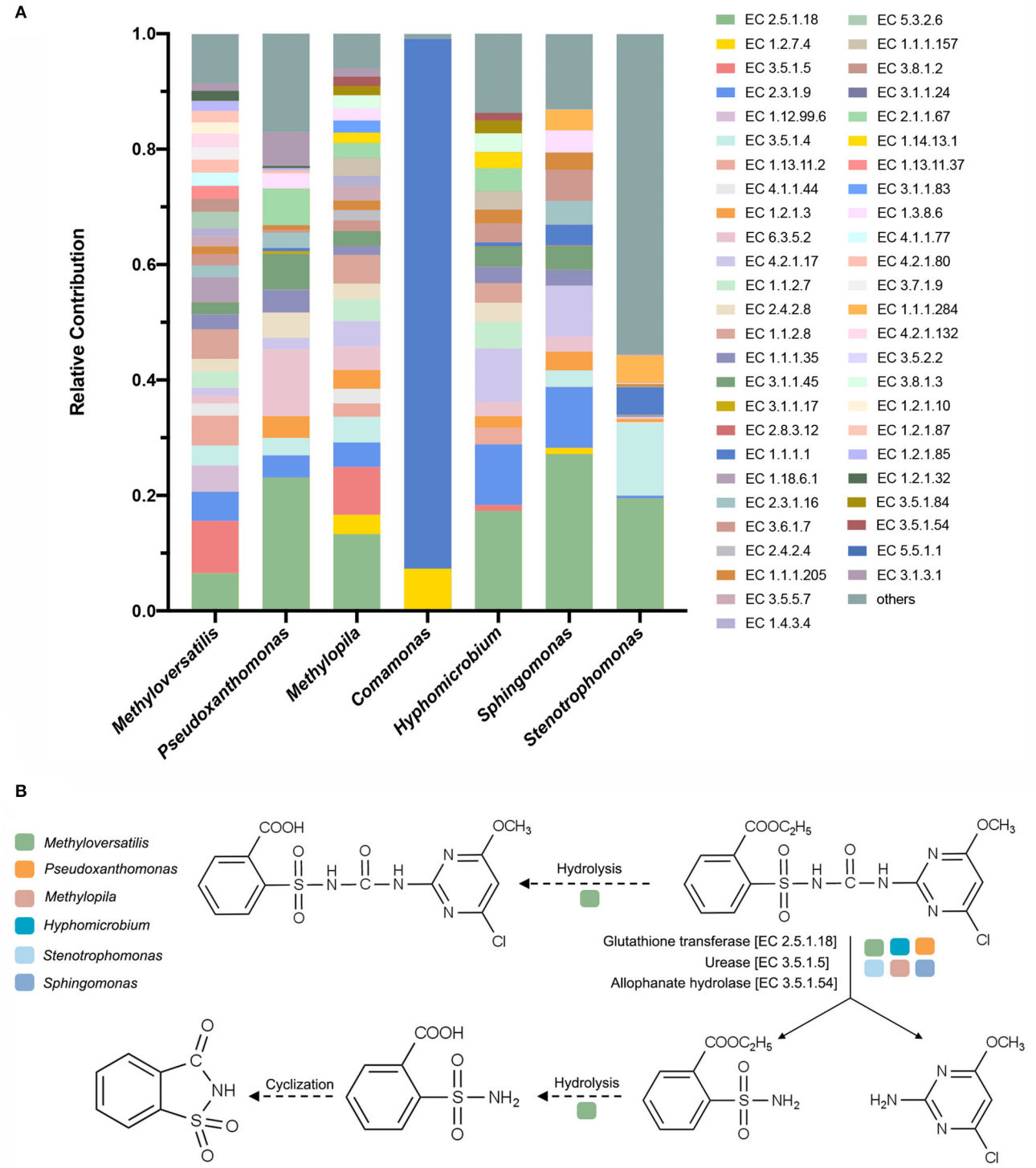

FIGURE 6 | (A) Analysis of the functional contribution of seven possible degrading genera at the enzyme level; **(B)** proposed degradation pathway of chlorimuron-ethyl by MC-L1. Solid lines indicate the enzymes and pathways present in the MC-L1; dashed lines indicate the inferred pathways.

alkanesulfonates, alkylnitronate, phenols, acetone, urea, and methane sulfonic acid (meta-cleavage pathway) (Kittichotirat et al., 2011). *Sphingomonas* can mineralize chloroacetanilide and substitute urea herbicides, as well as aromatic compounds, such as phenol and chloramphenicol (Sørensen et al., 2013; Ruan et al., 2018; Cheng et al., 2019; Zhang et al., 2020a).

Notably, *Sphingobium*, *Sphingomonas*, *Hyphomicrobium*, *Bosea*, and *Afipia* were enriched in the chloramphenicol-degrading bacterial consortium, indicating that these genera contain important functional genes and special co-metabolic networks for degrading benzene ring structures and chlorides (Zhang et al., 2020a). Network analysis can help reveal these non-random interactions and define the functions of the genera (Fuhrman, 2009).

In summary, we identified six functional genera in MC-L1 that are directly involved in CE degradation and identified the possible reactions involved in each genus (**Figure 6B**). The main reaction steps are as follows: glutathione transferase [EC 2.5.1.18], urease [EC 3.5.1.5], and allophanate hydrolase [EC 3.5.1.54] in *Methyloversatilis*, *Pseudoxanthomonas*, *Methylopila*, *Sphingomonas, Hyphomicrobium*, and *Stenotrophomonas* directly catalyzed cleavage of the CE carbamide bridge to produce 2-amino-4-chloro-6-methoxypyrimidine and ethyl o-sulfonamide benzoate. Some enzymes in *Methyloversatilis* may then catalyze the hydrolysis of the ethyl ester on the benzene ring by undergoing a hydrolysis reaction to produce o-sulfonamide benzoic acid, which in turn undergoes a cyclization reaction to form o-sulfonate benzoic imide.

Correlation Network Analysis of Bacterial Consortium L1

To explore changes in the co-occurrence patterns of the consortium at different time points, five complex networks were constructed for different groups using OTU data (**Figure 7A**). As shown in **Table 1**, although all five networks showed close interactions, the complexity of the networks decreased with degradation, whereas the NO network was the simplest. This may be because at the start of degradation, microorganisms can use methanol to rapidly increase biomass and degrade CE through co-metabolism; however, as the substrate concentration decreases, the interactions gradually weaken, which is consistent with the pattern of microbial carbon utilization, that is, simple alcohols are utilized before the complex organic matter is used. The number of edges with positive and negative correlations was similar across the five networks; however, the day 7 group clearly showed more positive correlation edges than negative (**Table 1**), indicating a cooperative relationship of mutualism or commensalism. At the end of degradation, CE is essentially catabolized into bioavailable metabolites, and the bacteria responsible for downstream biodegradation may perform cross-feeding by exchanging metabolic products or using the metabolites of other bacteria to grow and flourish (Woyke et al., 2006; Faust and Raes, 2012). The transitivity and average degree of the network were highest on day 1 and lowest in NO; however, the average shortest path length of the network was highest in NO and lowest on day 1. These results suggest that the apparent reduction in bacterial network complexity was closely associated with the loss of degradation function, indicating a reduction in interactions between the bacterial communities.

Based on the high-degree node (degree $\geq$20), 28, 26, 19, 10, and 6 keystone taxa were selected from the five complex networks. The number of keystone taxa in NO and day 7 groups was far smaller than that in the early and middle degradation stages. These results indicate that the abundance of CE in the early degradation stages created many trophic levels or resource cascades so that some keystone taxa cooperate to achieve co-metabolism. In these keystone taxa, 10 OTUs belonging to six genera appeared in four groups, among which three genera, namely, *Chitinophaga*, *Starkeya*, and unclassified *Alcaligenaceae*, were dominant and appeared in networks of four time points, thus participating in the whole process of degradation (**Supplementary Table S3**). In the keystone taxa, eight OTUs belonging to seven genera appeared in the three groups, whereas only five genera appeared in the consortium with degrading ability. These five genera, namely, *Variovorax*, *Sphingomonas*, *Pseudomonas*, *Dyadobacter*, and *Aquamicrobium*, accounted for a small proportion of the community composition. Keystone species are commonly considered to exert a large effect on the ecosystem, which may not be proportional to their abundance because their impact on the community is shaped by their interactions with other members (Berry and Widder, 2014). Specifically, *Sphingomonas* was related to not only the degradation according to KEGG functional analysis but also a keystone taxon in the degradation process based on the results of network analysis. This may be because *Sphingomonas* can catalyze the amide bond hydrolysis reaction, providing a substrate for downstream microorganisms. In addition, there were 4, 4, 2, and 1 unique OTUs in the five networks, respectively.

In the function-taxon correlation network, there were 47 taxon nodes, 49 functional nodes, and 1,593 edges, of which 965 were positively correlated and 628 were negatively correlated (**Figure 7B**). Generally, close ecological interactions, functional differentiation, and metabolite exchange in consortia may allow coexisting species to cycle nutrients efficiently, improve the overall resource utilization efficiency, and acquire robust tolerance and resilience to environmental disturbances (Kato et al., 2008; Bernstein and Carlson, 2012). The attributes of the taxon and function nodes in the networks of all samples are shown in **Supplementary Table S4**. Among all functional nodes, 15 nodes associated with "XBM" ranked first, followed by 8 nodes related to "amino acid metabolism". Among all taxon nodes, seven nodes had a degree distribution higher than 40; three of these were related to "amino acid metabolism". The degree of the six pathways was 33–37. This result indicates that the performance of functions related to CE degradation by MC-L1 requires the collaboration of multiple strains. The synergistic metabolism among strains of the consortium resulted in a higher degradation efficiency compared with that of a single bacterium.

Among all taxon nodes, there were 29 nodes with a degree distribution higher than 40; the highest degree was 46. The degree of correlation between these nodes and "XBM" was 14. *Methyloversatilis* was not only a keystone taxon in the co-occurrence network but also extremely important in the function-taxon correlation network. In addition, as a dominant genus, *Methyloversatilis* was identified in K–W test analysis as a possible degrading genus. Interestingly, some bacteria such as *Afipia*, *Agrobacterium*, *Shinella*, and *Pseudacidovorax* which have not been predicted to be associated with degradation, showed a high degree. This indicates that the degradation of CE in

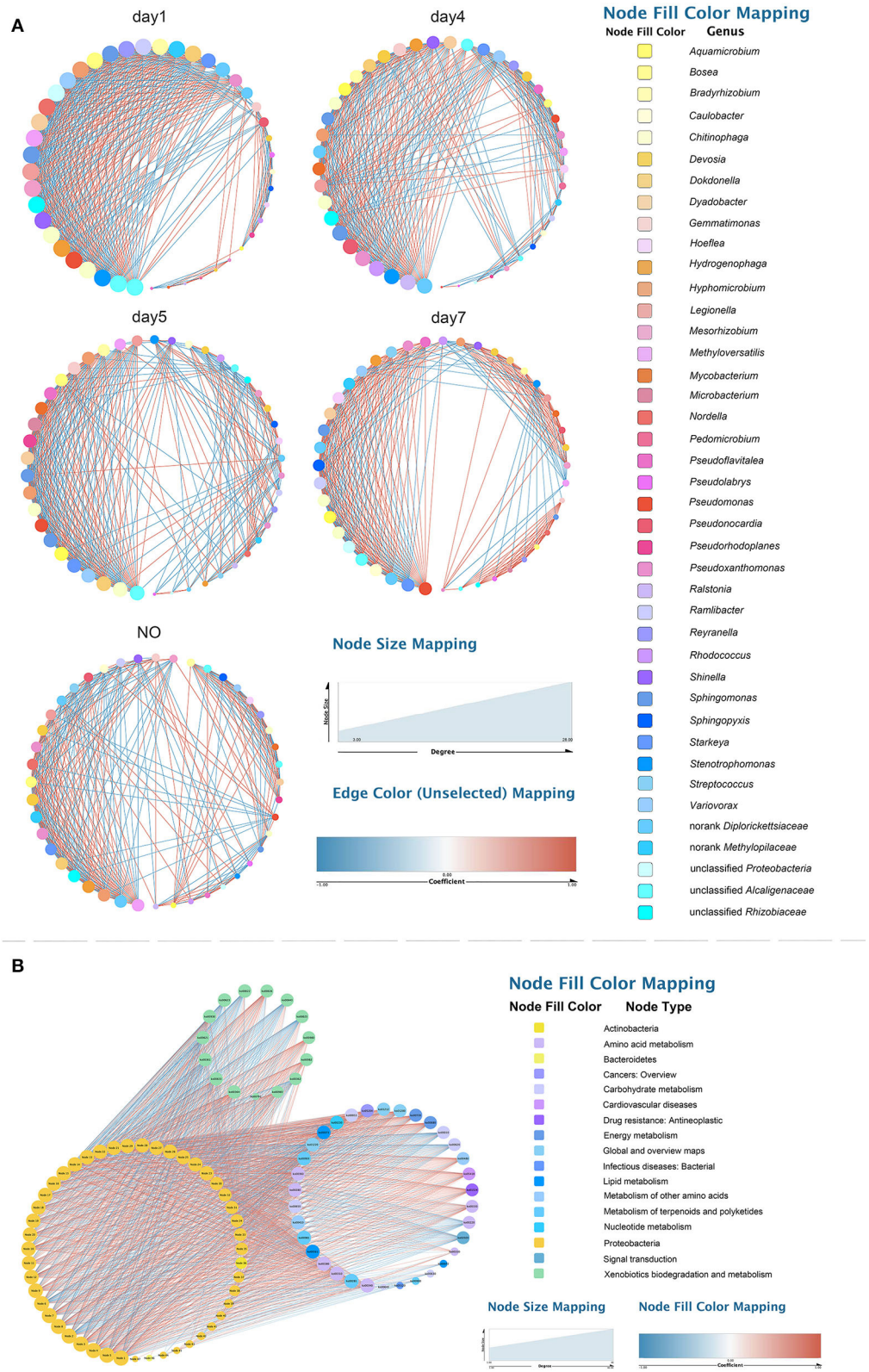

FIGURE 7 | Networks of the microbial consortium in different time points and incapable consortium **(A)** co-occurrence network analysis; **(B)** species/function correlation network. Node size is proportional to node degree. Node colors represent various phylogenetic genera. Red lines indicate positive interactions, and blue lines indicate negative interactions.

TABLE 1 | Attribute table of five co-occurrence networks.

Sample	Node	Edge	Positive edge	Negative edge	Transitivity	Avg. shortest path length	Avg. degree	Keystone numbers
Day 1	45	457	226	231	0.90402534	1.96969697	20.31	28
Day 4	45	384	190	194	0.81281407	2.22828283	17.07	26
Day 5	46	351	176	175	0.84092098	2.29178744	15.26	19
Day 7	44	321	218	103	0.85828877	2.23890063	14.59	10
No	44	311	163	148	0.80847688	2.36363636	14.14	6

MC-L1 requires not only "functional bacteria" with degradability (showing a direct effect on hydrocarbons) but also "auxiliary bacteria" without degradability but that promote/inhibit the degradation process *via* synergistic growth and metabolism. The abundance of "auxiliary bacteria" must be controlled within a suitable range. *Pseudoxanthomonas*, *Stenotrophomonas*, and *Sphingobium* (*Sphingomonas*), which were considered degrading bacterial strains according to KEGG analysis, showed had a high degree, specifically in the degree centrality, closeness, and betweenness centrality.

In summary, network analysis provides information on co-occurrence and functions, providing a foundation for analyzing the interactions between bacteria. Further studies should focus on the isolation and cultivation of these strains, based on which the construction, characterization, and modeling of artificial synthetic microbial communities can be performed to further improve the degradation efficiency and range of degradable substrates of the new synthetic consortium for application in the remediation of herbicide contamination.

CONCLUSION

In this study, we obtained the novel microbial consortium L1 using enrichment culture. The microbial consortium L1 degraded 98.04% of 100 mg L^{-1} CE within 6 days, which is superior to all CE consortia reported to date. In addition, 16S rRNA high-throughput sequencing and metagenomic sequencing were performed to comprehensively characterize changes in the diversity and structural and functional interactions of microbial consortium L1 during degradation and to predict the metabolic enzymes, pathways, and degrading genera of the chlorimuron-degrading consortium. These findings provide insight for further exploration of new microbial resources that can degrade sulfonylurea herbicides and a theoretical basis for the rational design of optimized artificial consortium models to investigate the mechanisms of microbial community formation and connection between the microbial community structure and ecological functions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: NCBI Sequence Read Archive (SRA); PRJNA788363 and PRJNA788073.

AUTHOR CONTRIBUTIONS

XiaL performed the experiments, analyzed the data, and wrote the manuscript. CL, YD, and ZY performed the experiments. XinL, XW, and ZS assisted the manuscript checking. WG, XuL, and TL helped to modify the graphs. MX provided assistance and guidance throughout the research. HZ designed the experiments and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Mechanism and kinetics of chlorpyrifos co-metabolism by using environment restoring microbes isolated from rhizosphere of horticultural crops under subtropics

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The indiscriminate use of organophosphate insecticide chlorpyrifos in agricultural crops causes significant soil and water pollution and poses a serious threat to the global community. In this study, a microbial consortium ERM C-1 containing bacterial strains *Pseudomonas putida* T7, *Pseudomonas aeruginosa* M2, *Klebsiella pneumoniae* M6, and a fungal strain *Aspergillus terreus* TF1 was developed for the effective degradation of chlorpyrifos. Results revealed that microbial strains were not only utilizing chlorpyrifos (500 mg L^{-1}) but also coupled with plant growth-promoting characteristics and laccase production. PGP traits, that is, IAA (35.53, 45.53, 25.19, and 25.53 μg mL^{-1}), HCN (19.85, 17.85, 12.18, and 9.85 μg mL^{-1}), and ammonium (14.73, 16.73, 8.05, and 10.87 μg mL^{-1}) production, and potassium (49.53, 66.72, 46.14, and 52.72 μg mL^{-1}), phosphate (52.37, 63.89, 33.33, and 71.89 μg mL^{-1}), and zinc (29.75, 49.75, 49.12, and 57.75 μg mL^{-1}) solubilization tests were positive for microbial strains T7, M2, M6, and TF1, respectively. The laccase activity by ERM C-1 was estimated as 37.53, 57.16, and 87.57 enzyme U mL^{-1} after 5, 10, and 15 days of incubation, respectively. Chlorpyrifos degradation was associated with ERM C-1 and laccase activity, and the degree of enzyme activity was higher in the consortium than in individual strains. The biodegradation study with developed consortium ERM C-1 showed a decreased chlorpyrifos concentration from the 7th day of incubation (65.77% degradation) followed by complete disappearance (100% degradation) after the 30th day of incubation in the MS medium. First-order degradation kinetics with a linear model revealed a high k^{-day} value and low $t_{1/2}$ value in ERM C-1. The results of HPLC and GC-MS analysis proved that consortium ERM C-1 was capable of completely removing chlorpyrifos by co-metabolism mechanism.

KEYWORDS

chlorpyrifos, biodegradation, plant growth promotion, consortium, ERM C-1

Introduction

Chlorpyrifos, an acetylcholinesterase inhibitor, causes neurological diseases in sensitive groups, particularly youngsters and the elderly population (Farkhondeh et al., 2020; Huang et al., 2021; Rahman et al., 2021). Chlorpyrifos pollution in the environment, including soil, air, and water, has been extensively investigated due to increased usage and greater diffusion impacting even unanticipated sites (Bhatt et al., 2021; Farhan et al., 2021; Huang et al., 2021; Kumar et al., 2021; Rahman et al., 2021). The half-life of chlorpyrifos in the soil is generally 60–120 days, while it can range from 15 to over 365 days depending on the soil type and other environmental factors (Kumar et al., 2021). In comparison to other approaches, such as physical and chemical remediation, biodegradation of chlorpyrifos in the soil is usually regarded as a low-cost, environmentally friendly, and less energy-demanding biotechnological strategy with a high benefit-to-cost ratio for cleaning up the polluted soil ecosystems (Bhatt et al., 2021; Elshikh et al., 2021; Kumar et al., 2021).

Microorganisms having the ability to degrade chlorpyrifos have been reported mostly from polluted environments. The most common pesticide-degrading microbes described in the literature are *Bacillus*, *Burkholderia*, *Rhodococcus*, *K. pneumoniae*, and *Pseudomonas* sp.; however, *Pseudomonas* sp. has the most reports for the degradation of aromatic compounds (Bhatt et al., 2021; Farhan et al., 2021; Huang et al., 2021; Kumar et al., 2021; Rahman et al., 2021). Recently, Chishti et al. (2021) tested chlorpyrifos degradation in different soil-slurry systems using *Enterobacter* sp. SWLC2 strains. Similarly, Uniyal et al. (2021) carried out a microcosm study of chlorpyrifos biodegradation using rhizobacterial consortium ECO-M on apple. Lin et al. (2022) have developed a bacterial consortium, ZQ01, for the effective degradation of acephate and their toxic intermediate product methamidophos.

Elshikh et al. (2021) and Kumar et al. (2021c) have reported that *Bacillus cereus* and *Klebsiella pneumoniae* were the most prominent biodegraders of chlorpyrifos in submerged fermentation and in the soil, respectively. Apart from bacteria, fungal strains have also been reported for chlorpyrifos degradation individually and in combination. A recent report has suggested the application of fungal strains *Byssochlamys spectabilis* C1 and *Aspergillus fumigatus* C3 for chlorpyrifos degradation in liquid CDM and in the soil (Kumar et al., 2021). Furthermore, chlorpyrifos metabolism by microorganisms is known to be a three-step process in which chlorpyrifos is first converted into a bioavailable form by reducing the surface tension of the pesticide with the help of biosurfactants produced by bacterial strains (Kumar et al., 2015). These surface tension-reducing microorganisms have the property to enhance the bioavailability of hydrophobic pesticides for microbial activity. As pesticides become bioavailable, microbes further secrete other catabolic enzymes and couple with sugar or amino acid molecules (dechlorination), and finally, get converted into non-toxic compounds like carbon dioxide and water. Several catabolic enzymes like dioxygenases, hydrolases/esterases, glutathione S-transferases (GSTs), and cytochrome P450 take part in the complete metabolism of chlorpyrifos. Additionally, the mixed-function of GSTs with oxidases is involved in the second metabolic phase (Foong et al., 2020; Ambreen and Yasmin, 2021; Aswathi et al., 2021; Nandhini et al., 2021; Soares et al., 2021; Uniyal et al., 2021). Phosphotriesterases (PTE), organophosphate hydrolases (OPH), methyl parathion hydrolase (MPH), and chlorpyrifos hydrolase (CPH) are the most often described enzymes for chlorpyrifos biodegradation (Zhao et al., 2021; Bhende et al., 2022).

Numerous investigations on chlorpyrifos degradation using pure microbial cultures have been carried out in recent years (Gaonkar et al., 2019; Farhan et al., 2021). However, only a few microbial strains have been identified that can effectively degrade chlorpyrifos in both aqueous and soil environments (Ambreen and Yasmin, 2021; Kumar et al., 2021; Uniyal et al., 2021). Furthermore, the application of single culture or artificial consortia as a remediating agent has not been found to be the most appropriate approach in the bioremediation process occurring in the natural environment, as it occurs in real nature and depends on the collaborative metabolic functions of diversified indigenous microbial communities (Kumar et al., 2021b).

An efficient degradation system can be established by developing a consortium of distinct domain microorganisms isolated from a real polluted environment with the competence to utilize the chemical of interest as the carbon source due to their ability for synergistic metabolism. With the understanding of knowledge aforesaid, the effort has been made to develop the microbial consortia among two different domain microbes for the effective remediation of chlorpyrifos from subtropical agriculture land, which is not possible by individual single microorganisms.

Materials and methods

Chemicals

Analytical grade Chlorpyrifos (purity 99.9%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, United States). Other chemicals used in this study were also analytical grade quality and purchased from Merck, India. Media used were purchased from Hi-Media Laboratories, Mumbai, India. Mineral salt medium (stocktickerMSM) used in this study contained (g L^{-1}): $(NH_4)_2SO_4$ 2.0, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.01, $FeSO_4.7H_2O$ 0.001, $Na_2HPO_4.12H_2O$ 1.5, and KH_2PO_4 2.0. The stock solution of chlorpyrifos (2150 g L^{-1}) and 3, 5, 6-trichloro-2-pyridinol (100 mg L^{-1}) was prepared in a mixture of acetone + n-hexane (50:50) and passed through 0.22-μm syringe filters.

Sampling, isolation, and characterization of chlorpyrifos-degrading microorganisms

For sampling, a survey was conducted to determine the pesticide application in subtropical regions. Two locations, ICAR-CISH farm (26° 45′ - 27° 10′ N, 80° 30′–80° 55′ E) and progressive farmers field of Malihabad region (26° 91′–27° 21′ N, 80° 70′–80° 95′ E), were selected on the basis of frequent pesticide applications. Nine rhizosphere soil samples were collected from three different crops, namely, *Solanum lycopersicum*, *Solanum melongena,* and *Capsicum annum* (bell pepper) plant. Twenty grams of soil sample was transferred into a 250 mL Erlenmeyer flask containing 50 mL of MS medium amended with 100 mg L^{-1} of chlorpyrifos. The flasks were incubated at 30 ± 2°C with shaking at 110 rpm. After 5 days of incubation, 5 mL (pioneer acclimatized) of culture aliquot was transferred to a freshly prepared MS medium containing (100 mg L^{-1}) chlorpyrifos. The flasks were again incubated for 5 days under the previously mentioned conditions. By following the above-mentioned method, enriched culture was shifted periodically into the liquid MS medium with increasing concentrations of chlorpyrifos up to 200, 300, 400, and 500 mg L^{-1} over 5 following days at 30 ± 2°C temperature and 100 rpm shaking. Five successive transfers were performed to screen for potent chlorpyrifos-degrading strains. From the last flask, 1 mL of culture was serially diluted, and 100 μL was spread on solid Luria-Bertani (LB) agar and potato dextrose agar plate amended with 500 mg L^{-1} chlorpyrifos for bacterial and fungal growth, respectively. Furthermore, plates were incubated at 30 ± 2°C for bacterial growth and 28 ± 2°C for fungal growth. The grown colonies with uniform morphologies were purified on solid agar plates. Fungal and bacterial colonies with a high proclivity for tolerating chlorpyrifos toxicity were chosen for further experiments. Four microorganisms (three bacterial strains T7, M2, and M6, and one fungal strain TF1), capable of growing at maximum chlorpyrifos concentration (500 mg L^{-1}), were selected for further characterization. Selected microbes were identified through ribotyping by amplifying the 16S rRNA gene for bacterial strains using forward 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1392R (5′-GGTTACCTTGTTACGACTT-3′) primers, while for fungal strain, ITS regions were amplified using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-GCTGCGTTCTTCATCGATGC-3′), respectively. The amplified genes were sequenced using Sanger dideoxy sequencing method. The obtained sequences were submitted to the NCBI Genbank database[1] under the accession numbers MW172266, MW228078, MW228061, and MZ268151, respectively. The phylogenetic analysis was performed using MEGA version 5.2 software. After the BLAST analysis, the FASTA form of a sequence of most similar organisms along with nearest-neighbor sequences from the NCBI database was downloaded. Apart from this, one analog sequence of other genera was also taken for the out-group purpose. The downloaded sequences were aligned by the inbuilt ClustalW alignment tool of MEGA version 5.2 software.

The evolutionary history was inferred by using the maximum likelihood method and the General Time-Reversible Model with bootstrap replications of 1,000 (Nei and Kumar, 2000). The tree with the highest log-likelihood value is shown in **Supplementary Figures 1–3**. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with a superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Plant growth-promoting attributes of chlorpyrifos-degrading microorganisms

The selected microbes were tested for plant growth-promoting attributes qualitatively and quantitatively following the standard methods described by previous researchers. Phosphate solubilization was assessed by the method of Nautiyal (1999), while Zn solubilizing activity was tested following the method of Mumtaz et al. (2017). K solubilization was assessed by the method described by Bagyalakshmi et al. (2017), HCN production by Lorck (1948), and ammonia production by Cappuccino and Sherman (2005). Moreover, IAA production was checked by following the method described by Bric et al. (1991) on their respective media. All the tests were performed in triplicates.

Laccases production by chlorpyrifos-degrading microorganisms

In order to test the activity of laccases, Luria-Bertani agar plates supplemented with guaiacol (0.01% w/v) as a substrate for laccase and 0.35 mM $CuSO_4$ were inoculated with the selected isolates. Plates were incubated at 30 ± 2°C for 24 h, and the development of yellow to brown colored zones around the colonies indicates a positive test. Furthermore, quantitative estimation of the activity of laccases was done as per the method described by Abd El Monssef et al. (2016) which was later

1 www.ncbi.nlm.nih.gov

modified by Kumar et al. (2021). Briefly, fungal and bacterial strains were grown separately in their respective liquid media, and the supernatant was reacted with sodium acetate buffer (10 mM, pH 4.5), guaiacol (1 mM), and $CuSO_4$ (2 mM). Blank with water served as control. The optical density in terms of absorbance of the reaction mixture was measured at 410 nm with the help of a spectrophotometer (Evolution 201, Thermo Fisher Scientific, Waltham, MA, United States). The activity of laccases was estimated (U mL^{-1}) using the following equation:

$$E.A = A \times \frac{V}{t \times \varepsilon \times v} \quad (1)$$

where E.A is the enzyme activity in units per mL, A is the absorbance at 410 nm, V is the total mixture volume (mL), v is the enzyme volume (mL), t is the incubation time (min), and ε is the extinction coefficient of guaiacol (0.674 μm cm^{-1}).

Screening of biosurfactant production by chlorpyrifos-degrading microbes

Chlorpyrifos-degrading microorganisms (T7, M2, M6, and TF1) were evaluated for biosurfactant production using liquid MSM fed with glucose 2% (w/v) and yeast extract 0.25% (w/v). It was assumed that biosurfactants contribute to the increase of the aqueous phase partitioning of chlorpyrifos, resulting in increased chlorpyrifos bioavailability for microbial activity.

Emulsification activity (E_{24})

The E_{24} activity was checked by homogenizing 2 mL of chlorpyrifos (21,500 g L^{-1}) and 2 mL of cell-free supernatant followed by high-speed vortexing for 2 min. The emulsification activity after 24 h was determined using the following formula:

$$E_{24}\ (\%) = \frac{\text{Total height of the emulsified layer (cm)}}{\text{Total height of the liquid layer (cm)}} \times 100$$

Foaming test

The foaming test was performed by following the method of Abouseoud et al. (2008), which was later modified by Ratna and Kumar (2022). The production of foam by 96-h old culture, when shaken gently for 2 min, indicated positive results for foam production. The stability of the foam was monitored by observing it for 2 h.

Drop-collapse test

The test was performed by following the qualitative drop-collapse test described by Bodour and Miller-Maier (1998). Chlorpyrifos concentration of 21,500 gL^{-1} was used for this study. About 2 μL of chlorpyrifos was inoculated into 96-well microplates and allowed to equilibrate for 24 h. After 48 h, 2 μL of culture supernatant was transferred to the chlorpyrifos-coated well sections, and the drop size was measured using a magnifying glass after 1 min. A flat drop was considered as a positive result for biosurfactant production, while rounded drops were regarded as negative results, indicating a lack of biosurfactant production.

Development of microbial consortium for chlorpyrifos degradation

The consortium was developed based on the compatibility test between the microbes. Two methods, dual culture plate and crowded plate assay, were followed for the compatibility test (Muniaraj et al., 2008; Xu and Kim, 2014). Microbes showing compatible growth were used to develop the microbial consortium. Three bacterial strains (*Pseudomonas putida* T7, *Pseudomonas aeruginosa* M2, and *Klebsiella pneumoniae* M6) and one fungal strain (*Aspergillus terreus* TF1) were combined to develop a consortium and named as environment restoring microbes (ERM C-1). For this, bacterial and fungal strains were individually inoculated in a liquid nutrient broth medium, and flasks were incubated at 30 ± 2°C for 72 h. Furthermore, cell biomass was collected after centrifugation at 12,879 g for 5 min. The cell pellets were washed two times with sterile distilled water and finally suspended in sterile saline solution (0.9% NaCl). The cell density (absorbance) was maintained at 1.0 OD (600 nm) with the help of a spectrophotometer (Evolution 201, Thermo Fisher Scientific United States). It was assumed that both domain microbes (bacteria and fungi) were maintained in equal quantity while preparing the consortium (Uniyal et al., 2021).

Biodegradation study of chlorpyrifos in liquid MS medium, and natural and sterile soil system

For chlorpyrifos biodegradation, a lab-scale batch experiment was performed in 250 mL Erlenmeyer flasks containing 100 mL MSM amended with 500 mg L^{-1} chlorpyrifos. Selected strains were inoculated individually and with the developed consortium. Flasks without inoculated microbes served as control. Furthermore, flasks were wrapped with brown paper to evade photolysis and were incubated in a rotatory shaker at 30 ± 2°C and 120 rpm speed. About 20 mL of culture aliquots were withdrawn from every flask at 7-, 15-, and 30-day intervals, and the supernatant was used for the extraction of residual chlorpyrifos and further measured using HPLC (Nexer-R, SIL-30ACMP, Shimadzu Japan) and GC-MS (TQ 8050 Nexis Shimadzu Japan) techniques. The bacterial growth was measured at 600 nm, while the fungal growth was

measured at 310 nm with the help of a spectrophotometer (Abdul Manan and Webb, 2018).

For biodegradation of chlorpyrifos in sterile and natural soil system, 250 g of agriculture soil was filled in sterile plastic-wrapped paper pots. Chlorpyrifos (500 mg kg^{-1}) was amended to the soil (sterile and natural) and mixed thoroughly to ensure a uniform concentration of chlorpyrifos. Individual strains and developed consortium (Section Screening of biosurfactant production of chlorpyrifos degrading microbes) were inoculated at a concentration of 1×10^8 cfu g^{-1} into chlorpyrifos-spiked soils. The sterile soil pots were wrapped with 100 mm of clinging plastic wrap to avoid environmental contamination. The samples (20 g) were recovered in triplicates at 7-, 15-, and 30-day intervals and extracted with acetonitrile. The chlorpyrifos residue was analyzed with the help of HPLC and GC-MS (Bhatt et al., 2021).

Kinetics of chlorpyrifos biodegradation

In order to estimate biodegradation kinetics in different systems, first-order degradation kinetics was applied to the experimental data. The first-order degradation equation is mentioned below:

$$Ct = C_0 e^{-kt} \quad (2)$$

where Log "C" (chlorpyrifos residue in the particular medium) is calculated against time "t" to determine the "k" value, while "C_0" denotes the initial concentration of chlorpyrifos in different systems (MSM, SS, and NS). "Ct" represents chlorpyrifos concentration at reaction time "t," and "k" represents the constant rate of chlorpyrifos degradation day^{-1}. For the estimation of the half-life of chlorpyrifos biodegradation in different systems, a graph was plotted between time "t" and constant "k." The equation for the half-life is as below:

$$t_{1/2} = \frac{Ln2}{k} \quad (3)$$

Soil enzyme dynamics of chlorpyrifos-treated soils

Two soil enzymes, dehydrogenase (DHA) and fluorescein diacetate (FDA), directly related to soil microbial activities were assessed in chlorpyrifos-treated soils. The DHA activity was tested by following the standard protocol reported by Casida et al. (1964), while FDA activity was checked by the method developed by Schnrer and Rosswall (1982), later modified by Adam and Duncan (2001). Dehydrogenase activity is normally found in soil as part of the oxidative reactions that occur within live cells, and hence its measurement represents only intracellular enzyme activity, making it a good indication of microbial activity. However, the hydrolysis activity of fluorescein diacetate is a non-specific assay in which lipase, esterase, and protease classes of enzymes hydrolyze fluorescein by cleaving the ring, so it was assumed that they may also take part in the cleavage of bonds present in chlorpyrifos ring.

Statistical analysis

For the kinetic study of chlorpyrifos biodegradation, a statistical analysis software package (Origin Pro 2018b, MA, United States) was used. Three replicates of each sample were used in the statistical analysis of the biodegradation data. The data were also validated by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) to compare the mean values. The IBM-SPSS program (version 25, IBM, New York, NY, United States) was used to perform a *post-hoc* DMRT test analysis. Statistical significance was calculated using the lowest significance differences (LSDs) at $P \leq 0.05$ to analyze the differences among treatments.

Results

Isolation and characterization of chlorpyrifos-degrading microbes

In this study, a sum of 21 different bacterial and four fungal isolates was screened from nine different samples at the primary stage in the presence of 100 mg L^{-1} of chlorpyrifos amended medium. Further screening was done on the basis of the growth of microbes at a maximum concentration (500 mg L^{-1}) of chlorpyrifos and plant growth-promoting traits. Results revealed that out of 21 bacterial and four fungal isolates, three bacterial strains (T7, M2, and M6) and one fungal strain (TF1) were capable of growing at the maximum concentration (500 mg L^{-1}) of chlorpyrifos and showed multiple plant growth-promoting characteristics. Apart from this, all these isolates (T7, M2, M6, and TF1) were also tested for growth in the presence of 250 ppm of TCP. Observed results showed a luxuriant growth of all the four isolates in the presence of TCP amended medium. Finally, identification of the strains, including both bacteria and fungi, was carried out using 16S rRNA and ITS molecular approaches, respectively. The molecular identification through homology searching and BLAST analysis (**Table 1**) revealed that the isolated bacterial strains, that is, M2 and T7, belonged to *Pseudomonas,* while strain M6 belonged to *Klebsiella* genera and fungal strain TF1 belonged to *Aspergillus* genera. The phylogenetic position of these strains with other related organisms has been depicted in **supplementary files** (**1, 2** and **3**).

TABLE 1 Homology search of isolated Plant growth promoting rhizobacteria (PGPRs).

Isolated PGPRs strains	Identification	GenBank accession no.	Similar organism	Accession number	Sequence similarity (%)
T7	*Pseudomonas putida*	MW172266	*Pseudomonas putida*	DQ112329	97%
M2	*Pseudomonas aeruginosa*	MW228078	*Pseudomonas aeruginosa*	OK668300	97%
M6	*Klebsiella pneumoniae*	MW228061	*Klebsiella pneumoniae*	MT102629	97%
TF-1	*Aspergillus terreus*	MZ 268151	*Aspergillus terreus*	KM401402	96.84%

Plant growth-promoting characteristics of selected strains

To test the plant growth-promoting characteristics, the selected strains were grown on a specific medium to detect the positive characteristics. Results revealed that strains T7, M2, M6, and TF1 showed positive results for phosphate solubilization, potassium solubilization, zinc solubilization, IAA production, HCN production, and ammonia production. Furthermore, quantitative estimation revealed that T7 solubilized 52.37 ± 2, 49.53 ± 2, and 29.75 ± 2 μg mL^{-1} of phosphate, potassium, and zinc, respectively, while producing 35.53 ± 2, 19.85 ± 2, and 14.73 ± 2 μg mL^{-1} of IAA, HCN, and ammonium, respectively. Similarly, M2 solubilized 63.89 ± 2, 66.72 ± 2, and 49.75 ± 2 μg mL^{-1} of phosphate, potassium, and zinc, respectively, and produced 45.33 ± 2, 17.30 ± 2, and 16.37 ± 2 μg mL^{-1} of IAA, HCN, and ammonium, respectively. Strain M6 solubilized 33.33 μg mL^{-1} of phosphate, 46.14 ± 2 μg mL^{-1} of potassium, and 49.12 ± 2 μg mL^{-1} of zinc in an aqueous medium, while 25.19 ± 2 μg mL^{-1} of IAA, 12.18 ± 2 μg mL^{-1} of HCN, and 8.05 ± 2 μg mL^{-1} of ammonium were produced. Moreover, fungal strain TF1 showed positive PGP characteristics and solubilized 71.89 ± 2, 52.72 ± 2, and 57.75 ± 2 μg mL^{-1} of phosphate, potassium, and zinc, respectively, and 23.53 ± 2, 9.85 ± 2, and 10.87 ± 2 μg mL^{-1} of IAA, HCN, and ammonium were produced, respectively (**Figure 1**).

Estimation of laccase production by selected microorganisms

Microbial enzymes are considered a safe, eco-friendly, and green method for the breakdown of complex substances in water and terrestrial ecosystem. The microbial enzyme tests are employed in the soil to investigate the pattern of degradation of complicated compounds like chemical pesticides. The results revealed that bacterial strains (T7, M2, and M6) showed maximum laccase activity within 10 days of incubation, and later a decrease in enzyme production had been observed. Strains T7, M2, and M6 produced 25.53–52.37, 35.53–66.89, and 21.19–43.33 U mL^{-1} enzyme, respectively, between 5 and 10 days of incubation. However, fungal strain TF1 reached the maximum enzyme production (75.12 U mL^{-1}) after the 15th day of incubation, and thereafter the rate of enzyme production decreased, which might be due to the lack of substrate in the medium or due to the feedback inhibition. Furthermore, enzyme production was greater (87.57 U mL^{-1}) in the consortium ERM C-1 group than in groups treated with individual strains (**Figure 2**).

Biosurfactant production of chlorpyrifos-degrading microorganisms

The results of this study revealed that strains T7 and M2 were positive for emulsification activity (E24), foaming test, and drop-collapse test. It was noted that strains T7 and M2 showed a greater emulsification activity value (54.62 and 56.01%, respectively). Higher emulsification activity, foaming, and drop-collapse test value indicated that strains T7 and M2 could be promising isolates for biosurfactant production.

Biodegradation of chlorpyrifos in different media

Chlorpyrifos biodegradation was performed with developed consortium ERM C-1 and with individual strains in different media (i.e., mineral salt medium, natural soil, and sterile soil) at different time (7, 15, and 30 days) intervals. The results revealed that in the control treatment, 100% chlorpyrifos was available in the MS medium as well as in natural and sterile soil systems at zero days of incubation. However, over the time period, abiotic degradation of chlorpyrifos was observed and recorded as 2.07 ± 0.01, 3.57 ± 0.02, and 5.32 ± 0.02% at 7, 15, and 30 days, respectively. The abiotic degradation of chlorpyrifos

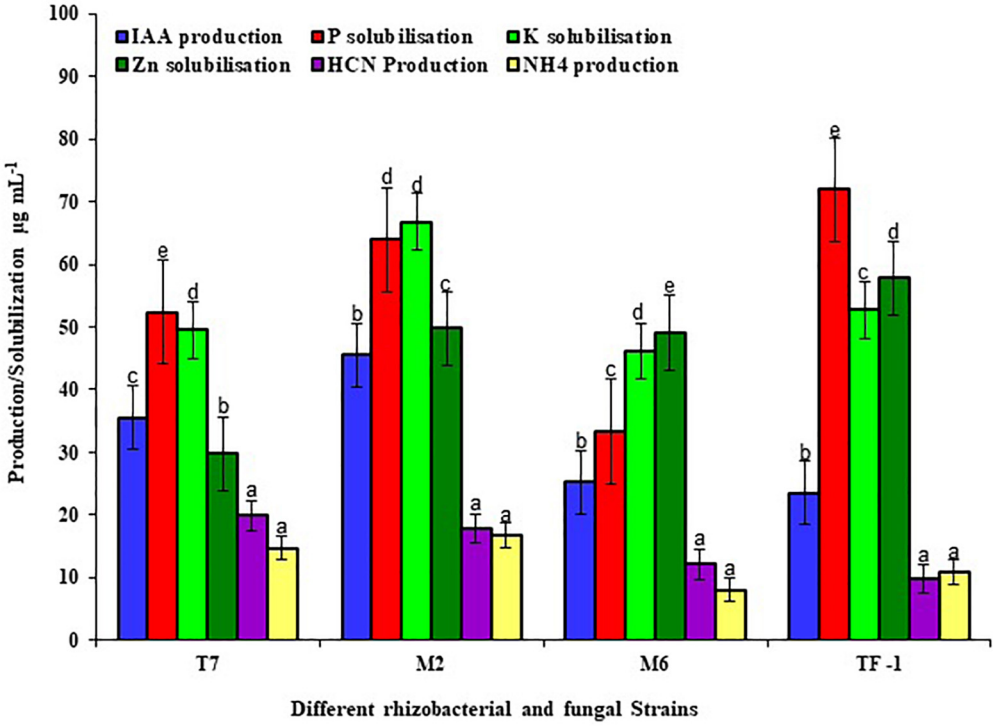

FIGURE 1
Different plant growth-promoting properties of rhizobacterial and fungal strains. The results were the average of five replicates (n = 5). Bars represent standard error. Significant differences based on the analysis variance (ANOVA) are shown by different letters above the error bars, followed by the *post-hoc* DMRT test ($p \leq 0.05$) using the software SPSS.

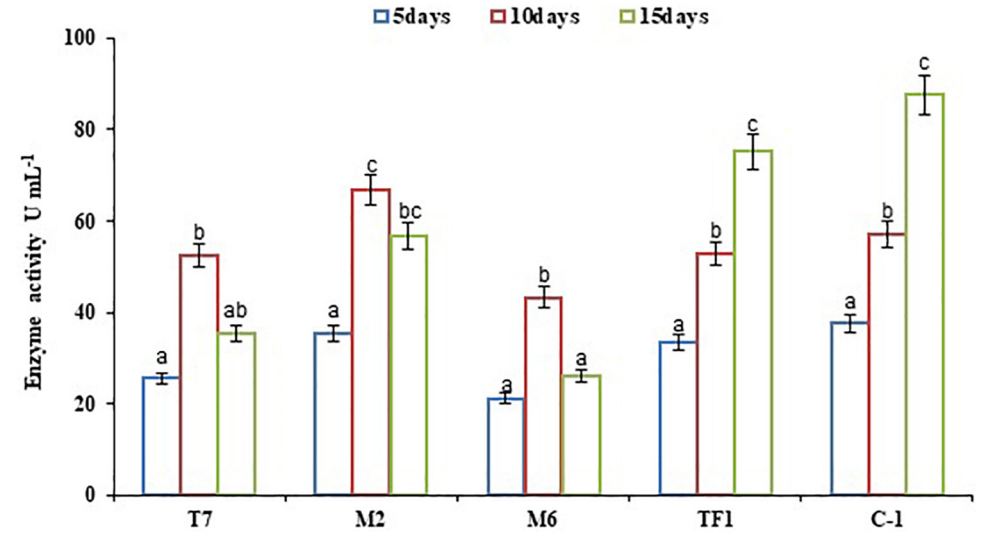

FIGURE 2
Laccase activity by individual strains and developed consortium. The results were the average of five replicates (n = 5). Bars represent the standard error. Significant differences based on the analysis variance (ANOVA) are shown by different letters above the error bars, followed by the *post-hoc* DMRT test ($p \leq 0.05$) using the software SPSS.

was taken into consideration to calculate the overall percentage of chlorpyrifos biodegradation at different treatments. Results showed the decrease in chlorpyrifos concentration (500 mg L^{-1}) was started on the 7th day of incubation, followed by full disappearance on the 30th day of incubation in liquid MS medium with ERM C-1 consortium treatment (**Figure 3**). Results from individual strains revealed that strains T7, M2, M6, and TF1 degraded 95.39 ± 1.05, 96.69 ± 1.21, 91.77 ± 1.11, and 92.97 ± 1.04% of chlorpyrifos, respectively, in liquid MS medium after 30 days of incubation. Furthermore, in different soil systems, 98.58 ± 1.11 and 92.16 ± 0.18% degradation values were noted in natural and sterile soil systems with consortium ERM C-1 treatment, respectively, after 30 days of incubation. The biodegradation patterns of individual strains in natural and sterile soil systems revealed that strain T7 degraded 79.51 ± 0.12 and 90.15 ± 0.27% of chlorpyrifos in sterile and natural soils, respectively, after 30 days of incubation. Strain M2 degraded 82.55 ± 0.22 and 91.89 ± 0.25% of chlorpyrifos in sterile and natural soil systems after 30 days of incubation. In the case of strains M6 and TF1, 65.31 ± 0.11 and 77.72 ± 0.16% values of chlorpyrifos degradation were recorded in the sterile soil system after 30 days of incubation, respectively. In the natural soil system, strains M6 and TF1 degraded 79.25 ± 0.13 and 83.15 ± 0.19%, respectively, at 30 days of incubation (**Figure 3**). The observed results clearly highlighted the ability of all the four strains to utilize chlorpyrifos as sole carbon and phosphorous source for their growth and metabolism. Moreover, GC-MS analysis of chlorpyrifos biodegradation in MS medium, sterile soil, and natural soil detected different intermediate compounds during the biodegradation process. The GC-MS analysis also revealed there was no TCP formation during the biodegradation of chlorpyrifos in any treatment after 30 days of incubation.

Biodegradation kinetics of chlorpyrifos in different media

The degradation kinetics of chlorpyrifos was assessed in three different media: MS medium, sterile soil, and natural soil systems. The degradation constant (k day^{-1}) and half-life ($t_{1/2}$) of chlorpyrifos in different media with different treatments (T7, M2, M6, TF1, and ERM C-1) were determined with the help of first-order biodegradation kinetics and linear model. The recorded results are depicted in **Table 2**. The theoretical half-life ($t_{1/2}$) of chlorpyrifos in the MS medium with control treatment was 229 days, while it was calculated as 3.5 days with consortium ERM C-1 in the MS medium. Furthermore, in natural and sterile soil systems, the half-life of chlorpyrifos was determined at 121 and 139 days with control treatment, respectively. However, soil treated with ERM C-1 recorded a half-life of 12 and 17 days in natural and sterile soil systems, respectively.

Soil enzyme dynamics of chlorpyrifos-treated soils

Two soil enzymes (DHA and FDA) were estimated in chlorpyrifos contaminated soil. Results revealed that chlorpyrifos decreased the soil DHA and FDA activity when compared to the control (natural) soil. Furthermore, it was observed that when chlorpyrifos contaminated soil was treated with different microbial strains (T7, M2, M6, and TF1) and developed consortium (ERM C-1), the activity of DHA and FDA was significantly ($\leq$0.05) increased (**Figures 4A,B**). The increase in DHA activity when compared to their respective controls was 52.07 ± 0.12 and 27.66 ± 0.11% when treated with consortium ERM C-1 in natural and sterile soils, respectively. In the case of FDA, 72.09 ± 0.27 and 71.34 ± 0.25% increase in the activity was observed in natural and sterile soils, respectively, with ERM C-1 treatment when compared to the corresponding control. The observed results clearly indicated that chlorpyrifos-degrading microbial consortium ERM C-1 significantly increased the soil enzyme activity.

Heat map analysis of different biodegradation patterns of chlorpyrifos contaminated soil

To analyze the degradation pattern in different media with different treatments, a heat map with clustal correlation analysis was performed. Results revealed that five different groups (ERM C-1, T7, M2, M6, and TF1) were constructed representing each treatment with different media (MSM, NS, and SS) at different incubation periods (7, 15, and 30 days). It was observed that when chlorpyrifos was treated with different strains and ERM C-1 in the MS medium and incubated for 30 days, the degradation pattern was ERM C-1>TF1>T7>M2>M6. Furthermore, the complete disappearance of chlorpyrifos with ERM C-1 treatment was observed in the MS medium after 30 days of incubation (**Figure 5**), while in the case of natural soil, the chlorpyrifos degradation pattern was ERM C-1>T7>M2>TF1>M6 after 30 days of incubation. In sterile soil, chlorpyrifos degradation pattern was observed as ERM C-1>M2>T7>TF1>M6 after 30 days of incubation. Consortium ERM C-1 was found to be most effective in chlorpyrifos degradation in both natural and sterile soil treatments after 30 days of incubation.

Discussion

Chlorpyrifos is an organophosphate insecticide that is commonly used to control pests in vegetable and fruit crops (Raj et al., 2021). The increased use of this pesticide poses a serious impact on the ecosystem. As a result, reclamation

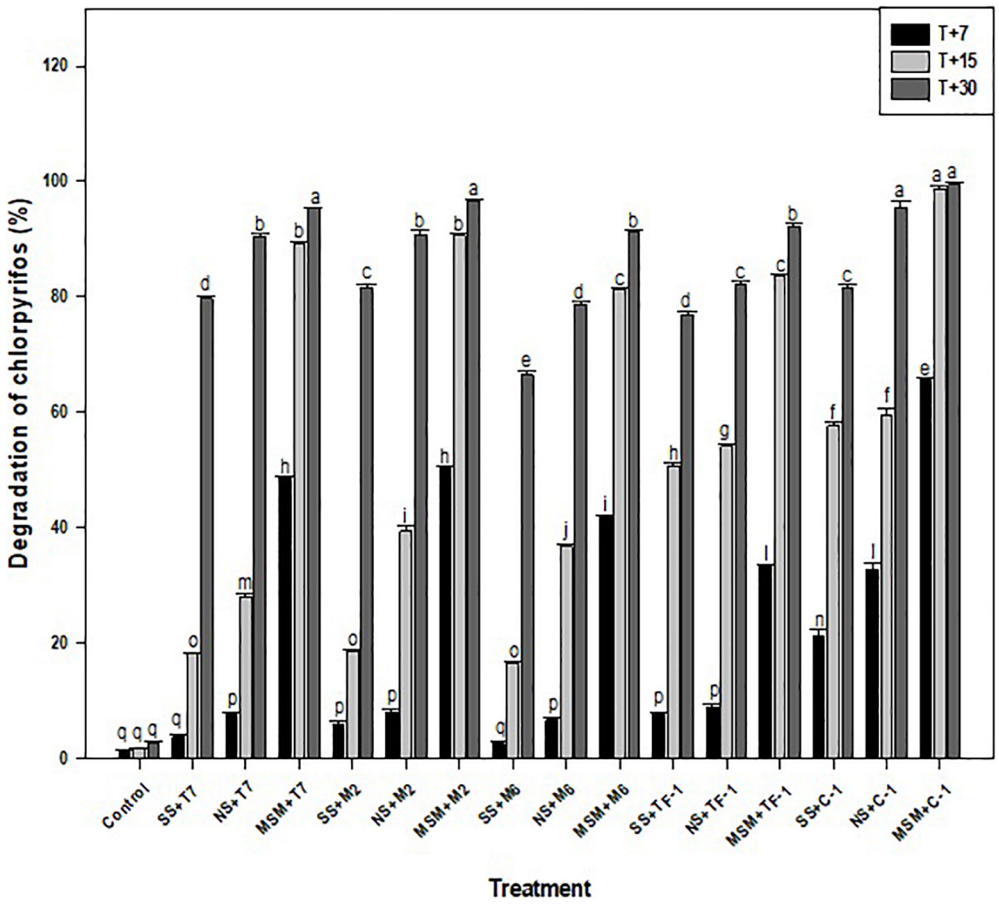

FIGURE 3
Chlorpyrifos biodegradation in different media at different time intervals. Data were the average of five replicates (n = 5). Bars represent standard error. Significant differences based on the analysis variance (ANOVA) are shown by different letters above the error bars, followed by the *post-hoc* DMRT test ($p \leq 0.05$) using the software SPSS.

of highly polluted arable land is urgently required (Bose et al., 2021). Physical and chemical procedures are being employed to detoxify numerous locations across the world, but they are costly and contaminate the same areas with secondary contaminants (Huang et al., 2021). In contrast, microbiological techniques are inexpensive, environmentally benign, and economically sound (Uniyal et al., 2021). Earlier studies of microbial chlorpyrifos remediation revealed that rhizosphere microbes are the most willing bio-agents because they produce extracellular enzymes that can participate in the bioremediation of various recalcitrant/xenobiotic compounds and support bacterial and fungal survival by providing essential nutrients (Bose et al., 2021; Raj et al., 2021; Uniyal et al., 2021). In this study, 21 chlorpyrifos utilizing/resistant bacteria and four fungal isolates were recovered from the agricultural soils contaminated by different pesticides. Furthermore, three bacterial and one fungal strain were thriving at 500 mg L^{-1} of chlorpyrifos concentration and showed great plant growth promotion and laccase activity in the presence of chlorpyrifos. Recently, Kumar et al. (2021c) reported the use of *Klebsiella pneumoniae* M11 for chlorpyrifos biodegradation and plant growth promotion. Ambreen and Yasmin (2021) isolated *Bacillus thuringiensis* MB497 from the agricultural soil which showed resistance to 200 mg L^{-1} concentration of chlorpyrifos and degraded up to 99% of chlorpyrifos in the M9 medium. Similarly, Yadav et al. (2020) reported that *Alcaligenes faecalis* (NBRI OSS2-5) degraded 94% of chlorpyrifos in MS medium at 10 days of incubation and produced exopolysaccharides which have good plant growth-promoting characteristics. Apart from bacteria, fungal strains have also been well-reported for chlorpyrifos degradation and laccase production. Recently, Kumar et al. (2021) reported that two fungal strains (*B. spectabilis* C1 and *A. fumigatus* C3) and their consortium degraded chlorpyrifos up to 96.7, 93.45, and 98.4%, respectively, in Czapek Dox medium (CDM) at 30 days of incubation, and individual strains were also able to produce laccase (2.23 and 1.74 U mL^{-1}, respectively). In this study, three bacterial strains, *P. putida* (T7), *Pseudomonas aeruginosa*

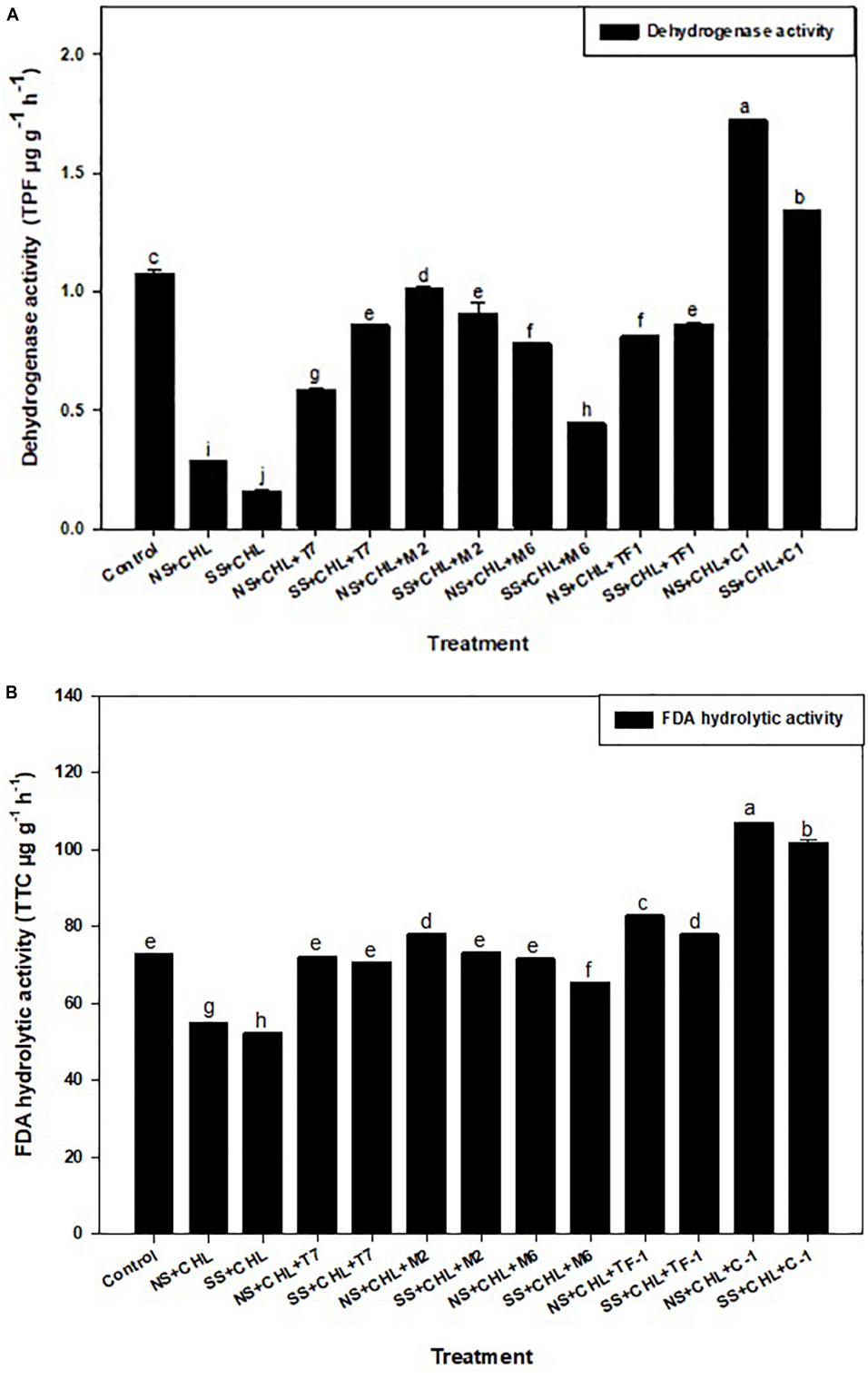

FIGURE 4
(A) Dehydrogenase activity of the soil treated by different strains and developed consortium in sterile and natural conditions. The recorded data were the average of five replicates (n = 5). Bars represent the standard error. Significant differences based on the analysis variance (ANOVA) are shown by different letters above the error bars, followed by the *post-hoc* DMRT test ($p \leq 0.05$) using the software SPSS. **(B)** Fluorescein diacetate (FDA) hydrolysis activity of the soil treated with different strains and developed consortium in sterile and natural conditions. The recorded data were the average of five replicates (n = 5). Bars represent standard error. Significant differences based on the analysis variance (ANOVA) are shown by different letters above the error bars, followed by the *post-hoc* DMRT test ($p \leq 0.05$) using the software SPSS.

TABLE 2 Chlorpyrifos degradation kinetics and half-life ($t_{1/2}$) in natural (NSS), sterile soil (SS), and mineral salt medium (MSM).

Treatments	Regression equation	$k^{(day-1)}$	R^2	$t_{1/2}$ (days)
SS + CHL	$\ln(Ct/C_0) = -0.011x + 4.981$	0.008 ± 0.003^a	0.935 ± 0.003^a	139.233 ± 0.33^f
NSS + CHL	$\ln(Ct/C_0) = -0.012x + 4.977$	0.007 ± 0.002^a	0.929 ± 0.002^a	121.447 ± 0.31^f
MSM + CHL	$\ln(Ct/C_0) = -0.0022x + 4.998$	0.002 ± 0.001^a	0.910 ± 0.003^a	229.753 ± 0.45^g
SS + CHL + T7	$\ln(Ct/C_0) = -0.019x + 4.968$	0.012 ± 0.002^c	0.980 ± 0.001^c	18.131 ± 0.18^d
NSS + CHL + T7	$\ln(Ct/C_0) = -0.018x + 4.955$	0.013 ± 0.002^c	0.981 ± 0.002^c	14.652 ± 0.15^c
MSM + CHL + T7	$\ln(Ct/C_0) = -0.019x + 4.858$	0.014 ± 0.001^d	0.999 ± 0.001^d	5.517 ± 0.04^a
SS + CHL + M2	$\ln(Ct/C_0) = -0.039x + 4.968$	0.013 ± 0.001^c	0.961 ± 0.002^b	16.611 ± 0.12^c
NSS + CHL + M2	$\ln(Ct/C_0) = -0.038x + 4.968$	0.012 ± 0.002^c	0.975 ± 0.006^b	13.174 ± 0.11^c
MSM + CHL + M2	$\ln(Ct/C_0) = -0.039x + 4.858$	0.014 ± 0.002^d	0.985 ± 0.006^c	5.958 ± 0.03^b
SS + CHL + M6	$\ln(Ct/C_0) = -0.013x + 4.945$	0.011 ± 0.003^b	0.991 ± 0.002^d	19.131 ± 0.18^d
NSS + CHL + M6	$\ln(Ct/C_0) = -0.012x + 4.829$	0.012 ± 0.003^c	0.978 ± 0.003^b	14.865 ± 0.12^c
MSM + CHL + M6	$\ln(Ct/C_0) = -0.011x + 4.747$	0.012 ± 0.003^c	0.991 ± 0.001^d	06.517 ± 0.04^b
SS + CHL + TF1	$\ln(Ct/C_0) = -0.049x + 4.878$	0.010 ± 0.004^b	0.970 ± 0.004^b	23.522 ± 0.21^e
NSS + CHL + TF1	$\ln(Ct/C_0) = -0.039x + 4.858$	0.010 ± 0.003^b	0.931 ± 0.005^a	21.747 ± 0.19^e
MSM + CHL + TF1	$\ln(Ct/C_0) = -0.051x + 4.823$	0.011 ± 0.003^b	0.971 ± 0.005^b	06.147 ± 0.07^b
SS + CHL + ERM C-1	$\ln(Ct/C_0) = -0.017x + 4.868$	0.016 ± 0.001^e	0.991 ± 0.001^d	17.678 ± 0.13^d
NSS + CHL + ERM C-1	$\ln(Ct/C_0) = -0.018x + 4.822$	0.018 ± 0.001^e	0.984 ± 0.004^c	12.746 ± 0.10^c
MSM + CHL + ERM C-1	$\ln(Ct/C_0) = -0.016x + 4.629$	0.019 ± 0.001^e	0.997 ± 0.001^d	03.513 ± 0.02^a

^{a,b,c,d,e,f,g}Indicates significant mean difference between the groups or variables.

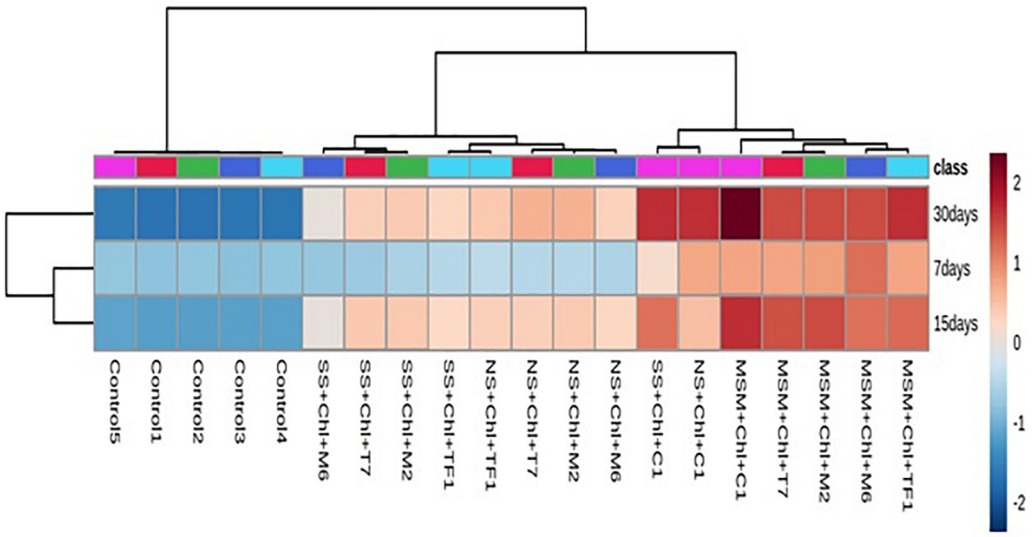

FIGURE 5
Heat map analysis of chlorpyrifos biodegradation by individual strains and developed consortium (ERM C-1) at different time intervals.

(M2), and *Klebsiella pneumoniae* (M6), and one fungal strain, *A. terreus* (TF1), showed the potential to degrade chlorpyrifos in MS medium, natural soil, and sterile soil up to 95–100% and showed great plant growth-promoting traits, which are noteworthy in this regard. Moreover, when the aforementioned strains were mixed together and developed as a consortium, the metabolic burden of the single strain splits into several parts and showed best results than the individual strains alone. Recently, Uniyal et al. (2021) have developed a consortium ECO-M comprising different bacterial strains (*Agrobacterium tumefaciens* ECO1, *Cellulosimicrobium funkei* ECO2, *Shinella zoogloeoides* ECO3, and *Bacillus aryabhattai* ECO4) for chlorpyrifos degradation in low-temperature mountainous agriculture system and reported 21.6–94.3% of degradation in 30 days of incubation. Similarly, in the present study, two different domain microorganisms (bacteria and fungus)

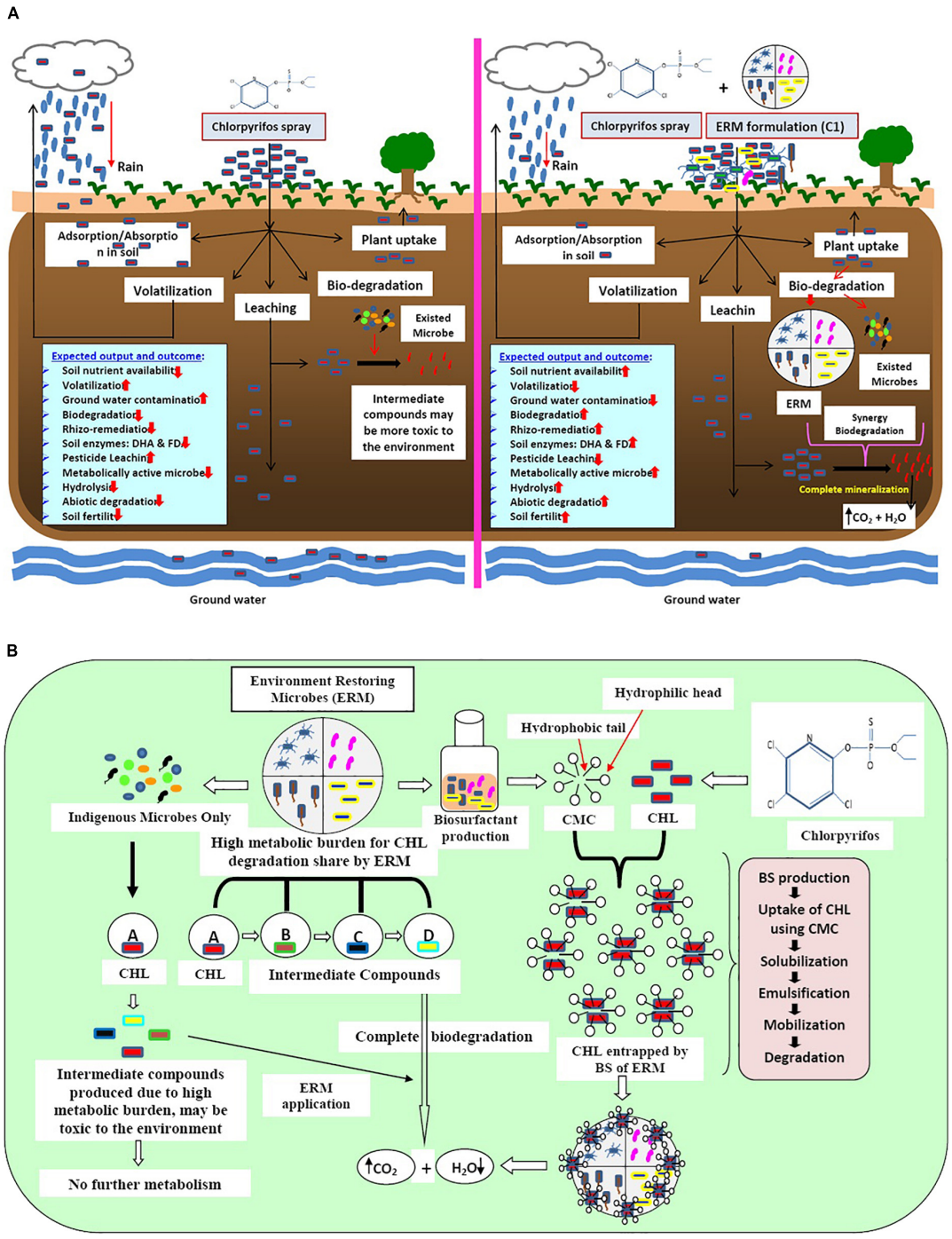

FIGURE 6
(A) Schematic diagram of the impact of pesticide (chlorpyrifos) and microbes (ERM- environment restoring microbes-C-1 formulation and already exited microbes) on soil, plant, and environment. **(B)** Mechanisms of action of bio-surfactant producing environment restoring microbes (ERM) in chlorpyrifos (CHL) biodegradation.

were mixed together and developed as consortium ERM C-1 for chlorpyrifos biodegradation and plant growth promotion to reduce pesticide load and enhance crop production in subtropical agricultural lands.

The chlorpyrifos biodegradation was examined at 7-, 15-, and 30-day intervals in different media, such as minimal salt, natural soil, and sterile soil. The maximum chlorpyrifos degradation was recorded in the group treated with consortium

rather than with individual strains, which might be due to the co-metabolism of strains where each strain produced a combination of catabolic enzymes responsible for more rapid and enhanced biodegradation of chlorpyrifos. Moreover, it might be attributed to the rapid growth of microbial strains during the first 15 days of the incubation period, or to the fact that a multi-strain consortium has a more efficient enzymatic system for chlorpyrifos biodegradation than the individual strain. The induction or activation of certain genes responsible for the production of catabolic enzymes in consortium treatment probably hydrolyzed the P–O ester bond causing rapid biodegradation in a short period of time, which might be another reason for chlorpyrifos biodegradation (Sun et al., 2020). The obtained results were encouraging and showed complete degradation in MS medium at 30 days of incubation while 98.56 ± 2.1 and 96 ± 1.7% in natural and sterile soils, respectively, at 30 days of incubation, which is more relevant than the previous studies. Similarly, Elshikh et al. (2021) formulated a bacterial consortium using two bacterial strains (*B. cereus* CP6 and *K. pneumoniae* CP19) and reported 93.4 ± 2.8% chlorpyrifos degradation by consortium treatment in liquid culture while 94.5 ± 3.3% in the soil at 16 days of the time interval.

Plant growth-promoting characteristics are the special feature found in plant rhizosphere micro-organisms and bulk agricultural soil, and some of these microorganisms also have pesticide-degrading abilities as a consequence of constant exposure to these substances (Kumar et al., 2021a,b). Previously, different microorganisms responsible for chlorpyrifos breakdown were identified from soil enrichment culture (Zhao et al., 2014; Govarthanan et al., 2020). In this study, all four strains (T7, M2, M6, and TF1) showed the best PGP traits, such as IAA, HCN, and NH_4 production and phosphate, potassium, and zinc solubilization ability in lab conditions. In the same way, Govarthanan et al. (2020) isolated a chlorpyrifos-degrading potent plant growth-promoting psychrophilic bacteria *Shewanella* sp. BT05 from brackish water, which showed IAA, HCN, and siderophore production and phosphate solubilization characteristics. Similarly, Zhao et al. (2014) reported *Acinetobacter calcoaceticus* D10 had the ability to degrade chlorpyrifos and showed IAA, siderophore, and phosphate solubilization activity in lab conditions.

Enzyme tests are used to investigate the mechanism by which microbes degrade complex substances. A polyphenol oxidase enzyme, laccase, has previously been reported to attack complex aromatic chemicals and produce simpler compounds, and some microbes have already been reported to be laccase producers and their linkage to chlorpyrifos biodegradation (Liu et al., 2016; Das et al., 2020; Srinivasan et al., 2020; Kumar et al., 2021). In this connection, different researchers have conducted studies on fungal and bacterial species capable of producing laccase and reported that *Bacillus halodurans*, *Azospirillum lipoferum*, *Pseudomonas desmolyticum*, *Bacillus pumilus*, *Bacillus subtilis*, *P. putida*, *B. spectabilis*, and *A. fumigatus* strains produced laccase enzyme (Gangola et al., 2018; Kumar et al., 2021). In this investigation, higher laccase production by consortium ERM C-1 than by individual strain might possibly be the reason for a greater chlorpyrifos degradation ability of ERM C-1. Srinivasan et al. (2020) reported *Bacillus* sp. for laccase production and chlorpyrifos degradation. Similarly, an engineered *P. putida* MB285 has been reported by Liu et al. (2016) for laccase production and complete chlorpyrifos degradation.

Biosurfactant is an amphipathic molecule made up of hydrophobic and hydrophilic moieties (Lal et al., 2018; Singh et al., 2018). *Bacillus* and *Pseudomonas* have previously been identified as potential biosurfactant producers for the removal of hazardous organic compounds and heavy metals from contaminated environments (Ambust et al., 2021; Ratna and Kumar, 2022). In this study, strains T7 and M2 showed biosurfactant production, and these strains were combined with other strains and included in consortium ERM C-1.

Pesticide biodegradation in the soil is an enzyme-driven transformation of organic compounds in a certain time duration. First-order degradation kinetics is frequently used to mimic the decline of residual pesticide mass in the soil system after its application (Bollag and Liu, 1990). The residual mass of pesticide diminishes exponentially with time "t" and the rate of degradation "*k*" or half-life continuously changes with the degradation process (Dykaar and Kitanidis, 1996). In this study, the values of degradation constant *k* were low and half-life ($t_{1/2}$) values were higher in the control condition, while an increase in *k* and decrease in $t_{1/2}$ values were observed with ERM C-1 treatment, indicating the rapid chlorpyrifos degradation in different media, which might be due to enzyme-catalyzed transformation of chlorpyrifos. The *k* and $t_{1/2}$ values of individual strains were lower than ERM C-1, indicating the combined effects of different enzymes present in the consortium treatment. Recently, Gaonkar et al. (2019) studied the biodegradation kinetics of dichlorvos and chlorpyrifos with *Pseudomonas aeruginosa* and *Taonella mepensis* in liquid MS medium, and similar findings were reported. Moreover, Kumar et al. (2021) performed biodegradation kinetics of chlorpyrifos with two fungal strains and reported decreased values of *k* and $t_{1/2}$ with consortium treatments.

Soil dehydrogenase (DHA) and fluorescein diacetate (FDA) activities are directly related to soil microbial activities and are considered as soil microbial indicators. DHA activity exists in soil as part of the oxidative processes occurring within living cells, and its measurement reflects intracellular enzymes solely; therefore, it is considered a direct indicator of microbial activity. However, FDA activity is a non-specific assay in which lipase, esterase, and protease classes of enzymes hydrolyze fluorescein by cleavage of the ring, so it is assumed that they may also take part in the cleavage of P-O bonds present in chlorpyrifos ring. In this study, an increase in DHA and FDA activities

with ERM C-1 treatment indicated that microbial consortium significantly increased the soil enzyme activity. Similar findings were reported by Kumari et al. (2021) with fipronil and atrazine degradation using biochip mixed biomixtures. Medo et al. (2021) reported reduced DHA and FDA activity with the application of two insecticides dimethachlor and linuron.

Proposed working mechanisms of ERM C-1

The application of chlorpyrifos in the field leads to various negative impacts on soil, water, and plant systems. Due to high hydrophobicity, chlorpyrifos adheres to the soil particles and forms clumps that entrap nutrients and restrict its movement toward the plant root. However, some amount of chlorpyrifos evaporates with rainwater while some quantity percolates down and contaminates groundwater. Consequently, the bioavailability of chlorpyrifos reduces greatly. In addition to all the negative impacts of chlorpyrifos on the whole system after its application, a compromised soil ecosystem has been generated in terms of plant nutrient uptake, leaching, and atmospheric pollution. However, trace quantities of chlorpyrifos remain available for microbial action leading to the partial degradation by indigenous microflora, subsequently resulting in the production of an intermediate compound like 3,5,6-trichloro pyridine-2-phenol (TCP), which showed a more toxic effect on the ecosystem than its parent compound. Production of such intermediate compounds is due to the reduction of metabolically active indigenous microbes (less metabolic versatility). On the other hand, when microbial formulation ERM C-1 was used, the produced intermediate compound was utilized immediately by high metabolically active microbes and metabolized completely into carbon dioxide and water. Simultaneously, ERM C-1 reduced the residual effect of chlorpyrifos and can establish better soil and plant health with high soil nutrient availability and soil enzymatic activities (**Figure 6A**).

Functioning of ERM C-1

ERM C-1 is constituted by high metabolically active microbes toward chlorpyrifos biodegradation, and hence enhances the bioavailability of chlorpyrifos by producing a special compound called biosurfactants (BS). Due to the high hydrophobic nature of chlorpyrifos, it persists in nature for a long duration. BS produced by ERM C-1 showed amphipathic nature and had a hydrophobic head and a hydrophilic tail. The hydrophobic part interacts with the benzene ring of chlorpyrifos, making it available for microbial attack by encapsulation process via critical micelle formation. When chlorpyrifos is available for ERM C-1 activity, the production of ring-opening enzymes like deoxygenase and other hydrolytic enzymes (hydrolases, oxidases, etc.) is initiated, and after the mineralization process, chlorpyrifos gets converted into CO_2 and H_2O. On the other hand, already existing but metabolically less versatile microbes degraded chlorpyrifos partially and produced intermediate compounds like TCP, which is more toxic than its parent compound. Excessive chlorpyrifos application caused a high metabolic burden to already existing microbes in the system which degrade chlorpyrifos partially, and toxic intermediate compounds prevail in the system. To address this problem, ERM C-1 application can be the best option because it showed the co-metabolism system by sharing metabolic burden through different microbes (**Figure 6B**). Therefore, the ERM C-1 application is recommended by this study for efficiently decreasing the chlorpyrifos concentration in the soil environment that has been affected by the excessive application of chlorpyrifos.

Conclusion

Prior research has not paid much attention to the question of whether combining the two domains of microorganisms (bacteria and fungus) is important to increase metabolic versatility for contaminant/pollutant removal. This study will look at novel ideas linked to the interactions between two domains of microbes in order to identify suitable co-metabolism methods for removing chlorpyrifos from the environment. The microbial strains (T7, M2, M6, and TF1) isolated from agriculture fields were able to thrive in chlorpyrifos (500 mg L^{-1})-supplemented medium. All four strains were used to develop consortium ERM C-1, which successfully degraded 100% of chlorpyrifos in the liquid MS medium. All the strains were able to produce extracellular laccase enzyme, which aided chlorpyrifos biodegradation. Plant growth promotion activities of the strains showed great potential, which can be an extra benefit to chlorpyrifos contaminated soil that is used for crop production. As shown by the kinetic constants, ERM C-1 demonstrated distinct capacities for chlorpyrifos degradation in different media. An *in-situ* pot experiment with natural and sterile soils, spiked with 500 mg kg^{-1} chlorpyrifos, revealed 98.58 $\pm$ 1.11 and 92.16 $\pm$ 0.18% degradation with ERM C-1 consortium. The conversion of chlorpyrifos into various metabolites was discovered using HPLC and GC-MS analyses. This is the first study to investigate chlorpyrifos biodegradation by employing a mixed fungal and bacterial consortia with a strong biodegradation capacity. In this study, *A. terreus* TF1 emerged as a powerful fungus that revealed great results and could be employed in a variety of bioremediation experiments. The consortium ERM C-1 can be further applied to degrade harmful compounds from a variety of sources. Further research can be conducted in the future by employing ERM C-1 on a broad scale

with varied pesticide concentrations and crops in different regions. This research might aid in the practical application of consortium ERM C-1 in the removal of chlorpyrifos in polluted environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/ (MW172266, MW228061, and MZ 268151).

Author contributions

GK: conceptualization, methodology, writing, and analysis. SL: writing, editing, methodology, and analysis. SS: analysis of results. SM, PS, PC, and AB: methodology and editing. NG: conceptualization and methodology. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.891870/full#supplementary-material

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Cloning of a novel tetrahydrofolate-dependent dicamba demethylase gene from dicamba-degrading consortium and characterization of the gene product

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Dicamba, an important hormone-type systemic herbicide, is widely used to control more than 200 kinds of broadleaf weeds in agriculture. Due to its broad-spectrum, high efficiency and effectively killing glyphosate-resistant weeds, dicamba is considered as an excellent target herbicide for the engineering of herbicide-resistant crops. In this study, an efficient dicamba-degrading microbial consortium was enriched from soil collected from the outfall of a pesticide factory. The enriched consortium could almost completely degrade 500mg/L of dicamba within 12 h of incubation. A novel tetrahydrofolate (THF)-dependent dicamba demethylase gene, named *dmt06,* was cloned from the total DNA of the enriched consortium. Dmt06 shared the highest identity (72.3%) with dicamba demethylase Dmt50 from *Rhizorhabdus dicambivorans* Ndbn-20. Dmt06 was expressed in *Escherichia coli* BL21 and purified to homogeneity using Co^{2+}-charged nitrilotriacetic acid affinity chromatography. The purified Dmt06 catalyzed the transfer of methyl from dicamba to THF, generating the herbicidally inactive metabolite 3,6-dichlorosalicylate (3,6-DCSA) and 5-methyl-THF. The optimum pH and temperature for Dmt06 were detected to be 7.4 and 35°C, respectively. Under the optimal condition, the specific activity of Dmt06 reached 165nmol/min/mg toward dicamba, which was much higher than that of Dmt and Dmt50. In conclusion, this study cloned a novel gene, *dmt06*, encoding an efficient THF-dependent dicamba demethylase, which was a good candidate for dicamba-resistant transgenic engineering.

KEYWORDS

dicamba, microbial consortium, gene clone, tetrahydrofolate-dependent demethylase, Dmt06

Introduction

Dicamba (3,6-dichloro-2-methoxybenzoate) is an important hormone-type systemic herbicide. It has been widely used to control more than 200 kinds of broadleaf weeds in the farmland of gramineous crops such as wheat, corn and rice (Stevens and Sumner, 1991; Huang et al., 2017). At present, the global annual usage of dicamba has reached 30,000 tons. Furthermore, the biotechnology giant Monsanto company has successfully developed transgenic soybean and cotton that were highly resistant to dicamba using the dicamba demethylase gene *DMO* (Behrens et al., 2007). These dicamba-resistant crops were approved by the US department of agriculture in 2015 and have been commercially planted since 2017 (Li et al., 2018). By 2019, the planting area of dicamba-resistant crops reached 50 million acres, accounting for 50% of the total planting area of soybeans in the United States.[1] Therefore, it has important theoretical and application value to explore dicamba-degrading and detoxifying strain and gene resources (Barrows et al., 2014).

Microbial metabolism was the main factor in the dissipation of dicamba in the soil (Krueger et al., 1989; Taraban et al., 1993; Werwath et al., 1998). So far, many dicamba-degrading strains, such as *Stenotrophomonas maltophilia* DI-6, *Pseudomonas* sp. DI-8 (Krueger et al., 1989; Wang et al., 1997), *Sphingobium* sp. Ndbn-10, *Rhizorhabdus dicambivorans* (formerly *Sphingomonas* sp.) Ndbn-20, have been isolated (Yao et al., 2015). The initial degradation step of dicamba was demethylation in all of these isolates, generating the herbicidally inactive metabolite 3,6-dichlorosalicylate (3,6-DCSA; Krueger et al., 1989; Behrens et al., 2007; Yao et al., 2015). Two main types of dicamba demethylases have been identified: (1) monooxygenase type demethylase: Herman et al. (2005) identified a dicamba monooxygenase DMO from *S. maltophilia* DI-6, DMO was a three-component monooxygenase using NADH as the electron donor. (2) tetrahydrofolate (THF)-dependent methyltransferase: Yao et al. (2016) and Chen et al. (2019) identified two dicamba demethylases Dmt and Dmt50 from *R. dicambivorans* Ndbn-20. Both Dmt and Dmt50 were THF-dependent methyltransferases. They catalyzed the methyl transfer from dicamba to THF, generating 3,6-DCSA and 5-methyl-THF. The activities of Dmt and Dmt50 were severely inhibited by the product 5-methyl-THF, resulting in significantly lower demethylation effect than DMO, which limited their application values.

In this study, a highly efficient dicamba-degrading consortium was enriched from the soil collected from the outlet of a pesticide plant. The enriched consortium was able to almost completely degrade 500 mg/L of dicamba within 12 h of incubation. A THF-dependent dicamba demethylase gene, named *dmt06*, was cloned from the total DNA of the consortium. Dmt06 was heterogeneously expressed in *Escherichia coli* and purified by affinity chromatography. Furthermore, the enzymatic characteristics of Dmt06 was also investigated. Our results indicated that Dmt06 showed higher demethylation activity than previously reported THF-dependent demethylases Dmt and Dmt50 (Yao et al., 2016; Chen et al., 2019), indicating that the Dmt06 has good potential application in the construction of dicamba-resistant crops.

1 http://www.isaaa.org

Materials and methods

Chemicals and media

Dicamba, 3,6-DCSA, NADH and THF were analytically pure. Methanol, acetonitrile, and acetic acid were chromatographically pure. All the chemicals were purchased from Sigma-Aldrich Company (Shanghai). Luria-Bertani (LB) broth and LB agar were obtained from Difco Laboratories (Detroit, MI). The minimal salt medium (MSM) consisted of the following components: 1.3 g K_2HPO_4, 0.86 g KH_2PO_4, 0.66 g $(NH_4)_2SO_4$, 0.097 g $MgSO_4$, 0.025 g $MnSO_4{\cdot}H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.0013 g $CaSO_4{\cdot}6H_2O$ per liter water, pH 7.0. For solid media, 15 g per liter of agar powder was added. All media were sterilized by autoclaving at 121°C for 20 min.

Bacterial strains, plasmids, and primers

The *E. coli* strains and plasmids used in this study are listed in Table 1, and the primers used in this study are listed in Table 2. All *E. coli* strains were aerobically cultured in LB broth or agar at 37°C. Antibiotics were added at the following concentrations: kanamycin, 50 mg/L; ampicillin, 100 mg/L.

Enrichment of dicamba-degrading consortium

Soil sample used in this study was collected from the outfall of a pesticide factory. 10 g of the soil sample was transferred into a 250 ml Erlenmeyer flask containing 90 ml MSM supplemented with 500 mg/L dicamba. The Erlenmeyer flask was incubated at 30°C and 180 rpm on a shaker. At certain intervals, the remained dicamba concentration in the

TABLE 1 Strains and vectors used in this study.

Strain or vector	Relative characteristics	Source or reference
E. coli DH5α	Host strain for cloning plasmid	TaKaRa
E. coli BL21 (DE3)	Host strain for expression vector	Vazyme Biotech
T vector	Clone vector; ampicillin^r (100 μg/ml)	TaKaRa
pET29a (+)	Expression vector; kanamycin^r (50 μg/ml)	Lab stock
pET29a*dmt06*	pET29a (+) derivative carrying *dmt06* gene	This study

TABLE 2 Primers used in this study.

Primer	DNA sequence (5′–3′)[a]	Purpose
27F	5′-AGAGTTTGATCCTGGCTCAG-3′	Amplification of the 16S rRNA gene
1492R	5′-GGTTACCTTGTTACGACTT-3′	of the dicamba degrader
pET-*dmt06*-F	TAAGAAGGAGATATACATATGGGAGAAGGACGGTCCCTTCA	Amplification of *dmt06* gene for
pET-*dmt06*-R	AGTGCGGCCGCAAGCTTCGGCGACCCGGCGGCCGTCGCC	expression in *E. coli* BL21
dF1	CTC(G)TTCG(A)ACCAGT(A)CC(G)CACCACATG	Amplification of the conserved
dF2	TCGGCGACT(G)GC(G)ATCCTG(T)TAC(T)TA(G)	regions of *dmt06* gene
dR1	GGC(A)TGGATC(G)C(G)CC(G)TACCCG(C)CTG(C)GCCG	
dR2	TC(G)AAGTTC(T)GAC(T)CAC(T)GACTTCATCGG	
uSP1	AGTACACGATGCAGTCGCCGACCACGT	Amplification of the upstream
uSP2	AACCCGGCGAAGGTGTTGATGCCGA	sequence of *dmt06*
uSP3	CTTTCAGGAACAGCTNNNNNNNNNGGTGGG	
dSP1	AGTGGAGCTGTCCGGCCCGTAC	Amplification of the downstream
dSP2	GACACCGTACGGTCGGCCATTCTC	sequence of *dmt06*
dSP3	AGAAGTACGGAATCGNNNNNNNNNGCACCC	

[a]The underlined bases indicate that they were overlapped and were used to construct plasmids by the In-Fusion.

microbial consortium was determined using high-performance liquid chromatography (HPLC) as described below. When ~70% of the added dicamba was degraded, 10 ml of the enrichment culture was transferred into 90 ml fresh medium. The transfer was repeated for 5–6 times until the enriched consortium acquired high dicamba-degrading ability.

Determination of the dicamba demethylase activity in the cell extract of the enriched consortium

To investigate which type of dicamba demethylase was in the enriched consortium. The bacterial cells of the final transferred consortium were collected by centrifugation at 6000 rpm for 10 min. After wishing twice with MSM, the cells were resuspended with ice-cold phosphate-buffered saline (PBS) buffer (50 mM, pH 7.4), and then disrupted by pulse sonication on ice with 15 s burst and 10 s pause for 15 min (Auto Science, UH-650B ultrasonic processor, 40% intensity), and the cell lysate was centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected as the cell extract. The protein concentration of the cell extract was quantified by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard (BCA Protein Assay Kit, Sangon Biotech Shanghai Co., Ltd.). The demethylase activity of the cell extract toward dicamba was determined in a 300 µl mixture containing 100 mM PBS buffer, 2.0 mM NADH or 2.0 mM THF, 0.5 mM substrate, and 50 µl crude enzyme. The mixture was incubated for 5 min at 30°C, then the reaction was terminated by boiling at 100°C for 1.0 min. The conversion of substrate was analyzed by HPLC. One unit of dicamba demethylase activity was defined as the amount of enzyme that catalyzed the conversion of 1.0 nmol of dicamba per min.

Cloning of the dicamba demethylase gene from the enriched consortium

The total DNA of the enriched consortium was extracted by Sodium dodecyl sulfate (SDS) high-salt method (Sambrook and Russell, 2001). To clone the dicamba demethylase gene from the total DNA of the enriched consortium, four degenerate primers including two forward primers and two reverse primers (Table 2) were designed according the conserved region of the reported THF-dependent demethylase gene sequences. These primers were paired for PCR amplification using the total DNA of the enrichment as a template. Amplification conditions: 94°C 3 min; 94°C 30 s, 55°C 30 s, 72°C 2 min, 33 cycles; 72°C 10 min. The acquired fragment was ligated into T-vector and transformed into *E. coli* DH5α. Sequencing of the fragment in the T-vector was performed using the ABI 3730xl DNA sequencer (Applied Biosystems). Then, the upstream sequence and downstream sequence of the acquired sequence were amplified by SEFA-PCR method, a DNA walking technology developed in our Lab (Wang et al., 2007), using the primers listed in Table 2.

Expression of the dicamba demethylase gene and purification of the product

The dicamba demethylase gene was amplified by PCR using 2 × Phanta Master Mix (Vazyme Biotech Co., Ltd) using the primers listed in Table 2 and the total DNA extracted from the enrichment as the template. The PCR products were inserted into the NdeI-HindIII site of pET29(+) to generate the recombinant plasmid pET-*dmt06* using a one-step cloning kit (Vazyme Biotech). Then, pET-*dmt06* was transformed into *E. coli* BL21 (DE3) for expression. *Escherichia coli* BL21 (DE3) cells harboring pET-*dmt06* was grown in 100 ml of LB broth supplemented with 50 mg/L kanamycin at 37°C. When the absorbance at 600 nm reached 1.0, the cultures were

induced with 0.4 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) for 10 h at 16°C. Then, cells were harvested by centrifugation at 12,000 rpm for 5 min. After washed twice with 100 mM PBS buffer (pH 7.4), the cells were resuspended in ice-cold PBS buffer, and then disrupted by sonication as described above, and the undisrupted cells and cell debris was removed by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was charged onto 1-ml His-bind resin columns (HiTrap Talon crude; GE Healthcare Life Sciences), which had been activated by Co^{2+} and equilibrated with binding buffer. Following washing with binding/washing buffer containing different concentration of imidazole, the target protein was eluted with 5 ml of elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, 100 mM imidazole (pH 8.0)]. The resultant fractions were dialyzed overnight at 4°C to remove imidazole in PBS buffer (100 mM, pH 7.4). The purities and molecular weights of the expressed protein was determined by 12% SDS-polyacrylamide gel electrophoresis (PAGE). The protein concentration of the cell extract was quantified by the BCA method. The dicamba demethylase activity was determined as described above. The metabolite was identified by mass spectrometry (MS) as described below.

Study on the enzymatic characteristics

The temperature range was investigated at 50 mM PBS buffer (pH 7.4) under different temperatures (4°C–70°C), and the relative activity was calculated by assuming that the activity at 30°C was 100%. The pH range was investigated at pH values from 3.6 to 10.6 in three different buffering systems: 20 mM HAc-NaAc buffer (pH 3.6–5.8), 50 mM PBS buffer (pH 5.5–8.5), and 20 mM glycine-NaOH buffer (pH 8.6–10.6). The activity observed at pH 7.4 in PBS buffer was set as 100%, each value was the average from three independent experiments. For pH stability investigation, the enzyme was preincubated in buffers with different pH (pH 3.6–10.6) at 30°C for 4 h. Then, the remaining activity was assayed under the optimal condition. For thermostability investigation, the enzyme was preincubated in a water bath at different temperatures (30°C–70°C) for 120 min, and then the residual activity was assayed. To investigate the effects of potential inhibitors on demethylase activity, the enzyme mixture was preincubated for 30 min at 35°C, then the chemical agents (EDTA and SDS, final concentration of 5.0 mM) and metal ions (Li^+, Na^+, Mg^{2+}, K^+, Hg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Ba^{2+}, Al^{3+}, Cd^{2+}, Ag^+, Fe^{2+}, Fe^{3+}, final concentration of 1.0 mM) were individually added, and the reactions were performed at 35°C for 30 min. Dicamba demethylase activity was assayed as described above and expressed as a percentage of the activity obtained without addition of the above compound.

Chemical analysis

The collected samples were freeze-dried and dissolved in 1.0 ml of methanol. The solution was filtered through a 0.22 μm-pore-size Millipore membrane to remove particles. The concentrations of dicamba and metabolite were analyzed on an UltiMate 3,000 titanium system (Thermo Fisher Scientific) equipped with a C_{18} reversed-phase column (internal diameter, 4.6 mm; length, 250 mm; Agilent Technologies). The mobile phase was a mixture of ultrapure water (58.4%), acetonitrile (31.7%), methanol (7.5%), and 2.4% acetic acid (Yao et al., 2015). The flow rate was 1.0 ml min^{-1}. A VWD-3100 single-wavelength detector was used to detect the UV absorption, the wavelengths for dicamba and 3,6-DCSA were 275 nm and 319 nm, respectively. The MS analysis was performed according to the method described by Liu et al. (2020).

Accession Number. The gene dmt06 sequence is deposited in the GenBank database under accession number ON828423.

Results

Enrichment of an efficient dicamba-degrading microbial consortium

In this study, we used dicamba as a sole source of carbon to enrich dicamba-degrading microbial consortium. It took 11 days to degrade ~70% of the added 500 mg/L of dicamba for the first round of consortium. During the acclimation, the degradation ability of the consortium became stronger and stronger. After six rounds of transfer, the enriched consortium could almost completely degrade the added 500 mg/L of dicamba within 12 h incubation (Figure 1). HPLC analysis indicated that an intermediate metabolite was accumulated during the degradation, the retention time of the metabolite was equal to that of the 3,6-DCSA standard, and this metabolite disappeared with prolonged incubation (Figure 2). The results indicated that the enriched microbial consortium could efficiently degrade dicamba, and the first

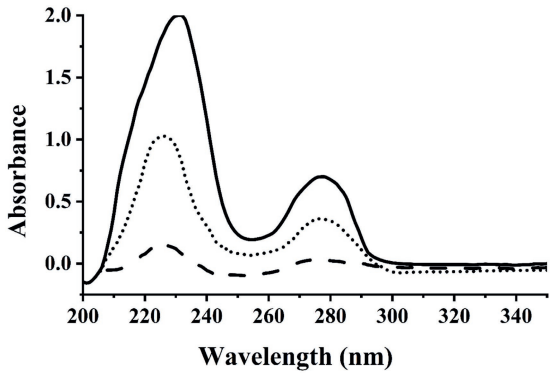

FIGURE 1
UV scanning detection of dicamba degradation by the enriched consortium. The sixth generation of the enriched consortium was inoculated into MSM supplemented with 500mg/L of dicamba, which then was incubated in at 30°C and 180 rpm on a shaker. Solid line indicated the sample collected at 0h, dotted line indicated the sample collected at 6h, dashed line indicated the sample collected at 12h.

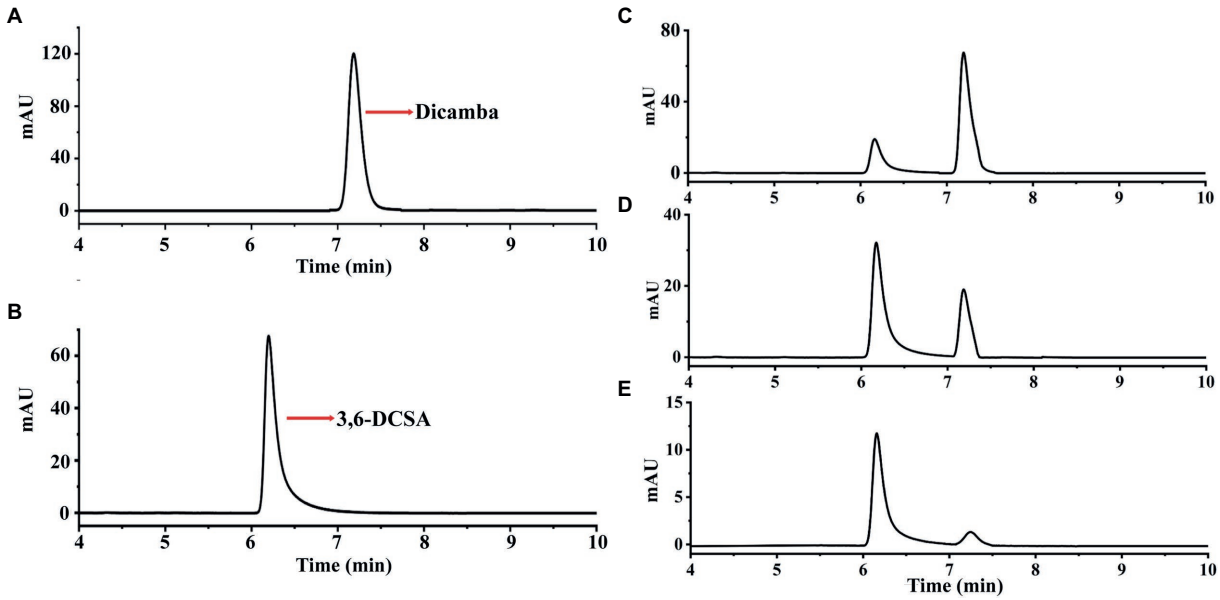

FIGURE 2
HPLC analysis of dicamba degradation by the enriched consortium. **(A)** The dicamba standard. **(B)** The 3,6-DCSA standard. **(C)** Sample collected at 4h. **(D)** Sample collected at 8h. **(E)** Sample collected at 12h.

TABLE 3 Demethylase activities in cell extract of the enriched consortium.

Demethylase activity (nmol/min/mg)		
Addition of NADH	**Addition of THF**	**No addition of NADH and THF**
0	1.09 ± 0.23	0

degradation step was demethylation to generate 3,6-DCSA, which could be further degraded by the enriched consortium.

Degradation of dicamba by the crude enzyme of the enriched consortium

To date, two types of demethylases catalyzing the demethylation of methyl group-containing aromatics have been reported, one was the NADH-dependent monooxygenase, and another was the THF-dependent methyltransferase. In order to determine which type of dicamba demethylase was in the enrichment culture, the cell extract of the enriched consortium was obtained by ultrasonic disruption, and the dicamba demethylase activity in the cell extract was assayed in the presence of NADH and THF, respectively. The results showed that the cell extract added with THF acquired the dicamba demethylase activity, while the cell extract added with NADH could not convert dicamba (Table 3). The results suggested that the dicamba-degrading bacteria in the enriched consortium employed a THF-dependent demethylase to convert dicamba to 3,6-DCSA.

Cloning of the THF-dependent demethylase gene from the enriched consortium

To date, four THF-dependent demethylase genes have been reported: syringate demethylase DesA (Masai et al., 2004) and vanillate demethylase LigM (Abe et al., 2005) from *Sphingomonas paucimobilis* SYK-6, and dicamba demethylase Dmt (Yao et al., 2016) and Dmt50 (Chen et al., 2019) from *R. dicambivorans* Ndbn-20. To clone the dicamba demethylase gene, four degenerate primers (Table 2) including two forward primers (dF1 and dF2) and two reverse primers (dR1 and dR2) were designed according the conserved regions of the four THF-dependent demethylase genes, and then the primers were paired to amplify the dicamba demethylase gene from the total DNA of the enriched consortium. The results indicated that one pair of primers (dF1 and dR1) successfully amplified a fragment with clear band on gel electrophoresis (Figure 3A), the size of the acquired fragment was ~600 bp, which was consistent with the theoretical value. Sequencing results showed that the sequence of this fragment was homologous with 191–818 bp of *dmt50* with a similarity of 74.3%. Then, to clone the whole gene, primers of SEFA-PCR, were designed in the upstream and downstream of the acquired fragment. After two steps of SEFA-PCR amplification, clear bands with length of ~1–1.5 kb were obtained at both upstream and downstream of the acquired fragment (Figure 3B).

The acquired SEFA-PCR products were sequenced and finally assembled a complete gene, which named *dmt06* in this study. *dmt06* was 1,422 bp in size, encoding a 473 amino acid protein. Blast in GenBank of the NCBI indicated that Dmt06 was most

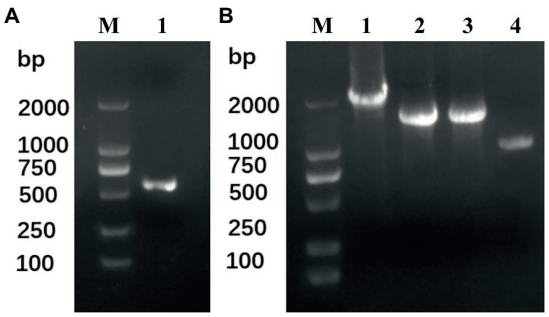

FIGURE 3
Amplification of the THF-dependent demethylase gene from the total DNA of the enriched consortium. **(A)** Fragment amplification from the total DNA of the enriched consortium using degenerate primers, Line M: DNA marker, line 1 amplified product using primer pair dF1 and dR1. **(B)** Amplified products from the upstream and downstream of the acquired fragment by SEFA-PCR, Line M: DNA marker, line 1: the first round SEFA-PCR from the fragment upstream, line 2: the first round SEFA-PCR from the fragment downstream, line 3: the second round SEFA-PCR from the fragment upstream, line 4: the second round SEFA-PCR from the fragment downstream.

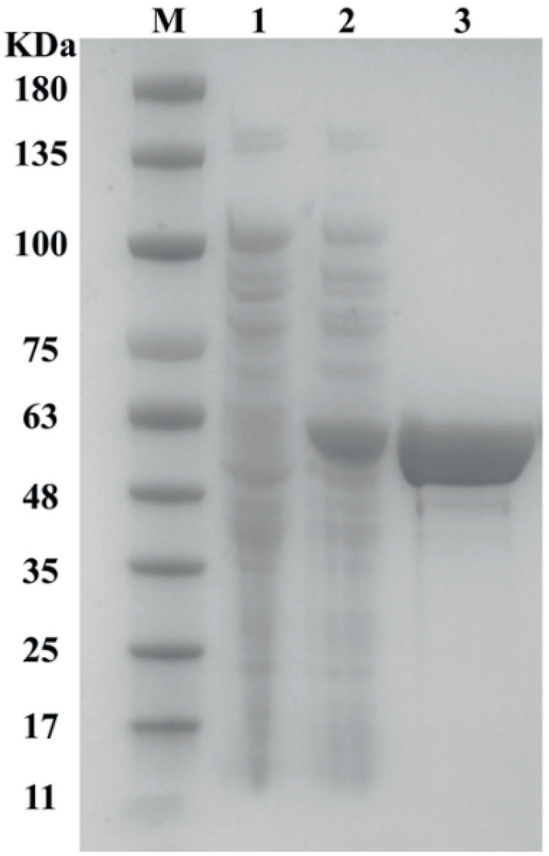

FIGURE 4
SDS-PAGE analysis of the purified Dmt06. Lane M: protein molecular marker; lane 1: crude extract of *E. coli* BL21 harboring pET29a; lane 2: crude extract of *E. coli* BL21 harboring pET-*dmt06*; lane 3: purified Dmt06.

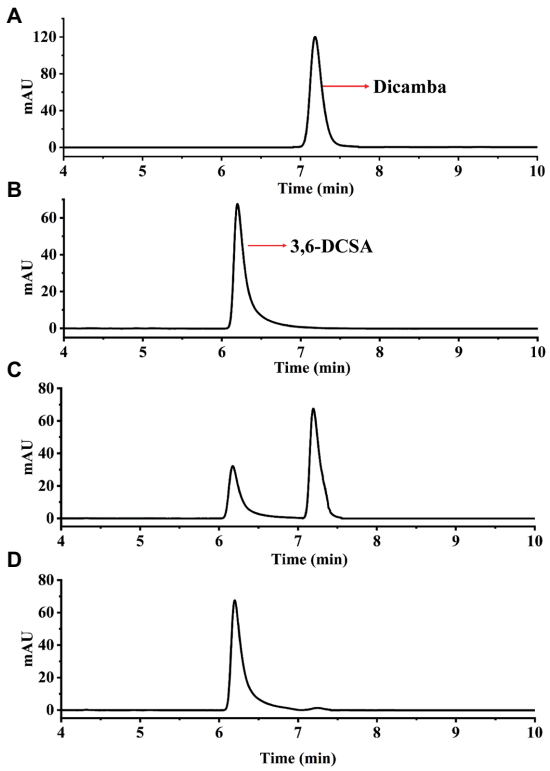

FIGURE 5
HPLC analysis of the product produced during dicamba conversion by Dmt06. **(A)** The dicamba standard. **(B)** The 3,6-DCSA standard. **(C)** Sample collected at 5min. **(D)** Sample collected at 10min.

related to some THF-dependent demethylases or aminomethyltransferase family protein. Interestingly, Dmt06 shared 100% identity with a putative aminomethyltransferase family protein from *Actinomadura parvosata* subsp. kistnae, and of the proteins with known function, Dmt06 showed the highest identity (72.3%) with dicamba demethylase Dmt50 from *R. dicambivorans* Ndbn-20, and shared 46.2% identity with Dmt.

Expression and purification of Dmt06

To investigate the function of Dmt06, the *dmt06* gene was ligated into plasmid pET29a(+) and expressed in *E. coli* BL21(DE3) under the induction of IPTG. Then, the recombinant Dmt06 was purified to homogeneity using Co^{2+}-charged nitrilotriacetic acid affinity chromatography (Figure 4). SDS-PAGE analysis indicated that the molecular mass of the denatured protein was ~55 kDa, which was consistent with the theoretical mass of the tagged protein (52.3 kDa). Dmt06 was stored at −80°C in 100 mM PBS buffer (pH 7.4) with 10% glycerol and 0.3 mM EDTA, 90% of its activity was retained when stored for 2 months, indicating that Dmt06 was very stable.

Enzymatic assays showed that Dmt06 could transfer dicamba to a product with a retention time at 6.25 min, which was equal to that of the 3,6-DCSA standard (Figure 5). MS analysis showed

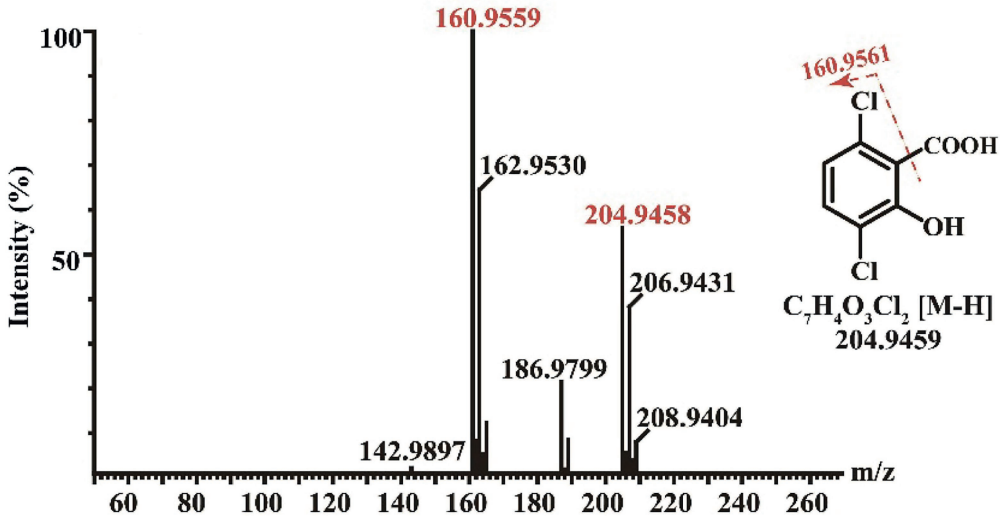

FIGURE 6
MS analysis of the product produced during dicamba conversion by Dmt06.

that the product had a prominent deprotonated molecular ion peak at m/z 204.95 $(M-H)^-$ with a fragment peak at m/z 106.95 (Figure 6), this ion peak characteristic was also consistent with that of 3,6-DCSA. Thus, the product was identified as 3,6-DCSA. The above results demonstrated that Dmt06 was a demethylase catalyzing the conversion of dicamba to 3,6-DCSA.

Enzymatic characteristics of the purified Dmt06

The purified Dmt06 displayed dicamba demethylase activity only in the presence of THF, indicating that Dmt06 was a THF-dependent demethylase. The activity of Dmt06 was detected from 10°C to 60°C and at pH ranging from 5.5 to 10.0. The optimum pH and temperature for Dmt06 was 7.4 and 35°C, respectively (Supplementary Figures S1, S2). In the stability studies, more than 70% of the activity of Dmt06 was retained after incubation at 50°C for 120 min, and <20% of its activity was retained after incubation at 60°C for 120 min. The results indicated that Dmt06 was stable at 50°C, but become unstable when temperature raised to 60°C. The effects of various metal ions and potential inhibitors on the activity of Dmt06 are shown in Supplementary Figure S3. The activity of Dmt06 was severely inhibited by 1.0 mM of Hg^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Ag^{2+}, and 5.0 mM of SDS, and was moderately inhibited by 1.0 mM of Mn^{2+}, Ni^{2+}, Cu^{2+}, Ba^{2+}, and Al^{3+}. On the other hand, 1.0 mM of Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, and 5.0 mM EDTA had no obviously effect on the activity of Dmt06. At the optimal condition, the incubation of 0.1 mg of Dmt06 for 5 min resulted in a specific activity of 165 nmol/min/mg toward dicamba. Dmt06 could not catalyze the methyl transfer of vanillate, syringate, isoproturon, and alachlor, indicating that Dmt06 possessed a very narrow substrate spectrum.

Discussion

The planting of glyphosate-resistant transgenic crops combined with the application of glyphosate can effectively kill weeds without damaging the crops, thus proving an efficient and low-cost weed control strategy (Barrows et al., 2014). Therefore, the planting area of glyphosate-resistant GM crops have been rapidly expanded since their commercialization in the early 1990s, reaching ~150 million hectares in 2018 (See footnote 1). However, long-term high-intensive use of glyphosate has resulted in increasing resistance of weeds (Cerdeira and Duke, 2006). It was reported that at least 41 kinds of weeds in 29 countries have developed strong resistance to glyphosate, which makes the weeding strategy of glyphosate-resistant transgenic crop ineffective. Therefore, it is necessary to screen new target herbicides and construct corresponding transgenic crops. Dicamba is a broad-spectrum herbicide that can effectively kill glyphosate-resistant weeds. In particular, dicamba has been commercially used for more than 60 years since it was developed in the 1960s (Tomlin, 2006), but so far, few weeds have developed resistance to dicamba. Therefore, dicamba is an ideal target herbicide for the next generation of herbicide-resistant engineering, in which the dicamba detoxification genes have great application potential.

Previous studies have indicated that dicamba could be degraded by soil bacteria, and the initial step of microbial degradation was demethylation to generate 3,6-DCSA, which was herbicidally inactive (Li et al., 2018). Thus, dicamba demethylase gene is an ideal herbicide-resistant gene for herbicide-resistant engineering. So far, a NADH-dependent dicamba monooxygenase DMO and two THF-dependent dicamba demethylases Dmt and Dmt50 have been identified.

Biological giant Monsanto has successfully used the oxygenase gene of *DMO* to construct dicamba-resistant soybean and corn, which have been commercially planted on a large scale. Compared with DMO, the advantage of the THF-dependent demethylases Dmt and Dmt50 is that they do not need reducing force NADH, so it is more energy-saving. In addition, the 5-methyl-THF generated during dicamba demethylation can be used for the synthesis of purine, pyrimidine, glycine and methionine, as well as the methylation of DNA, fatty acids and enzymes (Sullivan et al., 2021; Menezo et al., 2022). However, the disadvantage of Dmt and Dmt50 is that their activities were lower than that of DMO, which greatly limits their abilities to detoxify dicamba.

In this study, we enriched a highly efficient dicamba-degrading microbial consortium, the sixth generation of the enrichment could almost completely degrade 500 mg/L dicamba within 12 h incubation. In previous reports, *Sphingobium* sp. Ndbn-10 and *R. dicambivorans* Ndbn-20 degraded 2.25 mM (~497 mg/L) of dicamba within 36 h and 72 h, respectively, and *Pseudomonas maltophilia* DI-6 degraded 97% of 1,000 mg/L dicamba within 30 h (Krueger et al., 1989; Yao et al., 2016). Thus, the enriched consortium had a relatively high dicamba-degrading efficiency when compared with previous studies. To isolate pure strains that capable of degrading dicamba, the enriched consortium was serially diluted, the diluent was spread on LB plate and incubated at 30°C for 5 days. Colonies with different morphology were selected to test their dicamba-degrading abilities. We selected ~100 colonies, unexpectedly, none of them could degrade dicamba. The possible reason might be that the bacteria responsible for dicamba degradation in the enriched consortium were unculturable. Activities study of the crude extract of the consortium indicated that the degradation of dicamba was initiated by demethylation, which catalyzed by an unknown THF-dependent demethylase (named Dmt06 in this study). To clone the gene, a fragment of *dmt06* was firstly successfully amplified from the total DNA of the enriched consortium by PCR using two pairs of degenerate primers, which designed according to the conserved region of reported THF-dependent demethylases, and then the full length of *dmt06* was obtained by DNA walking using SEFA-PCR method. Interestingly, results of blastp in the Non-redundant protein sequences (nr) database of NCBI showed that Dmt06 shared 100% identities with a putative aminomethyltransferase family protein from *Actinomadura parvosata* subsp. kistnae, and ~70%–98% identities with a lot of putative aminomethyltransferase family proteins from indigenous soil bacteria *Rhizobiales*, *Nonomuraea*, *Rhizorhabdus*, *Proteobacteria*, *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Tianweitania*, and *Microbacterium*. The wide distribution of this gene in soil bacteria suggested that it may have important functions, e.g., it is possible that the gene participates in the demethylation process of some natural occurred methyl-containing aromatic compounds. Results of blastp in the UniProtKB/Swiss-Prot (swissprot) database of NCBI indicated that Dmt06 was most related to the two reported THF-dependent demethylases Dmt and Dmt50. However, the identities between them were <75%, these analyses suggested that *dmt06* might encode a novel THF-dependent methyltransferase gene that differs from *dmt* and *dmt50*. Furthermore, Dmt06 was also obviously different from Dmt and Dmt50 in size and enzymatic characteristics. E.g., Dmt06 has 473 amino acids, while Dmt and Dmt50 have 466 and 475 amino acids, respectively. Dmt06 had a relatively narrow pH range (5.5–10.0) than that of Dmt (5.0–10.0) and Dmt50 (3.6–10.0), Dmt06 could tolerate 50°C, which was higher than that of Dmt (45°C) but much lower than that of Dmt50 (75°C). The optimal pH and temperature for Dmt06 (7.4 and 35°C, respectively) were also different from that of Dmt (8.0 and 30°C, respectively) and Dmt50 (8.0 and 45°C, respectively). Most important, the specific activity of Dmt06 reached 165 nmol/min/mg toward dicamba, which was significantly higher than that of Dmt (114 nmol/min/mg; Yao et al., 2016) and Dmt50 (146 nmol/min/mg; Chen et al., 2019), indicating that Dmt06 has stronger dicamba detoxification ability than Dmt and Dmt50. Thus, *dmt06* is a potential candidate for the engineering of dicamba-resistant transgenic crops and bioremediation of dicamba residue pollution in environment. At the same time, the sequence comparison and structure analysis of Dmt06, Dmt and Dmt50 can provide a basis to elucidate the catalytic mechanism of the THF-dependent demethylase. In the future, we will study the structure and the key active sites of this new dicamba demethylase, and improve the activity of Dmt06 through directed evolution technology and rational protein design.

Conclusion

In conclusion, this study cloned a novel THF-dependent dicamba demethylase gene *dmt06* from an efficient dicamba-degrading microbial consortium. Dmt06 were synthesized in *E. coli* BL21 and purified as His-tagged enzymes. Enzymatic assay showed that the dicamba demethylation activity of Dmt06 was much higher than that of Dmt and Dmt50, indicating that it has good application value in the dicamba-resistant transgenic engineering.

Data availability statement

The data presented in the study are deposited in the NCBI GenBank repository, accession number ON828423.

Author contributions

NL and LC conceived the presented idea. NL and JH contributed to the writing and prepared the figures and tables. EC, CY and HZ participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.978577/full#supplementary-material

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Current status of pesticide effects on environment, human health and it's eco-friendly management as bioremediation: A comprehensive review

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Pesticides are either natural or chemically synthesized compounds that are used to control a variety of pests. These chemical compounds are used in a variety of sectors like food, forestry, agriculture and aquaculture. Pesticides shows their toxicity into the living systems. The World Health Organization (WHO) categorizes them based on their detrimental effects, emphasizing the relevance of public health. The usage can be minimized to a least level by using them sparingly with a complete grasp of their categorization, which is beneficial to both human health and the environment. In this review, we have discussed pesticides with respect to their global scenarios, such as worldwide distribution and environmental impacts. Major literature focused on potential uses of pesticides, classification according to their properties and toxicity and their adverse effect on natural system (soil and aquatic), water, plants (growth, metabolism, genotypic and phenotypic changes and impact on plants defense system), human health (genetic alteration, cancer, allergies, and asthma), and preserve food products. We have also described eco-friendly management strategies for pesticides as a green solution, including bacterial degradation, myco-remediation, phytoremediation, and microalgae-based bioremediation. The microbes, using catabolic enzymes

for degradation of pesticides and clean-up from the environment. This review shows the importance of finding potent microbes, novel genes, and biotechnological applications for pesticide waste management to create a sustainable environment.

KEYWORDS

pesticides, water, plants, DNA damage, cancer, allergy, biodegradation

Introduction

Pesticides are chemical compounds that are used to eliminate insects, rodents, fungi, and weeds. They include insecticides, herbicides, nematicides, fungicides, molluscicides, rodenticides, plant growth regulators, and other compounds (Zhan et al., 2020; Bhatt et al., 2021a; Zhang et al., 2021). It is generally used to prevent illnesses spread by vectors, including crop protection, food preservation, and significant roles in commercial as well as food based industrial practices, i.e., aquaculture, agriculture, food processing, and storage (Mieldazys et al., 2015; Sharma et al., 2019). Any living bodies, either animals or plants, which are harmful for human or animals are known as pests. Pesticides are substances that are used to either kill or prevent the growth of pests.

According to the United States Code of Federal Regulations (CFR), a pesticide is any component or mixture of compounds intended for use as a plant regulator, defoliant, or desiccant (United States Environmental Protection Agency, 2004). Pesticides are defined by the Food and Agriculture Organization (FAO) of the United Nations as substance or mixture of substances attended for controlling, preventing, destroying any pest, animal, or human disease causing vectors, undesirable plants, or animal species affecting food production, managing, selling, storage, and transportation (World Health Organization, 2015). Since ancient times, a variety of chemical compounds have been used to control pests. Sulfur compounds are well known example of such insect and mite control pesticides (Gyawali, 2018). Pyrethrum, a plant (*Chrysanthemum cinerariaefolium*) derived pesticide, has been used for over 2000 years (Unsworth, 2010). Salty water and chemical compounds (organics as well as inorganic) were widely used to control pests' populations until the introduction of dichloro diphenyl trichloroethane (DDT) by Paul Herman Muller in 1939 as a potent pesticide (Abubakar et al., 2020). However, use of DDT is helpful to increasing the food productivity and shelf-life of food products. Thus, the global demand for DDT increased day by day, which opened the door to synthesizing new chemical substances that act as pesticides. DDT was replaced by organophosphates (OPs) and carbamates (CMs) in the United States in 1975 (Barnhoorn et al., 2009).

The global pesticide consumption in 2019 was approximately 4.19 million metric tons, where China was by far the largest pesticide-consuming country (1.76 million metric tons), followed by the United States (408 thousand tons), Brazil (377 thousand tons), and Argentina (204 thousand tons) (Fernández, 2021). In southeast Asia, WHO reported an annual increase in pesticide usage with 20% of developing countries as pesticide-consumers, including Cambodia, Laos, and Vietnam (Schreinemachers and Tipraqsa, 2012; Schreinemachers et al., 2015). India belongs to one of the major pesticide producing countries in Asia, having 90 thousand tons annual production of organochlorine pesticides including benzene hexachloride and DDT (Khan et al., 2010; Pozo et al., 2011). Between 2010 and 2014, the average cost/benefit ratio was 0.645 g of total pesticides per kilogram of crop yield, with an average yearly consumption of 2.784 kg ha^{-1}. Japan (18.94 kg ha^{-1}) had the greatest average pesticide usage from 2010 to 2014, followed by China (10.45 kg ha^{-1}), Mexico (7.87 kg ha^{-1}), Brazil (6.16 kg ha^{-1}), Germany (5.12 kg ha^{-1}), France (4.85 kg ha^{-1}), United Kingdom (4.03 kg ha^{-1}), United Status (3.88 kg ha^{-1}), and India (0.26 kg ha^{-1}) (Zhang, 2018).

Herbicides account for 47.5% of pesticide contributions, followed by insecticides 29.5%, fungicides 17.5%, and other types of insecticides 5.5%, as shown in **Figure 1** (Gill and Garg, 2014; Zhang, 2018; Sharma et al., 2019). Pesticides are classified based on a variety of variables. The most often used criteria for pesticide classification are the mode of entry, chemical makeup, and the target it kills. On the other hand, the WHO and Globally Harmonized System (GHS) classified pesticides based on their toxicity or harmful effects, prioritizing public health.

The main advantages of pesticides are the expected immediate gains after application, e.g., eliminating caterpillars, which has the primary benefit of raising cabbage yields and quality. The three major outcomes result in 26 key advantages, ranging from the preservation of recreational grass to the saving of human lives. Secondary benefits are those that arise as a result of the primary advantages but are less obvious or immediate. They might be subtle, less visible at first glance, or long-term in character. As a result, proving cause and effect for secondary benefits is more difficult, although they can still be strong pesticide reasons. Increased productivity of cabbage leads to an increase in economic wealth, which

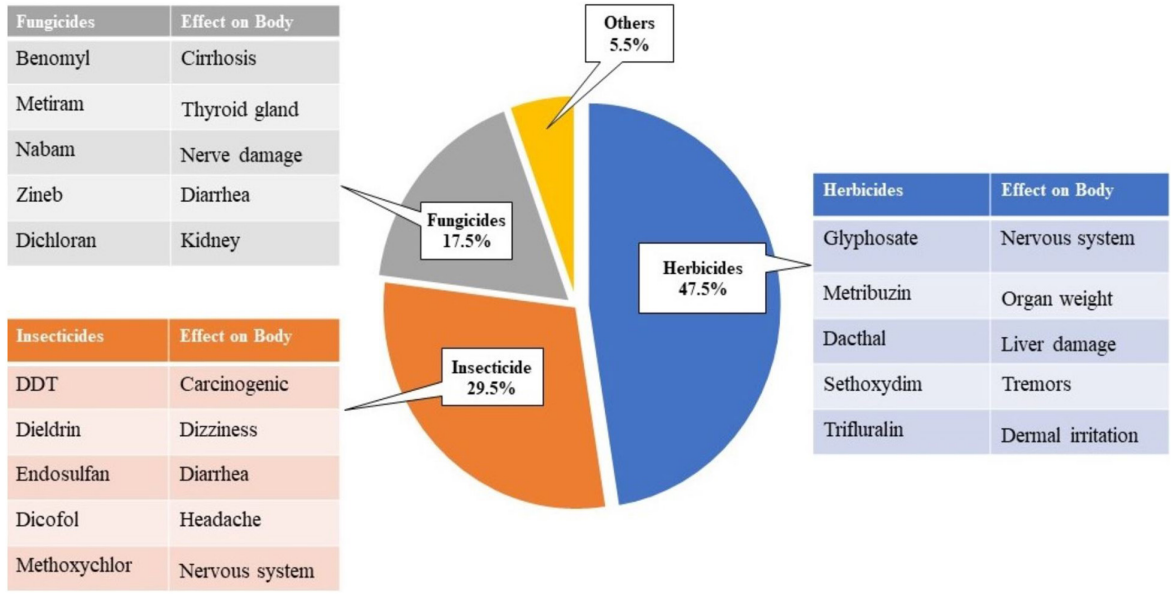

FIGURE 1
Percentage distribution of pesticides (Nicolopoulou-Stamati et al., 2016; Alengebawy et al., 2021).

helps to improve children's health and education systems. Secondary benefits have been identified, including healthier individuals and permanently cultivated land that conserves biodiversity. This accomplishment was aided by the use of high-yield seed types, advanced irrigation technologies, and agricultural herbicides (Bureau, 1993). Similarly, most nations' productivity and output have increased significantly, such as wheat yields in the United Kingdom and maize yields in the United States. A multitude of factors have been blamed for increased productivity, including better cultivars, machinery use, and fertilizer usage. Pests, insects, diseases, and weeds can substantially reduce the production of harvestable crops; as a result, pesticides have played a crucial role in food production and processing. Warren (1998) also highlighted the huge increase in food production in the United States over the 20th century. Pesticides are used to increase agricultural output and food preservation while ignoring their associated risks. Overuse, exposure, and harmful consequences can all be mitigated by applying it judiciously and utilizing different pesticide categories (World Health Organization, 2009). Many detrimental effects have been seen as a result of widespread pesticide usage, and effective waste management strategies are necessary to address pesticide issues.

Pesticide biodegradation is a new way of environmentally acceptable pesticide pollution control for a long-term environmental benefit. Microorganisms play a significant role in the breakdown of pesticides and have been recognized for their influence and many uses in human welfare. Several recent studies have demonstrated the potential of microorganisms, isolated from sewage or soil to degrade pesticides. These microbes include several bacterial and fungal strains, actinomycetes, algae, etc. (Kafilzadeh et al., 2015). The process of pesticide biodegradation, which involves bacteria and enzymes, is described in detail in the biodegradation portion of this review. The entire process of pesticide synthesis or formulation, manufacturing or mass industrial production, detrimental effects on the environment and human health, and biodegradation of pesticides has been shown in **Figure 2**. To date, there is scant information about the detailed classifications, toxicity, and remediation of pesticides in the environment. Therefore, this review article exploring the new dimensions for removal of pesticides from the environment. This review discusses the impact on living systems, bioremediation approaches, and complete residual removal of pesticides from the environment.

Classification of pesticides

The pesticides show the toxicity in the living systems on the basis of their chemical formulations and quantity in an instance. Pesticides are a broad category of products that include antiseptics, disinfectants, anti-bacterial, fungicides, algicides, rodenticides, and herbicides (Garcia et al., 2012). Pesticides are classified into two major categories based on their physical and chemical properties. Pesticide classification by nature of pesticide (synthetic and natural) and acting on pest type is illustrated in **Figure 3**. Organic chemicals made up the majority of synthetic pesticides, which were grouped into the following four groups: Organophosphates, organochlorines,

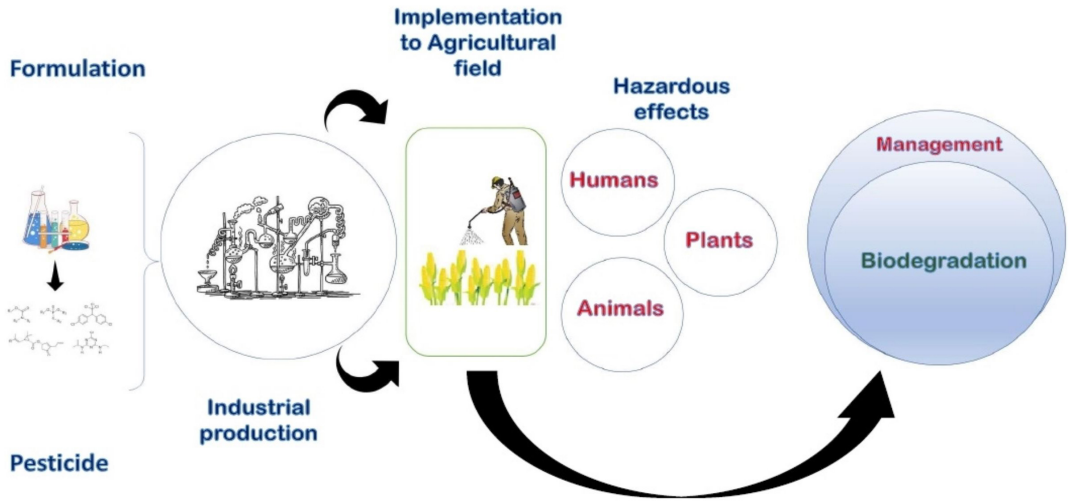

FIGURE 2
Thematic diagram of the synthesis, production, uses, effects, and eco-friendly management of pesticides.

carbamates, and pyrethroids. Some widely used pesticides and their structures are shown in **Table 1**. Naturally occurring pesticides, also known as biopesticides, are formed by living creatures such as plants, bacteria, and fungus (Abubakar et al., 2020; Bhatt et al., 2020a, 2021b).

Classification of pesticides on the basis of toxicity

The amount of pesticides used (dose) and exposure period (time) are the two most important factors for pesticide toxicity that define the acute and chronic toxicity of pesticides. Acute toxicity refers to a pesticide's toxicity to animals, plants, and humans following a definite short-term exposure of pesticide. A pesticide with a high acute toxicity is fatal, even if only a tiny quantity is absorbed into body. The World Health Organization (WHO) recognizes only acute toxicity for pesticide categorization and based on lethal dosage (LD50) divided into two types, i.e., acute cutaneous (dermal) toxicity (e.g., extremely: less than 50-mg/kg body weight of rat; highly: 50-200-mg/kg body weight of rat; moderately: 200-2,000-mg/kg body weight of rat, etc.) and acute oral toxicity (e.g., extremely: less than 5-mg/kg body weight of rat; highly: 5-50-mg/kg body weight of rat; moderately: 50-2,000-mg/kg body weight of rat, etc.) are shown in **Table 2** (World Health Organization, 2009).

The deadly impact of pesticide exposure that persists over time is known as chronic toxicity. Chronic toxicity of pesticides is a worry for the general population and those who work with pesticides directly because of possible exposure to pesticides. Pesticides are now classified into "WHO Hazard classifications" according to the widely used "WHO Recommended Categorization of Pesticides by Hazard." Following a change in 2009, such a classification was merged with the "Globally Harmonized System (GHS) Acute Toxicity Hazard Category" shown in **Table 3** (Mieldazys et al., 2015). Pesticides are also classified based on pest type, mode of action, and disease management strategies as shown in **Table 4**. Another type of classification is based on its mode of entry, which is divided into the following five categories: (1) Systemic pesticides (absorbed by animals or plants and transferred to other locations, such as in plants, entering into untreated tissues of roots, stems, or leaves *via* multidirectional movement through the vascular system), (2) non-systemic or contact pesticides (they require physical contact with the pest for their action), (3) stomach toxicants (it enters the digestive tract and is absorbed inside the insect's body; such toxicants are effective for vector control and are used for mosquito or black fly management by malathion application), (4) fumigants (these pesticides are used as poisonous gases or vapor that enter the pest respiratory system *via* spiracles and kill it), and (5) repellents (it is used to keep pests away from treated objects) (Yadav and Devi, 2017).

Migration and behavior of pesticides in the ecosystem

When pesticides are administered to a specific area or plant by a farmer, they have the potential to migrate and degrade into the environment and using indigenous microbial strains and physicochemical factors. They show a variety of effects on non-targeted plants as well as kingdom animalia after entering into the ecosystem (Tudi et al., 2021). Pesticides are degraded in our ecosystem by a variety of physical and microbiological

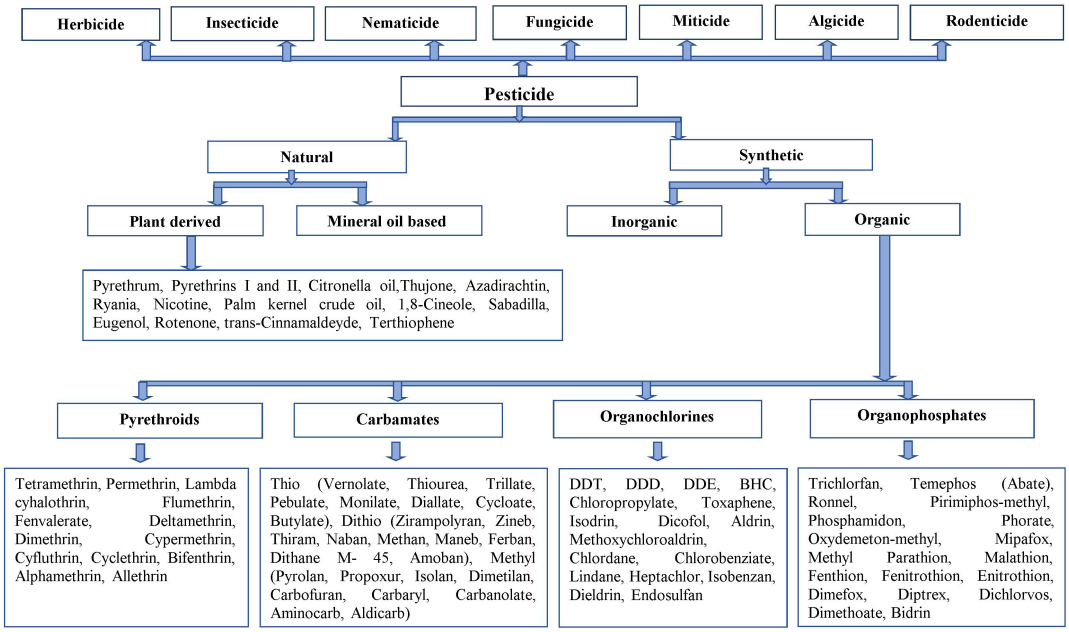

FIGURE 3
Classification of pesticides (Jayaraj et al., 2016; Hassaan and El Nemr, 2020; Malhotra et al., 2021; Souto et al., 2021; Parra-Arroyo et al., 2022).

processes, including light, temperature, moisture, oxygen, and microorganisms. Pesticides degrade into new chemical entities called metabolites, which can be hazardous or non-toxic depending on their chemical composition (Liu et al., 2015; Marie et al., 2017). Pesticides and their metabolites are transported from a targeted to a non-targeted area *via* adsorption, leaching, volatilization, or surface runoff (Tudi et al., 2021). Because there is an attraction between soil particles and pesticides in sorption systems (attraction influenced by soil organic matter and soil texture), pesticides linger in the soil for a long period of time and have a harmful effect on the soil and ecosystem (Qin et al., 2014).

Impact of physical and chemical factors on the transformation of pesticides in soil and water

Physical and chemical properties such as molecular weight, ionizability, lipophilicity, polarizability, and volatility of pesticides decide their behavior and biological activity in soil (Bailey and White, 1970; Pignatello and Xing, 1995; Gevao et al., 2000; Beulke et al., 2004). In general, pesticide fate in a soil ecosystem depends on the abiotic transformation related to its physicochemical properties and also on biological transformation related to the presence of live organisms (Różański, 1992). The physical properties make them resistant, reducing losses while chemical structures determine the persistence of pesticides in soil or the environment. These physical and chemical properties of chemical compounds are linked to their movement in soil and aquatic systems and robustness under adverse conditions (Pereira et al., 2016).

Some crucial processes, including adsorption, degradation, and movement, control the behavior and fate of pesticides in soil. Depending on how the pesticide moves, these processes are further classified into leaching, transmission, runoff, microbial and plant absorption. Pesticide transformations in the soil system may vary. Adsorption processes are based on physical forces such as van der Waals or chemical nature, such as electronic interactions (Gevao et al., 2000). Degradation of the pesticides leads to formation of free and bound residues with some altered molecular structures, which are difficult to extract (Roberts, 1984; Gevao et al., 2000). Through diffusion and volatilization, pesticides can dissipate into the atmosphere and wind or runoff leading to subsequent contamination of water bodies. The physical and chemical properties of soil and pesticides, along with other environmental conditions, are mainly responsible for their adsorption by target and non-target organisms, a phenomenon known as bioaccumulation. Chemical and physical characteristics have an impact on leaching, and vertical downward shifting from soil systems. Through the leaching process, pesticides can reach up to groundwater level, making water vulnerable to pollution. Leaching of pesticide into the groundwater in sufficient quantities can pose a hazardous risk to animal and human health. The soil with a sandy nature and low organic content acted as an unstable holding system and weakly absorbed or persistent compounds were most likely to leach-out easily. The

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TABLE 1 Generally used pesticides and their chemical structures.

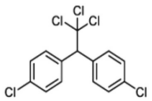
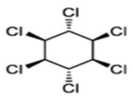
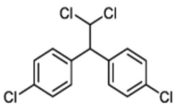
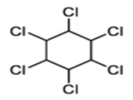
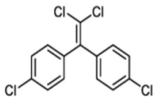
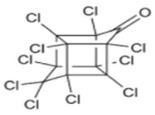
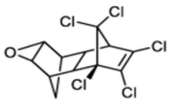
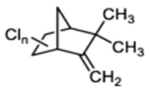
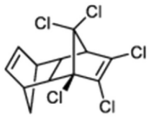
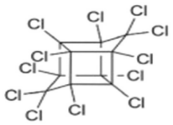
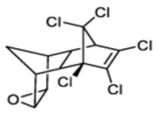
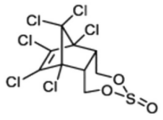
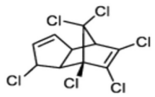
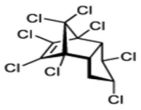
Name	Structure	Name	Structure
DDT (Dichlorodiphenyltrichloroethane)		Lindane	
DDD (Dichlorodiphenyldichloroethane)		HCH	
DDE (Dichlorodiphenyldichloroethylene)		Chlordecone	
Dieldrin		Toxaphene	
Aldrin		Mirex	
Endrin		Endosulfan	
Heptachlor		Chlordane	

TABLE 2 Pesticides classification according to WHO guidelines (World Health Organization, 2009).

Class	LD_{50} of rat		Hazardous level
	Dermal	Oral	
Ia	Less than 50 mg/kg body weight	Less than 5 mg/kg body weight	Extremely
Ib	50–200 mg/kg body weight	5–50 mg/kg body weight	Highly
II	200–2000 mg/kg body weight	50–2000 mg/kg body weight	Moderately
III	Above 2000	Above 2000	Slightly
U	5000 or above	5000 or above	Unlikely to present acute hazard

TABLE 3 Pesticides classification according to the Globally Harmonized System GHS (World Health Organization, 2009).

Category	Classification criteria			
	LD_{50} of rat dermal	Hazardous description	LD_{50} of rat oral	Hazardous description
1	Less than 50 mg/kg body weight	Lethal if come in skin contact	Less than 5 mg/kg body weight	Lethal if consumed
2	50–200 mg/kg body weight	Lethal if come in skin contact	5–50 mg/kg body weight	Lethal if consumed
3	200–1000 mg/kg body weight	Toxic if come in skin contact	50–300 mg/kg body weight	Toxic if consumed
4	1000–2000 mg/kg body weight	Harmful if come in skin contact	300–2000 mg/kg body weight	Harmful if consumed
5	2000–5000 mg/kg body weight	Possibly harmful if come in skin contact	2000–5000 mg/kg body weight	Possibly harmful if consumed

TABLE 4 Pesticides classification according to pest type, functions, and management strategies.

Type of pesticide	Type of pests	Functions	Pests and disease management	References
Aldicarb	Nematicides	Inhibit nematodes (plants parasites)	Damage tissue *via* oxidative stress, and also binds and inhibits acetylcholinesterase (AChE) (controlling acetylcholine neurotransmitter)	Yadav and Devi, 2017; Hassaan and El Nemr, 2020
	Insecticides	Inhibit insects and other arthropods also		
Atrazine	Herbicides	Destroy weeds and other plants, photosystem-II (PSII)–inhibiting	Use to control grasses and broadleaf weeds in sorghum, corn, and sugar cane crops	Gupta and Crissman, 2013; Yadav and Devi, 2017
Avitrol	Avicides	Chemicals that lethal to small seed-eating birds	Used for population management of certain birds (crows, gulls, cowbirds, blackbirds, starlings, grackles, pigeons, sparrows, red-winged blackbirds)	Yadav and Devi, 2017; NIPHM, 2018; Hassaan and El Nemr, 2020
Azoxystrobin	Fungicides	Kill fungi (blights, rusts, molds, and mildews), azoxystrobin act fungal mitochondrion, binds to cytochrome bc_1 complex and inhibit electron transport thorough oxidative phosphorylation.	Uses to kill Oomycetes, Ascomycetes, Deuteromycetes, BasidiomycetesAnd it controls disease like apple scab rusts, rice blast, powdery and downey mildew.	Yadav and Devi, 2017; Hassaan and El Nemr, 2020
Benzoxazin	Ovicides	Prevention of mites and insects egg growth	In pest managmeent	Yadav and Devi, 2017; Hassaan and El Nemr, 2020
Bifenazate	Acaricides	Control spiders and mites that feed on plants and animals by altering their growth and development. Target site of Bifenazate is mitochondrial, particularly the Q_o site of that encoded for cytochrome b	Bifenazate uses as an acaricide on strawberry, flowering plants, and nursery ornamentals	Van Nieuwenhuyse et al., 2012; Hassaan and El Nemr, 2020; Authority et al., 2021
Boric acid	Desiccants	Act on plants by drying their tissues	Use to bed bug control	Hassaan and El Nemr, 2020; United States Environmental Protection Agency [US-EPA], 2022
Copper complexes	Bactericides	Prevent bacteria with greater doses, copper works as a broad-spectrum biocide by interfering with nucleic acids, disrupting enzyme active sites, interfering with the energy transport system, cell membranes integrity disrupted	Copper complexes are used to prevent infection of seedlings from plant pathogens by seed treatment	Yadav and Devi, 2017; Hassaan and El Nemr, 2020
Copper sulfate	Algaecides	Control or kill growth of algae	Alter the algal growth and photosynthesis	Lamichhane et al., 2018; Hassaan and El Nemr, 2020
Dichlorobenzene	Moth balls	Inhibit molds and moth larvae and prevent cloths damage	Commonly used to control moths, molds, and mildew	Yadav and Devi, 2017; Eastmond and Balakrishnan, 2010
Fipronil	Termiticides	Fipronil inhibits termites by acting as a GABA antagonist and leads to excessive CNS excitation and causes death	Used in seed coatings and granular soil treatments to control unwanted arthropods in many kinds of food, horticultural, and turf plants	Cannon and Ruha, 2013; Beasley, 2020; Hassaan and El Nemr, 2020
Methiocarb	Repellents	Repel pest vertebrates and invertebrates by its taste or smell	Use as a seedling bird repellant and also effective against frit fly larvae.	Finch et al., 2014; Yadav and Devi, 2017
Methoprene	Larvicides	Prevents larvae growth	Uses as mosquito larvicide, also effective against horn flies, mushroom flies in compost, dipteran pests of livestocks, nuisance flies, highly selectivity for insects and no acute toxicity is expected in humans	Ramaseshadri et al., 2012; Monteiro and Jurado, 2014; Yadav and Devi, 2017
Metaldehyde	Molluscicides	Prevent mollusk's (snail's) usually disturbing growth of plants or crops	Use in vegetables and gardens, to kill slugs, snails, other garden pests	Yadav and Devi, 2017; Hassaan and El Nemr, 2020
Rotenone	Piscicides	Toxic and act on fishes	Uses in fisheries and fish management strategies (where unbalanced population of fish)	Gupta, 2014; Hassaan and El Nemr, 2020
Scytovirin	Virucides	Acts against viruses	Control of viral infections and diseases	Hassaan and El Nemr, 2020
Tebuthiuron	Silvicides	Specific to woody vegetation and act on it	Uses to manage the undesirable plants or unwanted forest species and apply to eliminate trees and brush or "entire forest"	Yadav and Devi, 2017
Trifluromethyl nitrophenol (TFM)	Lampricides	Target larvae of lampreys by uncoupling mitochondrial oxidative phosphorylation and ATP production reduces which ultimately leads to death	TFM used to control invasive sea lamprey (*Petromyzon marinus*)	Birceanu and Wilkie, 2018; Hassaan and El Nemr, 2020

chemical, physical, and biological factors of soil with pesticides applied for agriculture practices may influence the leaching process (Steffens et al., 2013). The various agriculture practices are responsible for pesticides translocation in soil or water and the period of their persistence in that environment can be short or longer for weeks, months, or even years due to a number of factors, which include climate change, texture of soil, pH, temperature, moisture, and the content of mineral and organic compounds (Bailey and White, 1970; Gevao et al., 2000; Gupta and Gajbhiye, 2002). Additionally, the leaching and seepage of chemical compounds depends on their mobility as well as persistence, which increases the risk of water pollution (Pereira et al., 2016).

Pesticide impact on the natural system

Pesticides safeguard around a third of all agricultural goods globally, yet their extensive usage has negative consequences for ecosystems (Zhang et al., 2011). Pesticides harm and accumulate in more other places than crops due to poor management/mishandling, or a lack of information (misuse and overuse). Label instructions on how to use and safety recommendations such as donning rubber gloves and protecting eyeglasses from exposure are not effectively followed by users (EPA Common cause of pesticide incidents) (Qu et al., 2019). Pesticides have a wide range of effects on non-targeted creatures, resulting in environmental issues (Rosell et al., 2008). In the case of air pollution by persistence organic pesticide (POP), is caused by ground and spray. Pesticides that are semi-volatile in nature adsorbed on aerosol particles. The half-lives of these particles are few days to more than a month, it depends on gas-phase reactivity. POP (which are present in the air) undergo a transformation from their native form to a highly toxic form *via* oxidation and photochemical reactions. The migration of these pesticides (POP) depends on the low solubility in water, climate-weather, temperature and humidity (Woodrow et al., 2018). Current use pesticides (CUPs) are more biodegradable in nature as well as less toxic and persistent as compared to previously used organochlorine pesticides (Chen et al., 2020).

Pesticide impact on the soil system

Pesticides are generally used to protect the crop, but there are several ways in which they can also contaminate the soil. Some of the common reasons include inappropriate use, a lack of information on how to use them in terms of amount, a high amount of runoff into water bodies, and pesticides that are adsorbed, desorb, and broken down during their passage through soil, and these phenomena are dependent on pesticide properties such as persistence, bio-accumulation, and toxicity. Because of this process, the soils become secondary sources of the pollutants with respect to air soil exchange (Pokhrel et al., 2018). According to the report, in European countries, the distribution of 76 pesticide residues was evaluated in 317 agricultural top soil samples, either they contained one pesticide or more than one (Silva et al., 2019).

The bioavailability of pesticides in the food web, pesticide uptake, toxic kinetics, dispersion, metabolism, and excretion all have an impact on species. Pesticides are used excessively and arbitrarily on various crop species, causing harm to beneficial biota such as microorganisms, honey bees, predators, birds, plants, and small animals (Alengebawy et al., 2021).

Pesticide impact on the aquatic system

Persistence organic pesticide and CUP pesticides enter into the water bodies through a variety of mechanisms, including atmospheric precipitation, chemical or pesticide manufacturing industries releasing unprocessed chemical waste into running water sources (rivers) and other water bodies, where these pesticides travel for miles and contaminate aquatic or water bodies, negatively impacting aquatic ecosystems (Socorro et al., 2016). These pesticides accumulate and transmit from lower to higher trophic levels in aquatic systems, affecting aquatic flora and fauna directly, from which these pesticides have an impact on human health through intake or other means (Woodrow et al., 2018). Chen et al. (2020) studied the aquatic system of shanghai, China and reported a high concentration of CUP (napropamide, atrazine, and chlorpyriphos).

Effect of pesticide on water eco system

Water is one of the essential elements for all forms of life on earth. About 71% of the water is covered by the earth's surface. Groundwater constitutes about 30% of the world's freshwater resources (Marsala et al., 2020). Groundwater quality is under threat due to fast population growth, urbanization, industrialization agricultural pesticides, and population stress (Jayaraj et al., 2016; Wagh et al., 2020). Pesticides may get into groundwater as a result of agricultural runoff from the field or even direct application. The presence of pesticides in water sources is a cause for worry. Pesticides are a type of hazardous chemical that poses a health risk to humans. In many places in the world, groundwater is the most significant source of drinking water. Pesticide pollution is generated from poorly managed agricultural operations and contaminates the surface

and ground water. It reduces the quality of drinking water available (Khatri and Tyagi, 2015).

Among the pesticides, organochlorine pesticides (OCPs) have been widely used across the world to control agricultural pests and vector borne diseases (malaria and dengue). Organochlorine pesticides are non-volatile compounds. The problem with using them is that they linger for a long time in natural systems. The use of these substances in an indiscriminate manner has the potential to affect the environment, drinking water systems, and human health. The OCPs' exposure over time can result in cancer, birth deformities, neurological impairment, reproductive problems, and immune system disease (Agbeve et al., 2014; Fosu-Mensah et al., 2016).

The entry of pesticides into both ground and surface water should be protected. Surface runoff and leaching carry pesticides into water bodies. These pesticides are taken up by plants in the soil, reduced into different chemical forms, and then leached into groundwater. High rainfall increases the risk of pesticides contaminating water. Pesticides that enter groundwater impair the quality of the water, making it unsafe for human consumption as well as for flora and animals. Eliminating pesticides from groundwater is a challenging process. Pesticides in drinking water have negative consequences for both individuals and the ecosystems. According to WHO, around 1 million people are poisoned acutely because of pesticide contact (Hassaan and El Nemr, 2020). To improve production, pesticides will always be a part of human existence and the environment. For pest management, an Integrated Pest Management (IPM) method should be used, which is meant to cause the least amount of environmental disruption by pesticides.

Effects of pesticides on aquatic animals

Pesticide exposure does not just harm target creatures; it also affects a variety of non-target organisms, with fish being the most notable one. Acute exposure to several pesticides resulted in the mortality of fish in certain cases, whereas lower exposure to the same chemicals resulted in deadly alterations. In many species of fish exposed to various pesticides, changes in hematological parameters such as red blood cells, white blood cells, or plasma and serum level modifications lead to histological abnormalities affecting the liver, kidneys, gills, muscles, brain, and gut (Tahir et al., 2021). Furthermore, genotoxicity has been documented in numerous cases caused by several pesticides. Fish are the lowest rung of the aquatic food chain; thus, they mirror the state of water quality and contamination. Submissive phenomena allow them to collect and store compounds such as heavy metals and pesticides, allowing contaminants in their environment to be recognized. Fish ingest a higher amount of pesticide-infected algae, phytoplankton, and other aquatic plants, causing toxic toxins to progressively accumulate in the tissues and organs of the fish. A small number of these compounds can be regulated by metabolism, while the rest bio-accumulate in the organs and organ systems of fish. Different pollutants are absorbed by the fish's gills, skin, and alimentary canal, which then disseminate into various organs and tissues, altering physiological and natural phenomena (Banaee et al., 2011). Because the gills are completely exposed to water, they are the most polluted organs. Toxicants enter the body through the gills, increasing oxygen demand. As a result, monitoring any hazardous stress in the aquatic environment is an important metric (Panigrahi et al., 2014).

The following components of a global bicycle should be addressed when determining the principal pathways of pesticide exposure to aquatic systems and biota: (1) The water column, which is frequently the first to be exposed to pesticides, (2) Algae, mosses, vascular hydrophytes, leaf litter, and branches are examples of organic substrates, (3) Inorganic substrates ranging from fine silt to coarse sand particles (Murthy et al., 2013). Pesticide levels in interstitial water and sediments are often lower than in the water column, and lithic biotopes are typically less polluted than the standing waters. Pesticides have toxic effects on aquatic creatures, including fish, at sub-lethal and deadly dosages (Khafaga et al., 2020).

Hematological causes by pesticide in fish

Fish hematological research has grown in importance as a reliable and sensitive index for assessing biological and pathological changes caused by natural or anthropogenic factors such as microbial infection or levels of contamination in aquatic sources. As a result, hematological parameters are regarded as a crucial tool for determining the body's functioning condition in response to various stresses (Ali and Rani, 2009). Pesticides changed the hematological parameters of fish in a relatively short time (Rezania et al., 2018). As a result, the hematologic index may be used to efficiently monitor the health and reaction of fish and aquatic creatures to various toxicants, displaying the ecological position of the environment and a typical way to determine the contaminant's sub-lethal effects (Pimpao et al., 2007). According to Rios et al. (2002), the blood parameters of fish were altered by several genetical and environmental factors. Pesticides affect a variety of fish characteristics, with a focus on blood parameters.

Pesticide-induced behavioral changes in fish

In several fish species, including *Tor putitora* and *Cyprinus carpio*, pesticides can cause schooling behavior, mucus

formation *via* skin's goblet cells (sliminess), motionlessness, transformations in migration activities, tumbling toward base, jumping, non-responsiveness with hyperexcitability, irregular activities, greater opercular rate (respiration increases), and modifications in body color. Furthermore, they have the ability to change and disturb aquatic vertebrate swimming behavior, such as that of fish and amphibians, as well as impair their growth rates (Stehle and Schulz, 2015). Pyrethroid exposure, decreased the function of the dopamine active transporter, resulting in unpredictable behavior (Wang et al., 2020).

Malformations and reproductive disorders caused by pesticides in fish

Pesticides may cause reproductive issues in brown trout (*Salmo trutta*) and in Atlantic salmon (*Salmo salar*) (Jaensson et al., 2007). In addition, additional studies discovered a range of developmental abnormalities in fish exposed to the herbicide (Dawar et al., 2016). Pyrethroids have been found in various studies to be harmful to fish reproductive and early embryonic stages. Pyrethroids such as bifenthrin and permethrin can cause egg proteins (choriogenin and vitellogenin) to be delayed in juvenile fish (Brander et al., 2012). Deltamethrin [second-generation (type II) pyrethroid neurotoxin insecticide] at concentrations of 20 and 40 g/L was shown to be damaging to the development of the swim bladder in zebrafish embryos reported by Wu et al. (2020).

Common effects of pesticides on fish

Pesticides have been shown to have effects on the activity of acetylcholinesterase (AChE), causing an impact on the neurological system and triggering numerous neurotoxic effects (neurotoxicity) in fish (Sharbidre et al., 2011). Fish species such as *Rhamdia quelen*, *C. carpio*, *Colisa fasciatus, Oreochromis mossambicus,* and *Labeo rohita* are affected by pesticide exposure and have also shown the alteration in AChE activity (Joseph and Raj, 2011). In addition, cypermethrin (CYP) caused neurotoxicity and apoptosis in the *Catla catla* brain (Jindal and Sharma, 2019). Pesticides also harm fish's endocrine systems (Brodeur et al., 2013). When used in large numbers, these chemical compounds may induce molecular toxicity in fish such as *Cirrhinus mrigala*, *Carassius auratus* (goldfish), and *L. rohita* (Ullah et al., 2014). According to histopathological examinations, they have a negative effect on the endocrine systems of *Oncorhynchus mykiss* and *L. rohita* (Dey and Saha, 2014). Pesticides also cause oxidative stress in *T. putitora*, *Lepomis macrochirus*, *Hoplias malabaricus*, *Oreochromis niloticus*, *Clarias gariepinus*, and *L. rohita* by affecting antioxidant defense enzyme activities and reducing the lipid peroxidation marker malondialdehyde, glutathione-*S*-transferase, glutathione reductase, and glutathione level (Muthukumaravel et al., 2013).

Effect of chemical pesticides on plants

Nowadays, chemical pesticides are widely used by farmers on agricultural land to control weeds, insects, bacteria, fungus, mollusks, rodents, etc. To combat their needs, an increasing population is demanding more foods. Pesticides are used for better crop production (Tomer, 2013). The pesticide defends crops in agricultural land and also minimizes the risk of damage during post-harvest storage. It is very effective and successful in controlling a number of diseases in plants as well as humans, such as malaria and typhoid, but on the other hand, it decreases the soil quality of agricultural land, which is the reason that their negative effects are kept in mind. In 1960, most of the technologically advanced countries banned or restricted the use of pesticides. Ideally, a synthetic or chemical pesticide must be toxic or lethal to the targeted or non-target species. Because of extensive use of pesticides, the pests and insects are going to develop resistance to transformed pesticides like DDT and escape from it.

Effect of pesticides on vegetables and fruits

The use of pesticides provides a protective layer against pod infection by other pod-feeding insect pests, but damaged pods may not yield seeds or be of poor quality and unfit for use (Mugo, 1998). The usage of chitosan at an early developmental stage boosted plant growth and development and produced higher seed output in rice and soybeans (Chibu et al., 2002). Similar work has been done by Boonlertnirun et al. (2005) in rice and Rehim et al. (2009) in maize and bean.

Pesticides impact on plant growth and metabolism

Although all pesticides are designed to eliminate or prevent certain plant or animal species, it is a great deal to know about the increasing biological as well as physiological effects of these chemicals on their target organisms. Simultaneously, there are many advantages and potential risks to the use of agrochemicals. Chemically treated seeds are often exposed to substantially greater chemical concentrations than the mature

plants during cultivation, so these benefits are countered by the danger of phytotoxicity. Herbicides suppress or control plant weeds by a variety of mechanisms with biological processes such as photosynthesis activity, mitosis cell division, function of enzymes, root and leaf development, DNA and protein synthesis, cell membrane destruction, or encouraging uncontrolled growth. The use of pesticides involves a variety of enzymatic and non-enzymatic alterations in biochemical and physiological antioxidants that can have an initial effect on plant growth from germination and ultimately affect the production of plant yield, e.g., vegetables, fruits, and seeds (Choudhury, 2019; Yengkokpam and Mazumder, 2020).

Effect of pesticides on plant growth and development

Plant (crop) growth and development do not proceed normally and lead to growth due to the life cycle of the crop, which increases seed size, dry matter accumulation, food storage material in leaves, stems, fruits and roots (Jan et al., 2012). Despite the fact that plant development is influenced by a variety of environmental, genetic, exogenous, and endogenous variables, as well as hormonal situations. Plant development, on the other hand, is an essential phase in determining their producing capability. Brecke and Duke (1980) introduced glyphosate to reduce leaf dry matter accumulation in *Phaseolus vulgaris* L. Basantani et al. (2011) observed an overall decrease in germination rate, dry weight, and root length of *Vigna radiata* after treatment with glyphosate (10 mm). Mishra et al. found that spraying high quantities of pesticides (dimethoate) shortens root and shoot length. Due to increasing levels, dimethoate concentrations in the root are higher than in the shoot (Mishra et al., 2008). Murthy et al. (2005) conducted similar research on *Glycin max L.*

Effect of pesticides on plant physiology

In the field of pesticide studies, the plant growth is hampered by pesticide accumulation in plants and causes a variety of metabolic disorders, such as chlortoluron affected the plant photosynthetic electron transport chain mechanism (Fuerst and Norman, 1991; Sharples et al., 1997), and Barry et al. (1990) was observed that the PS II reaction center was disrupted. During the photosynthetic pathway, uracil-type herbicides prevent the hill reaction and photosystem II. Reduction of total chlorophyll as well as chlorophyll a, b, and carotenoid content is increased with the increasing application of fungicide doses to plant leaves (Tort and Turkyilmaz, 2003). Sharma et al. (2018a) stated that employment of herbicide causes noxious effects on plants like necrosis, stunting, burns, chlorosis and twisting of leaves. However, Donald (2004) has observed in his experiment that excessive application of pesticides can cause a major reduction in structural vegetation of diversity. Most scientists have been recorded that use of pesticides adversely affects the plant growth and development (Sharma et al., 2015, 2016a,b,c; Shahzad et al., 2018).

Effect of pesticides on plant defense systems

The use of pesticides causes oxidative stress due to the formation of reactive oxygen species (ROS), which can finally lead to growth deficiency and reduced efficiency of photosynthesis in plants. Plants improve the toxicity because of pesticides by increasing the activity of their antioxidative defense system, which includes non-enzymatic antioxidants and antioxidative enzymes (Xia et al., 2009; Sharma et al., 2015, 2017a,b, 2018b). Plant proteins, chlorophyll pigments, and photosynthetic efficacy are all reduced by oxidative stress (Xia et al., 2006).

Effect of pesticides on human health

The human body gets exposure to pesticides either directly or indirectly. By using pesticides on crops, humans come in direct contact with them and they affect the skin, eyes, mouth, and respiratory tract, and cause acute reactions such as headache, irritation, vomiting, sneezing, and rashes on the skin. The severity of these pesticides on humans depends upon exposure time and concentration. Generally, pesticides are released from the body in the form of excretion (urinary, biliary, and secretory gland). The consumption of such vegetables and fruits that are grown in pesticide contaminated soil and water used for long-term, accumulation increase the concentration of toxins inside the body organs and causes chronic diseases such as neurotoxicity, cancer, necrosis, asthma, reproductive disorder, cardiac disease, diabetes, etc. (Kalyabina et al., 2021). The quaternary nitrogen compounds such as paraquat are associated with neurodegenerative diseases like Parkinson's, but their molecular mechanism are still not well known (Franco et al., 2010). Similarly, pesticide group of carbamates inhibits the acetylcholinesterase (AChE) activity and is used as a biomarker of neurotoxicity (Gupta et al., 2016). The cancer problem is caused by the various pesticides, but breast cancer is the most common in all cancer types and is associated with organophosphorus (malathion and parathion) that affect cellular growth and proliferation (Calaf, 2021). Similarly, autoinhibitory M2 muscarinic receptors on parasympathetic

neurons that innervate airway smooth muscle are implicated in the case of asthma by organophosphorus (Calaf, 2021). It also reduces fertility and creates genital tract anomalies in both males and females by affecting the action of endocrine hormones, their release timing, and imitating these hormones. According to several studies, organophosphorus reduces paraoxonase activity and increases the risk of coronary artery disease (Kabir et al., 2015). In several African nations, hunger and undernutrition are the most serious concerns.

Role of pesticides in genetic damage

The DNA is an important biomolecule present in living organisms that carries hereditary information and controls the biological synthesis of proteins and enzymes. It acts as the key molecular target of drugs and environmental chemicals such as pesticides. Pesticides interact with DNA and cause conformational changes that could induce gene mutations and lead to adverse health consequences such as carcinogenesis. The acute effects of such chemically synthesized compounds on human health are generally tested and reported before the market launch of these pesticides (Van der Plaat et al., 2018). However, the long-term effect of chronic exposure to pesticides has become a major concern in the last decade.

Pesticide exposure is of the following three types. (1) Direct occupational: Farmworkers who mix and spray the pesticides in agriculture fields; (2) Direct non-occupational: Rural-resident people who live near agriculture fields; (3) Indirect exposure: People who stay far from agriculture areas but get exposed to pesticides through agriculture products, the food chain and contaminated water. Occupational exposure is the most dangerous one as it is linked to a broad range of immediate effects or diseases such as lung disease and airway obstruction. A study conducted in the Dutch population reflects a significant association between the airway obstruction in farmworkers and the corresponding genomic methylation of 31 CpGs (Van der Plaat et al., 2018). Alteration in the genomic methylation pattern affects the expression and repression of genes.

Paredes-Céspedes et al. (2019) reported a notable increase of %5mC in the CpG sites of the WRAP53α gene, "antisense" gene of the p53, in mestizo urban fumigation sprayers who generally use organophosphate insecticides and pyrethroids. Such genetic modifications could act as carcinogenic agents. Differentially methylated CpGs have been found to be unique to the active ingredients of marketed pesticides such as mesotrione, dicamba, acetochlor, picloram atrazine, malathion, glyphosate, and metolachlor (Hoang et al., 2021). Occupational and non-occupational pesticide exposure, as well as chronic and high pesticide exposure in human beings, lead to altered genomic methylation. Various pesticides, including DDT, vinclozolin, methoxychlor, chlorpyrifos methyl, and organochlorine, have been reported to increase or decrease the epigenetic methylation pattern in human beings (Mahna et al., 2021).

The possible genetic damage initiated by occupational pesticide exposure is much greater than that caused by smoking and alcohol consumption (Nascimento et al., 2022). This points to the commonly unacceptable fact that pesticide exposure is much more dangerous than quitting smoking. The random effect of DNA damage in the pesticide-exposed group is roughly 4.63 times more than in the control-exposed group, according to a meta-analytical evaluation addressing probable DNA damage arising from pesticide exposure to farmers (Nascimento et al., 2022). A total of 42 studies were included in the study, with a total number of individuals 2,885 and 2,543 in the exposed and control groups, respectively. In contrast to previous studies, this study found that DNA damage induced by pesticides was not affected by the usage of personal protective equipment, pesticide type, or an individual's age and gender.

Non-farm employees who reside near agricultural grounds are exposed to pesticides through passive exposure and are thus at risk of pesticide-induced genetic destruction. Non-occupational exposure to pesticides generally corresponds to a high blood concentration of pesticides and increased DNA damage. The pesticides, being oxidizing in nature induces DNA damage *via* oxidative stress (Doğanlar et al., 2018). The literature represents that aged people, females, and children are more vulnerable to non-occupational pesticide exposure. Increased micronuclei (MN) numbers, oxidative damage, and strand breaks in DNA were seen in the peripheral blood lymphocytes of toddlers living in pesticide-sprayed areas (Kapka-Skrzypczak et al., 2019).

Non-occupational exposure to pyrethroids, a key pesticide used in agricultural and commercial locations, occurs primarily *via* residues through contaminated air and diet. The presence of pyrethroids metabolites in the human urine, including CDCCA [*cis*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid], DBCA (*cis*-2,2-dibromovinyl-2,2-dimethylcyclopropane-carboxylic acid), TDCCA [*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid], and 3PBA (3-phenoxybenzoic acid) provides an indication of non-occupational pesticide exposure. The studies to estimate the effect of non-occupational pesticide exposure on human sperm are generally conducted on men recruited from infertility clinics with normal sperm concentrations. The presence of pyrethroid metabolites in human urine is linked to sperm DNA damage increasing and the quality of semen reduces (Meeker et al., 2008).

A positive association were examined with the medium DNA fragmentation index (M DFI) percentage and CDCCA 450th as well as the percentile of 3PBA 450th and high DNA fragmentation index (H DFI) (Jurewicz et al., 2015). Non-occupational exposure to pyrethroids also increases the risk of sex chromosome disomy in sperm nuclei. Radwan et al. (2015) reported disomy in sperm chromosome YY (3PBA), XY (3PBA,

TDCCA), 18 (3PBA, CDCCA), 21 (3PBA), and total disomy (3PBA). Those with higher levels of TDCCA and CDCCA have a consistent increased risk of XY, YY, XX, and disomy in the total sex chromosome (7–30%). Males with higher levels of 3PBA displayed an increased risk of YY disomy (28%), a decreased rate of XY disomy (16%), a decreased total disomy (7%), and an increased chromosome 18 disomy (Young et al., 2013).

In reality, human beings and animals are exposed to multiple pesticides and herbicides simultaneously, which may act independently or interdependently. The pesticides organophosphates (OP) and pyrethroids (PYR) act in synergism to increase the risk of germ cell abnormalities (Figueroa et al., 2019). Earlier, Salazar-Arredondo et al. (2008) also reported the chromatin as well as DNA damage in human spermatozoa caused by *in vitro* exposure to a mixture of various organophosphorus pesticides including CPO (chlorpyrifos-oxon), CPF (chlorpyrifos), DZO (diazoxon) or DZN (diazinon), and MePO (methyl-paraoxon).

The pesticides cause DNA damage by interacting with the DNA backbone in either of three ways (1) Intercalation, (2) Grove binding, and (3) Methylation. Extensive studies have been reported in the literature that show the type of interaction between DNA and pesticides (**Table 5**). The genetic damage caused by pesticides is generally studied in animal models such as mice or rats. Dinitroaniline herbicide, pendimethalin (PND), causes significant DNA damage in the liver and kidney cells of treated rats. This damage is shown to disturb the oxidative balance and activate apoptosis genes (Ahmad et al., 2018).

Pesticides' role in cancer

Several epidemiological and molecular research highlighted a close association between persistent pesticides exposure and increased risk of diseases such as neurodegenerative disorders, endocrine disruptors, respiratory complications, reproductive disorders, and birth defects (García et al., 2017; Larsen et al., 2017; Addissie et al., 2020; Bast et al., 2021; Bhadauriya et al., 2021; Witczak et al., 2021; Gea et al., 2022; Iteire et al., 2022). In addition, the carcinogenic, teratogenic, and mutagenic nature of these compounds are also believed to be a contributing source of cancer development in the human population.

It has been observed that a person with a direct exposure to pesticides is highly susceptible to several human malignancies such as cancer including head, neck, breast, thyroid, brain, colorectal, pancreatic, lung, leukemia, prostate, non-Hodgkin lymphoma and ovarian cancer (Obiri et al., 2013; Pardo et al., 2020; Leonel et al., 2021; Lerro et al., 2021). Several pathways have been discovered to date; however, the major molecular mechanism that is likely to cause pesticide-induced carcinogenesis involves oxidative stress, genetic and epigenetic changes, and endocrine disruptions (**Figure 4**). For instance, excessive production of ROSs

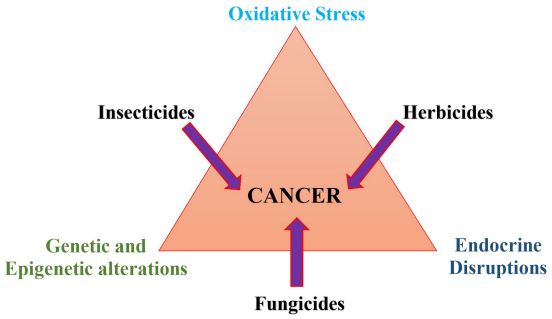


FIGURE 4
Major molecular mechanisms associated with pesticides-induced carcinogenesis.

as a result of pesticide exposure can disrupt the cellular equilibrium between pro and anti-oxidant molecules and induce oxidative stress to induce macromolecule damage, leading to dysregulation of several fundamental processes and subsequently stimulating cancer initiation, growth, progression, metastasis, and chemotherapeutic resistance (Pardo et al., 2020; Leonel et al., 2021; Lerro et al., 2021).

In a study by Želježić et al. (2018), herbicide terbuthylazine exposure was reported to form reactive terbuthylazine metabolites, which induce DNA cross-links in both *in vitro* and *in vivo* systems. Thakur et al. (2018) reported that oxidative DNA damage induced by two extensively used organophosphate pesticides, monocrotophos and chlorpyrifos, modulate the AP endonuclease 1-dependent base excision repair pathway to promote the proliferation of lung cancer. Similar toxic effects were also observed for widely used insecticide, neonicotinoid (dinotefuran, nitenpyram, and acetamiprid) exposure, which resulted in disturbance of amino acid metabolism, accumulation of lipids, and enhance oxidative stress in ICR mice *via* decreasing glutathione (GSH) level and increasing superoxide dismutase (SOD) level (Yan et al., 2020). Polymorphism in oxidative stress-related genes (catalase, glutathione peroxidase, glutathione-*S*-transferases, manganese superoxide dismutase, and paraoxonase) may not be directly linked to cancer; instead, they make people more vulnerable to pesticide-induced oxidative stress (Kaur et al., 2018; Moradi et al., 2018; Costa et al., 2019; Mbah Ntepe et al., 2020).

Endocrine disruptions are caused by agents/EDCs (endocrine disrupting chemicals) that affect the natural function of the endocrine (hormone) systems of a body by disrupting the synthesis, release, binding, specific activity or abolition of normal hormone, which are responsible for the growth, development, fertility, and homeostasis maintenance of a cell. Pesticides are well known for disrupting endocrine function *via* mimicking or delaying the release of natural hormones, thus, being accountable for decreased fertility, neurological or behavioral dysfunctions, thyroid gland

TABLE 5 Mode of interaction of various pesticides with DNA.

Pesticide	Pesticide group	Mode of interaction	References
Chloridazon or Pyrazon	Organochlorine herbicide	Intercalation via GC region	Ahmadi et al., 2011
Fenitrothion	Organophosphorus insecticide	Partially intercalation via NO_2 and the C Form conformation	Ahmadi et al., 2013
Permethrin, deltamethrin	Synthetic pyrethroid insecticides	Groove binding and partial intercalation	Ahmadi and Ghanbari, 2014
Methyl Thiophanate	Fungicide	Non-intercalative groove binding via AT region	Saquib et al., 2010
Propyzamide	Herbicide	Intercalation via AT region	Zhang et al., 2015
Edifenphos	Organophosphate pesticide	Electrostatic binding minor groove binding via AT region	Ahmad and Ahmad, 2018
Tau-fluvalinate, flumethrin	Synthetic pyrethroid pesticide	Hydrogen bonding and Van der Waals forces, minor groove binding via AT region	Tao et al., 2016
Dinitramine	Herbicide	Hydrophobic interactions,major groove binding	Daneshmehr et al., 2016
Resmethrin	Synthetic pyrethroid insecticides	Hydrogen bonds and Van der Waals forces, groove binding via GC region	Tao et al., 2015
Pendimethalin	Herbicide	Intercalation via GC region	Ahmad et al., 2016
Organophosphates	Pesticide	DNA methylation	Paul et al., 2018
Organophosphate, pyrethroids	Fumigation insecticide	DNA methylation	Paredes-Céspedes et al., 2019
Endosulfan	Pesticide	DNA hypomethylation	Mahna et al., 2021
Glyphosate	Pesticide	DNA hypermethylation	Mahna et al., 2021
Diazinon	Pesticide	DNA hypermethylation	Mahna et al., 2021
Fonofos, parathion, terbufos	Pesticide	DNA hypermethylation	Mahna et al., 2021

abnormalities, immunosuppression, and carcinogenesis (Kori et al., 2018; Pizzorno, 2018; Requena et al., 2019; Brandt et al., 2020; Montes-Grajales and Olivero-Verbel, 2020). Most of the pesticides work as agonists to activate numerous hormone receptors for instance androgen receptors, estrogen receptors, pregnane X receptors, nuclear hormone receptors, and aryl hydrocarbon receptors (Eve et al., 2020; Lacouture et al., 2022). Low dose of phenolic EDCs upregulated aromatase signaling and thus regulated aromatase-induced 17β-estradiol biosynthesis to support breast cancer cells proliferation (Williams and Darbre, 2019). Furthermore, thiacloprid and imidacloprid exposure stimulates CYP19 promoter activity, which increases estrogen biosynthesis *in vitro* in a similar manner to hormone-dependent breast cancer (Caron-Beaudoin et al., 2018). Recently, antagonistic effects of pesticides have also come into focus. For example, cypermethrin showed an inhibitory effect on the dihydrotestosterone activated interaction of the androgen receptor with its coactivators ARA70 and ARA55 (Zhen et al., 2020). Zhang et al. (2018) discovered a novel mechanism of endocrine disruption, where 16 pesticides showed anti-mineralocorticoid activity, among which 14 interfere with nuclear translocation of the mineralocorticoid receptor to promote hepatocellular carcinoma. Another novel pathway involves fungicides (Prochloraz, vinclozolin, and M2) competing with the androgen receptor, ZIP9, to block pro-apoptotic signaling in prostate cancer cells (Thomas and Dong, 2019). In another study, glyphosate was reported to inhibit aromatase signaling in a non-competitive manner while imidacloprid and thiacloprid inhibited estrogen receptor activity in MELN cells (Zhang C. et al., 2020). Overall, we observed that pesticides can alter the cellular metabolism in multiple ways to induce cancer risk. It was also observed that a person with direct or occupational exposure along with inherent genetic susceptibilities is more prone to disease.

Pesticide exposure causes allergies and asthma

The salubrious nature of pesticides makes them ideal candidates for modern agriculture techniques and enhanced crop-production. However, extensive usage of pesticides leads to serious health conditions due to their bio-magnification and persistent nature (Sharma et al., 2019). The vapors of pesticides can invade water, soil, air and finally enter the food chain, thereby threatening to human health (Sharma et al., 2017c). It has been found that food contaminated with pesticide residues leads to a higher level of toxicity compared to drinking or inhaling contaminated water or air (Margni et al., 2002). Pesticides can mimic or antagonizes natural hormones, thus disbalancing hormonal homeostasis,

reducing immunity, causing cancer and other reproduction-related problems (Yadav et al., 2015).

Studies have reported that acute or chronic exposure to such pesticides leads to airway diseases such as allergic rhinitis or asthma. The population at high-risk of developing health issues due to pesticide exposure includes mainly farm workers, pest control workers, or workers from agricultural industry, and the other environmentally exposed individuals residing near farms or agriculture fields or the individuals exposed to household pesticides (Ernst, 2002; Ndlovu et al., 2011).

More evidence of exposure to pesticides has been reported among farmers and their families along with insecticide producers or applicators across the globe, such as the United States, Canada, France, and Australia, with increased asthmatic conditions (Baldi et al., 2014). Such exposures may lead to decreased FEV 1 (forced expiratory volume in 1 s) of forced breath with exacerbation of asthma and also induction of autonomic function and altered immune response (Osteen and Fernandez-Cornejo, 2013; Henneberger et al., 2014). In relation to the use of domestic pesticides, exposure to insecticides has a particularly important role in the induction and worsening the asthma and asthma-like syndrome (Osteen and Fernandez-Cornejo, 2013). In countries such as the United States, where asthma morbidity is high due to cockroach sensitization, insecticides are used to control exposure, which in turn increases pesticide exposure, and asthma morbidity (Garthwaite et al., 2012).

Another study on farm operators showed a significant association between current asthma and lifetime allergic rhinitis by the use of carbaryl and 2,4-dichlorophenoxyacetic acid. Approximately 40% of 2.1 million farm operators had lifetime allergic rhinitis in 30% farmers and 5.1% has current asthma (Patel et al., 2018). Some synthetic insecticides, such as pyrethroid, used to control mosquitoes are known to cause asthma attacks, while permethrin and Sumithrin are key contributors to headaches, tremors, convulsions, asthmatic attacks, and can be lethal in more serious conditions (EPA et al., 2009; Amaral, 2014). Not much is known about specific pesticides responsible for allergic/asthmatic exposure. Studies from Canada, Spain, India, or South Africa demonstrated that pesticides belonging to class organophosphates and carbamates are particularly involved in causing asthmatic conditions (Hernández, 2015). These studies mainly performed lung function assays such as spirometry, and lung volumes/capacity, but none has involved primary inhalation challenge testing.

Effect of pesticides on asthma

Pesticide use and asthma incidences were reported in the common people as reported by some of the studies performed in the United States population. The US urban population was found to be chemically intolerant to at least three commonly used chemicals such as paints, pesticides, perfumes, or car exhaust. Subjects reported asthmatic and respiratory symptoms such as shortness of breath with wheezing and chest tightness (Baldwin et al., 1997; Amaral, 2014). A cross-sectional study of US National Health and Nutrition showed an association of residential pesticides with respiratory problems in children, mostly used in the kitchen or dining area (Xu et al., 2012). The incidence of such residential exposures has increased in the United States from 1.1 to 4.4 per million (Amaral, 2014; Hudson et al., 2014). Indoor air pollution, caused by pesticide spraying or the use of over-the-counter insecticides, has exacerbated symptoms such as irritation, lower respiratory pain, wheezing, dyspnea, and dry cough. In a randomized investigation of 25 asthmatic participants exposed to modest amounts of aerosols, asthmatic symptoms worsened when compared to a control group (given water). Asthmatic patients had a more than 15% decrease in FEV1 and severe bronchial responsiveness, with symptoms affecting the chest, nose, and eyes (Salome et al., 2000).

Previously, it has been reported that allergic asthma was relatively more common in children than in adults. The risk of environmental exposure to pesticides was higher for school children, especially those living near farms or rural areas (Matthews, 2005; De Barros Rodrigues et al., 2022). Children with acute symptoms have been reported due to pesticide drift near their schools, or they might be at even higher risk because of accidental contact while playing on agriculture farms with empty containers of contaminating materials (Buralli et al., 2020). In a longitudinal study, children living in agricultural communities had higher amounts of the dialkylphosphate (DAP) metabolite in their urine. The DAP metabolites are general to organophosphorus pesticides and are responsible for the temporal pattern of children's pesticide exposure upon pesticide spraying in an agricultural region (Koch et al., 2002). Other factors for children's hospitalization related to pesticide exposure are their increased respiratory rate, comparatively larger surface area of skin, and elevated metabolic rate (Sharma et al., 2019).

A few studies investigated the role of allergic asthma as well as other respiratory symptoms due to pesticide exposure among women. The studies were mainly focused on male workers, associated directly or non-directly with agricultural fields, but it was evident that women are also increasingly affected and at high-risk due to pesticide exposure (Ndlovu et al., 2011). In a study, Hoppin et al. (2008) evaluated pesticide and occupational exposures as risk factors for farm women. Out of 25,000 women with atopic and non-atopic asthma, who grew up on farms and used pesticides, were more likely to develop atopic asthma than the non-users. In an infant's environmental health birth cohort study of 266 mothers in Costa Rica, by performing a survey, they investigated the outcomes of respiratory and allergic conditions in mothers upon

exposure to pesticides and other environmental metabolites. The study found significant association of high asthma score and urinary levels of thiabendazole metabolite in women living near waste burning farms and women living in agriculture farms reported eczema and itch rash (Garry, 2004; Alhanti et al., 2021). Another study linked pesticide exposure to changes in the serum metabolome after eating fruits and vegetables (FVs). The study analyzed 171 women under infertility treatment and showed significant associations of metabolic pathways upon the eating of either high or low-to-moderate pesticide residue FVs. Different biological pathways were associated with the intake of high or low pesticide residues, including metabolism (energy, cellular receptor, enzyme, lipid, and vitamin) and intracellular signaling (Hood et al., 2022). There is a need to perform more such unique studies about associations between environmental and occupational pesticide exposures and respiratory and allergic diseases. Such an insightful study related to dietary intake of pesticides might provide information on potential mechanisms associated with human diseases.

A link between food allergies and pesticides

Food allergy affects up to 10% of the world population, with more severity in infants as compared to adults. It has been referred to as the "second wave" of the allergy epidemic, following asthma (Loh and Tang, 2018). In parallel, the use of pesticides such as organophosphates has been increased in agriculture and industries. This increased use of organic agents might prolong the allergic manifestations in atopic individuals by potential mechanisms such as epigenetic control of allergen expression, modifying proteins to make them even more allergenic; or increased polyamine production in stressed condition (Falak et al., 2012; Loh and Tang, 2018).

People who are exposed to chemicals either through chlorinated water or come into contact with foods that contain them or breathe polluted air are more likely to develop food allergies. Chemicals like dichlorophenols can alter the microbiota of the human body and in turn influence the body's immune system to trigger such reactions. In contrary to hygiene hypothesis, dichlorophenols can kill microbes and clear the environment such that young children become prone to developing allergy risks. In an international survey of the United States (NHANES) in the period 2005–2006, 2,200 children aged 6 were checked for dichlorophenol levels in their urine along with allergies to peanuts, eggs, milk, and shrimp. It was found that children with high levels of urine dichlorophenol were 80% more likely to develop allergies (Jerschow et al., 2012).

An ample number of studies have been performed related to pesticide exposure and asthma, but a lot more meticulous studies need to be accomplished. The previous data was generated accordingly self-reported or doctor-diagnosed asthma, which needs to be refurbished with bronchial responsiveness measurements and lung function. To strengthen the data, a detailed molecular and genetic phenotyping must be explored to study the effect of pesticides in different types of asthmatic conditions (Jerschow et al., 2012; Loh and Tang, 2018). Studies on different active and organic ingredients or new formulations along with potent agents might provide important insights, such as between asthma and exposure to pesticides. The recent cohort studies identified certain biomarkers directly linked to pesticide exposure and asthma, thus new biomarkers for the different and generally used pesticides can be considered. More robust measurement of pesticide exposure depending upon the biomarkers should be the focus of the future comprehensive studies. Their metabolic rate, bioactivity, life time, and threshold levels must be recorded to understand the pathophysiology of the underlying asthmatic or atopic conditions. Finally, more longitudinal studies offering a large sample size over a longer period of time can be a big step toward understanding the biological pathways at the gene level that can directly link pesticide exposure to disease development.

Pesticide effects on preserved food

Pesticides play a global role in the protection, preservation, comfort of food, fiber, and human health (Winteringham, 1971). However, the excessive and uncontrolled use and misuse of pesticides, as well as their long-run transportation and volatility, cause widespread environmental damage or contamination. Moreover, the occurrence of many highly toxic, non-patented, and eco-resistant chemicals creates severe health concerns that causes global impact simultaneously (Ecobichon, 2001). In India, the value addition and processing of ready-to-eat (RTE) or ready-to-serve (RTS) packaged products impact a lot on monitoring the levels of pesticide residues during the final consumption. However, during the processing of raw agricultural commodities (RAC), the levels of pesticides are mostly governed by the concentration level and physico–chemical characteristics of the product to be processed (Muralidhara et al., 2022). Researchers reported that pre- or post-processing steps are capable enough of reducing the load of pesticides in the final product. However, in certain specific cases, processing aids in the accumulation of pesticide residues (e.g., extraction of oil from oil seeds) (Kaushik et al., 2009; Muralidhara et al., 2022). Therefore, a maximum residual limit (MRL) of pesticides needs to be established in the case of food products attaining paramount

exposure to pesticides during their pre-harvesting phase (Scholz et al., 2017).

Processing factor (Pf) – During the processing of foods, there is a chance that the whole mass of pesticide residues can be assimilated into processed products. Therefore, the effect of pesticide residues on food products can be expressed by a term "processing factor" and can be calculated as follows.

$$\text{Processing factor (Pf)} = \frac{\text{Pesticide concentration in raw product}}{\text{Pesticide concentartion in processed product}}$$

The processing factor is an integral tool to generate data for global regulatory authorities monitoring the residual limits and also helps in assessing the risks by estimating the refined dietary exposure of pesticides in a processed food commodity before consumption (OECD, 2008).

Effect of pesticide residue on processing operations

Processing operations play a significant role in maintaining or lowering the pesticide limit in the final value-added processed products aiding enhanced shelf-life and better product quality; however, certain processing steps impact negatively by enriching the level pesticide residues in the final product by developing toxic metabolites or second- and third-generation derivatives. Post-harvesting operations such as washing, peeling, chopping, etc. help in reducing the pesticides on the surface of fruit and vegetable commodities (Yigit and Velioglu, 2020). The heat treatments such as pasteurization, sterilization, blanching, frying, boiling, cooking, etc. help in the reduction of pesticides by chemical reactions due to oxidation and hydrolysis of chemical compounds. Also, low moisture content, pH, and time–temperature combination during cooking also modulate the residual pesticide limit in the final product. Similarly, unit operations such as drying and grinding of samples, canning of food products, etc. abundantly reduce the residual limits by evaporating water and altering the physico–chemical nature of pesticides (Kaushik et al., 2009). However, the unit processing operations such as cereal grain processing, fruit processing, oil extraction, grape, egg drying, and so on have a high risk of increased levels of residual pesticides and are affected by a variety of factors such as the physico–chemical behavior of pesticide molecules, produced metabolites during the chemical process, their photostability, lipophilicity, thermal stability, and polarity (Scholz et al., 2017).

Determination of pesticide residues in food matrix

The determination of the residual pesticide limit in RTE/RTS foods involves a complex phenomenon and requires some special criteria. The extractability of a pesticide residue depends on the biochemical nature and behavior of food. The complexity of a matrix behavior is often increased by the processing operations involved, which impacts the performance method by decreasing precision as well as accuracy. Therefore, usage of matrix-matched calibrations and selective clean-up practices are necessary to avoid such issues (Law et al., 2019). The worldwide harmonization of maximum residual limits (MRLs) for pesticide residues in raw agricultural commodities has attained a high recognition. Similarly, in India, food technologists and central agency such as Food Safety and Standard Authority of India (FSSAI) are now emphasizing too much toward a sustainable growth in the processed food sector for making and consumption of value-added items with safe or lower residual limits of pesticides (Muralidhara et al., 2022).

Eco-friendly management of pesticides as bioremediation

Physical and chemical cleaning of pesticides release more toxic compounds, and both are harmful as well as costly. To maintain a sustainable environment with a healthy and productive ecosystem, eco-friendly approach as bioremediation methods is available to remove harmful contaminants (Desisa et al., 2022). Since plants, algae, fungi, bacteria, and their interactions are used to remove toxins *via* bioremediation, which serves as a cost-effective and environmentally benign method. Pesticide remediation today includes a variety of environment friendly techniques, such as phytoremediation, microalgae bioremediation, myco-remediation, and bacterial pesticide degradation (Singh et al., 2020).

Phytoremediation is an economical, solar-powered method that involves the removal or reduction of harmful chemicals from damaged sites using effective plant species. *Kochia* sp., *Triticum* spp., *Ricinus communis* and *Ceratophyllum demersum* are well-known plant species that have played a significant role in the removal of atrazine, lindane, chlorpyrifos, and endrin, respectively. The absorption of pesticides by plants results in the conversion of hazardous pesticides into less toxic compounds, which helps to remove toxic pollutants from polluted sites. Plants use various mechanisms to remove pollutants, including pollutant transpiration (phytovolatilization), clean-up through the rhizosphere microbiome (rhizo-degradation), enzymatic degradation (phytodegradation), and pesticide accumulation in different plant parts (phytoextraction). Such plants also

improve the landscapes, reduce soil erosion, and prevent pollutant seepage. In addition, phytoremediation serves as an economic, safe, and green approach for chemical waste treatment (Subashini et al., 2007; Gill and Garg, 2014; Mishra et al., 2015; Rissato et al., 2015; Kuppusamy et al., 2016; Main et al., 2017; Mir et al., 2017; Koranteng et al., 2018; Perez-Lucas et al., 2018; Singh et al., 2020).

Microalgae are also known as effective biosorbents of heavy metals and pesticides and can remove them from contaminated areas. *Chlamydomonas reinhardtii*, *Chlamydomonas mexicana,* and *Dunaliella* sp. have been reported for the removal of prometryne, atrazine, and mirex pesticides, respectively. Such photoautotrophic organisms exist in different forms in nature and are involved in the conversion of radiant energy (light energy to chemical energy). The use of microalgae results in the production of oxygen, which preserves the environment's balance. Oxygen generated from microalgae also helps the bacteria during the biodegradation process. Microalgae have been found to use chemical pollutants as an energy alternate and to accelerate the biodegradation process. It can be used to achieve a variety of objectives, including nutrient recovery from wastewater, biomass formation, removal of contaminants (bioaccumulation and biosorption), and being able to grow under stress conditions. In which, bioaccumulation is an energy-dependent active process involving living organisms that metabolize pollutants. Whereas biosorption is an energy-independent process that involves both dead and living organisms for the removal of contaminant form polluted environments. The use of such technology in a two-way manner, such as pesticide accumulation as well as conversion of toxic into less toxic compounds. The degradation is influenced by the introduction of potent microalgae, optimum conditions, and the chemical composition of pesticides. In addition, there are some major factors that alter the degradation process of pesticides, such as molecular weight, functional group, concentration, and water solubility. Under stress conditions, these microalgae act mixotropically and derive their energy from light and organic carbon, which gives them an advantage over bacteria and fungi during biodegradation (Velasquez and Dussan, 2009; Chojnacka, 2010; Mata et al., 2010; John et al., 2011; Subashchandrabose et al., 2011; Monteiro et al., 2012; Rath, 2012; Chekroun et al., 2014; Kabra et al., 2014; Torres et al., 2017; Singh et al., 2020).

Myco-remediation is another type of biological approach to pesticide waste management, where fungi can use such pollutants as a carbon source and convert them into less toxic compounds, thus cleaning them from the water and soil system. Fungi are ideal among microorganisms due to their structural morphology, which contains hyphae, that allows the transfer of small chemical molecules by microscopic pores easily. The mycelium networks have a multi-functional role, in addition to accelerating pesticide degradation, they also improve the plant's nutrient and water availability. Ligninolytic fungi are known to secrete a variety of extracellular enzymes that aid in the transformation of recalcitrant chemical compounds. While saprotrophic fungi excrete the most enzymes, followed by other fungi (soft rot, white rot, and brown rot). White-rot fungi (*P. Pleurotus ostreatus*, *Trametes hirsutus*, and *Cyathus bulleri*) are widely known for pesticide biodegradation due to their extracellular enzyme complex (e.g., laccase, manganese peroxidase, and lignin peroxidase) acting non-specifically. The consortium of potent fungal species was found to be suitable for chlorpyrifos and DDT biodegradation. The phyla Zygomycota, Ascomycota, and Basidiomycota are reported for biodegradation *via* attacking on functional groups (dehydrogenation, demethylation, hydroxylation, etc.). This process is also influenced by other factors such as optimal temperature, pH, moisture, nutrient, and water availability, all of which play a significant role in pesticide degradation. Nowadays, many developing countries cannot afford biopesticides or cannot avoid the use of chemical pesticides, so they need to use myco-remediation or other bioremediation approaches to control pesticide pollution in a parallel manner (Tortella et al., 2005; Huang et al., 2008; Sagar and Singh, 2011; Adenipekun and Lawal, 2012; Chen et al., 2012; Wu et al., 2015; Maqbool et al., 2016; Janusz et al., 2017; Singh et al., 2020).

Bacteria have been widely reported to degrade and remove pesticides as compared to other remedial approaches. *Pseudomonas*, *Azotobacter*, *Flavobacterium*, and *Arthrobacter* are the major bacterial genus involved in the removal of pesticides from polluted environments. The discovery of pollutant-degrading bacteria aided by advances in genetic engineering methods. These microbes use the pesticide for nutrients, generate H_2O and CO_2, and overcome the environmental risk associated with pesticides. In the soil system, such pesticides accumulate and act as electron donors and carbon sources for soil microorganisms. The environmental conditions, pesticide exposure time, and concentration, bacterial type, and growth factors (such as temperature, pH, moisture, nutrient, and water availability) all are important for efficient biodegradation. However, the presence of sulfate and chloride act as anion and bind strongly to microbes that blocks the microbial action on pesticides. The chemical structure is the first target of microbial degradation and converted into inorganic components that are further utilized by the microorganism. Advanced approaches such as bioaugmentation, bio-stimulation and natural attenuation are employed to increase the pesticide biodegradability, which includes potent bacteria, nutrient addition, and the introduction of native species to the contaminated site respectively. *Alcaligenes, Flavobacterium, Acinetobacter* are reported as endosulfan degrading bacteria. Similarly, *Stenotrophomonas* sp. also known for almost 100% removal of diazinon from the contaminated site. The bacterial system is well studied as compared to other bioremediation technologies. The diverse bacterial groups and their corresponding enzymes responsible

for degradation are explained in the "Biodegradation of Pesticide Pollutants" section (Gavrilescu, 2005; Singh and Walker, 2006; Arias-Estevez et al., 2008; Huang et al., 2008; Singh et al., 2011, 2020; Laura et al., 2013; Rani and Dhania, 2014; Adams et al., 2015; Deng et al., 2015).

Biodegradation of pesticide pollutants

Biodegradation of pesticides is mainly mediated by using microbial systems. Microbes are able to produce a specific group of enzymes that are able to catalyze the pesticides from contaminated sites. The pure culture and mixed cultures of the bacteria and fungi were found to be effective in the removal of pesticide residues from the water and soil environment. Microbial consortium was found with superior degradation abilities (Bhatt et al., 2021c). Singh et al. (1999) found that microbes have developed a number of metabolic routes to breakdown or detoxify a variety of environmental contaminants, including pesticides. Conde-Avila et al. (2021) reported bacteria from the genera *Streptomyces*, *Flavimonas*, *Burkholderia*, *Micrococcus*, *Sphingomonas*, *Brevibacterium*, *Flavobacterium*, *Pseudomonas*, *Agrobacterium*, *Arthrobacter*, *Enterobacter*, and *Bacillus* are associated with pesticide biodegradation. There is a diverse group of bacteria and fungi that are capable of degrading pesticides. The different phyla include Bacteroidetes, Basidiomycota, Chlorophyta, Cyanobacteria, Actinomycetota, Firmicutes, and Proteobacteria. The bacteria that fall under Actinobacteria have a tremendous capability to degrade several classes of chemical pesticides as most of the strains have high GC content and are actively used for the recycling of complex polymers. *Streptomyces*, *Nocardioides*, *Arthrobacter*, *Rhodococcus*, *Micrococcus*, and *Microbacterium* are members of the *Actinomycetota* phylum and can metabolize a variety of chemical compounds such as organochlorides, organophosphates, carbamates, triazinones, and others (Kim et al., 2017). Similarly, Firmicutes are also play a critical role in pesticide biodegradation. Among them, several strains possess endospores that are resistant to any adverse condition and are reported as extremophiles. There are a number of firmicutes that are capable of degrading pesticides, including *Paenibacillus polymyxa, Bacillus licheniformis*, *Bacillus thuringiensis*, *Bacillus pumilus*, *Bacillus subtilis*, and *Bacillus cereus* (Patil et al., 1970). Moreover, among the proteobacteria, α-, β-, and γ-proteobacteria have also been reported for their pesticide degradation activity.

Among the α-proteobacteria strains that have been reported are *Sphingomonas*, *Rhizobium*, *Methylobacterium*, *Azospirillum*, *Pseudaminobacter*, *Bosea*, *Mesorhizobium*, *Shinella*, and *Ochrobactrum*. Moreover, *Ralstonia*, *Alcaligenes*, *Burkholderia*, *Achromobacter*, and *Cupriavidus* are the reported bacterial strains among β-proteobacteria. Furthermore, reported bacterial strains among γ-proteobacteria are *Yersinia*, *Pseudomonas*, *Klebsiella*, *Acinetobacter*, *Serratia*, and *Xanthomonas* (Bhatt et al., 2020b; Kumar et al., 2021). Microbes and their enzymes associated with biodegradation of different types of pesticide are shown in **Tables 6**, **7**.

The basic stages of pesticide conversion were characterized by Kumar et al., 1996 as follows: (1) Mineralization: Carbon dioxide or methane as an end-product of complete degradation; (2) Detoxification: Conversion of toxic to non-toxic compounds; (3) Co-metabolism: Microbes involved in the metabolism process of compounds without benefiting themselves from these compounds; (4) Activation: Activation of compounds. During the beginning of 1064, hydrolases and oxygenases came in knowledge and Singh et al. also reported involvement of these enzymes in pesticide biodegradation (Bollag et al., 1968; Tiedje et al., 1969; Singh et al., 1999). Under both denitrifying and aerobic conditions, hydrolytic dehalogenation (the substitution of a halogen group by a hydroxyl) can occur, but only methanogenic and sulfonic circumstances result in reductive dehalogenation, which involves the substitution of a halogen group by a hydrogen group. Furthermore, biotransformation events such as polymerization and methylation may occur, resulting in more hazardous or recalcitrant compounds. Different methods for converting hazardous pesticides were used, depending on their chemical constituents and the microbes that were used for bioconversion (Singh et al., 1999). Factors such as the microbial culture, cultivation technique, size of inoculum, growth under elevated pesticide percentage, adaptation, rhizosphere interactions, and response against the environmental factors can all affect the pesticide degradation process (Conde-Avila et al., 2021). Research has concentrated on the practice of microbial cellular immobilization (CI) technology in several materials and supports the long-lasting survival of microbes. Now, research has shifted to the use of microbial cells as CI, which protects and allows them to be reused. Such a strategy enhances the possibilities of techniques lasting and succeeding in a pesticide-contaminated environment for a long period and has been found suitable for pesticide biodegradation (Colla et al., 2014; Pradeep and Subbaiah, 2016; Fernández-López et al., 2017; Conde-Avila et al., 2021).

The CI technology has served as an environmentally approachable processes for waste management practices. The use of CI of degrading microbes in the elimination and or degradation of pollutants, the CI system has developed as an eco-friendly alternative approach. There are certain disadvantages to CI technology, such as microbial interactions with the immobilization material and its impact on microbial survivability (Conde-Avila et al., 2021). When CI is utilized instead of free cells, the percentage of clearance and efficiency increases for pesticides including chlorpyrifos, atrazine, difenoconazole cypermethrin, carbaryl, endosulfan, and carbofuran. The benefits of utilizing CI are independently supported by the immobilization method or substance employed (Bhadbhade et al., 2002; Pattanasupong et al., 2004;

TABLE 6 Pesticides degrading microorganisms.

Type of pesticide	Example	Microorganism	References
Organophosphorus	Chlorpyrifos	*Bacillus* spp., *Pseudomonas* spp., *Arthrobacter* spp., *Micrococcus, Flavobacterium, Bacillus licheniformis, Cupriavidus* spp., *Burkholderia caryophylli, Brevundimonas diminuta, Spirulina platensis, Synechocystis*	Nandhini et al., 2021; Lourthuraj et al., 2022
Organophosphorus	Parathion	*Pseudomonas diminuta, Flavobacterium* spp., *Pseudomonas stutzeri, Arthrobacter* spp., *Agrobacterium radiobacter, Bacillus* spp., *Xanthomonas* spp.	Mali et al., 2022; Saravanan et al., 2022
Organophosphorus	Methyl parathion	*Pseudomonas* spp., *Bacillus* spp., *Plesiomonas* spp., *Pseudomonas putida,*	Dong et al., 2005; Singh and Walker, 2006; Parakhia et al., 2014
	Glyphosate	*Pseudomonas* spp., *Alcaligene* spp., *Bacillus megaterium, Rhizobium* spp., *Agrobacterium* spp., *Arthrobacter atrocyaneus, Geobacillus caldoxylosilyticus,*	Huch et al., 2022; Zhang et al., 2022
Organophosphorus	Coumaphos	*Nocardiodes simplex, Agrobacterium radiobacter, Pseudomonas diminuta, Pseudomonas monteilli, Flavobacterium* spp., *Nocardiodes Strain B-1*	Singh and Walker, 2006; Blatchford et al., 2012
Organophosphorus	Monocrotophos	*Pseudomonas* spp., *Bacillus subtilis, Arthrobacter* spp., *Peudomonas mendocina, Bacillus megaterium, Arthrobacter atrocyaneus, Pseudomonas aeruginosa, Clavibacter michiganense*	Kaur and Goyal, 2019
Organophosphorus	Fenitrothion	*Flavobacterium* spp., *Arthrobacter aurescenes; Burkholderia* spp.	Singh and Walker, 2006; Hong et al., 2007
Organophosphorus	Fenthion	*Bacillus* spp.	Aislabie and Lloyd-Jones, 1995
Organophosphorus	Diazinon	*Flavobacterium* spp., *Pseudomonas* spp., *Arthrobacter* spp.	Aislabie and Lloyd-Jones, 1995; Singh and Walker, 2006
Organophosphorus	DDT	*Alcaligene eutrophus*	Aislabie and Lloyd-Jones, 1995
Organochlorine	Aldrin	*Micrococcus, Bacillus polymyxa, Flavobacteria, Pseudomonas fluorescens, Phlebia aurea, Phlebia acanthocystis, Phlebia brevispora*	Bose et al., 2021
Organochlorine	Dieldrin	*Pseudomonas fluorescens, Phlebia aurea, Phlebia acanthocystis, Phlebia brevispora*	Bose et al., 2021
Organochlorine	Endosulfan	*Pseudomonas aeruginosa, Pseudomonas fluorescens, Mortierella* sp., *Trametes hirsute, Aspergillus niger*	Romero-Aguilar et al., 2014; Bose et al., 2021
Organochlorine	Alpha endosulfan	*Fusarium ventricosum,Klebsiella, Acinetobacter*	Siddique et al., 2003; Bose et al., 2021
Organochlorine	Beta endosulfan	*Fusarium ventricosum*	Siddique et al., 2003
Organochlorine	Dichlorodiphenyl-trichloroethane	*Trichoderma harzianum, Stenotrophomonas* sp., *Sphingobacterium* sp., *Pseudomonas* sp., *Trichoderma hamatum, Rhizopus arrhizus,*	Ortiz-Hernández et al., 2013; Russo et al., 2019; Bose et al., 2021
Organochlorine	Lindane	*Microbacterium* sp. P27, *Paracoccus* sp. NITDBR1, *Streptomyces* sp. A5, *Streptomyces* sp. M7, *Pleurotus eryngii, Pleurotus florida, Pleurotus sajor-caju, Phanerochaete chrysosporium*	Rigas et al., 2005; Xiao and Kondo, 2020; Zhang W. et al., 2020
Triazone	Atrazine	*Nocardia* spp. *Pseudomonas* spp., *Rhodococcus* spp.	Aislabie and Lloyd-Jones, 1995
Carbamate	Carbafuron	*Achromobacter* spp., *Pseudomonas* spp., *Flavobacterium* spp.	Aislabie and Lloyd-Jones, 1995
	EPTC	*Arthrobacter* spp., *Rhodococcus* spp.	Aislabie and Lloyd-Jones, 1995
	Carbafuron	*Achromobacter spanius, Diaphorobacter polyhydroxybutyrativorans*	Rahman et al., 2018
Avermectin	Emamectin Benzoate	*Achromobacter spanius, Diaphorobacter polyhydroxybutyrativorans*	Rahman et al., 2018
Neonicotinoid	Thiamethoxam	*Achromobacter spanius, Diaphorobacter polyhydroxybutyrativorans*	Rahman et al., 2018

Adinarayana et al., 2005; López-Pérez et al., 2006; Fuentes et al., 2013; Zucca and Sanjust, 2014; Abigail and Das, 2015; Chen et al., 2015; Tallur et al., 2015; Bhatt et al., 2016; Fernández-López et al., 2017). Because pesticides come in such a wide variety of chemical groups, the factors that influence their presence, transit, and mobility are complicated and difficult to anticipate. Extrinsic and intrinsic variables govern adsorption-desorption, biodegradation, volatilization, photodegradation and breakdown phenomena, which mediate pesticide occurrence, and migration (Conde-Avila et al., 2021). Soils with high organic matter reduce pesticide availability through adsorption to a larger percentage than sandy soils (Yanez-Ocampo et al., 2016).

Bioremediation procedures frequently include organic wastes and/or specialist strains with catabolic capabilities against contaminants to assist the breakdown of more persistent

TABLE 7 Bacterial enzymes, responsible for the degradation of pesticides (Ortiz-Hernández et al., 2013).

Pesticide	Enzyme	Bacteria
Gylphosate	Oxidoreductase (Gox)	*Pseudomonas* spp., *Agrobacterium* spp.
Endosulfan, aldrin, malathion, DDT, endosulfate	Monooxygenases (Esd)	*Mycobacterium* spp., *Arthrobacter* spp.
Hexachlorobenzene, Pentachlorobenzene	P450	*Pseudomonas putida*
Trifluralin	Dioxygenases (TOD)	*Pseudomonas putida*
Hexachlorocyclohexane	Haloalkane Dehalogenases (Lin B)	*Sphingobium* spp.
Chloro-S-trazina	AtzA	*Pseudomonas* spp.
Chloro-S-trazina	TrzN	*Nocardioides* spp.
Hexachlorocyclohexane (Gamma isomer)	Lin A	*Sphingobium* spp.
2,4-dichlorophenoxyacetic acid	TfdA	*Ralstonia eutropa*
Pyridyl-oxyacetic acid	TfdA	*Ralstonia eutropa*
Pyridyl-oxyacetic acid	DMO	*Pseudomonas maltophilia*
Phosphotriester	Phosphotriesterases (OPH/OpdA)	*Pseudomonas diminuta, Agrobacterium radiobacter, Flavobacterium* spp.

pesticides or to reduce their influence on microorganisms. Using genetically engineered strains to breakdown pesticides might be an effective method. Pesticide-exposed native species can develop the capacity to degrade toxic chemicals. Such technology was created to clean up pesticide-related pollutants (Barreiros et al., 2012; Nikel et al., 2014; Castillo et al., 2016; Bhatt et al., 2019a,b,c, 2020c, 2021d).

In comparison to pure cultures, the introduction of consortia or pesticide primed materials has been found to improve pesticide breakdown and mineralization capability in BPSs (bio-purification systems) (Sniegowski and Springael, 2015). Furthermore, Biobed bioremediation systems can be an ideal microcosm for developing specialized microorganisms capable of enhancing pesticide residue metabolization from wastewaters (Dunon et al., 2013). However, the bioaugmentation strategy for various pesticide biodegradations in wastewaters at high concentrations, as occurs in real-world scenarios, is still little known (Sniegowski and Springael, 2015).

Conclusion

Pesticide use has expanded extensively in the recent years, resulting in the environmental damage, particularly water and soil contamination. Pesticides come in a variety of forms, but organophosphates, organochlorine, carbamate, and pyrethroids are the most abundantly uses pesticides and have human and environmental concerns. Refined knowledge of various properties related to the physical and chemical background of pesticides are necessary to determine the impact and behavior of pesticide transformation in that environment. Such pesticides need proper management strategies for converting them to non-toxic compounds before releasing them into the environment. They are the most persistent and generally resistant to degradation under natural conditions. The scientific community has been working hard to come up with creative approaches to pesticide pollution reduction. Environmentally friendly management strategies include several bioremediation approaches and servers to solve pesticide problems or develop alternative green solutions. Bioremediation strategies such as phytoremediation, microalgae bioremediation, myco-remediation, and microbial degradation are also cost-effective and environmentally benign methods. Nowadays, microbial degradation methods are used extensively. Microorganisms and their enzymes play a key role in the breakdown of chemical compounds and synthetic pesticides. Although these methods are environmentally friendly, they have certain limitation such as metabolic routes followed by microbes are highly influenced by external factors. As a result, further study is needed in specific areas before this approach can be declared successful. Enzymatic degradation appears to be a viable method. It is becoming increasingly vital to do significant research to find enzymes capable of degrading synthetic pesticides. Microbial degradation occurs at a considerably slower rate and is not always as efficient or straightforward to carry out as traditional bioremediation technologies. It needed to find more potent microbes, novel genes, and bioremediation approaches for proper waste management of pesticide pollutants. Genetically engineered microorganisms and biotechnology also play a significant role in this area. The above discussion illustrates the utilization of pesticide degrading microorganisms in a constructive way to manage the pesticide pollutants in an eco-friendly manner. Hence, the further studies on the screening of effective microbial strains and enzymes are essential to reduce pesticide risks for the environment and human health.

Author contributions

VMP: conception and design of study and revising the manuscript critically for important intellectual content, approval of the version of the manuscript to be published. VMP and VV: analysis and/or interpretation of data. VMP, VV, BR, BK, NB, AS, SD, MY, RK, SS, AM, VP, NR, and JC: acquisition of data. VMP, BR, and BK: drafting the manuscript. All authors approved the version of the manuscript to be published.

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Contribution of insect gut microbiota and their associated enzymes in insect physiology and biodegradation of pesticides

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Synthetic pesticides are extensively and injudiciously applied to control agriculture and household pests worldwide. Due to their high use, their toxic residues have enormously increased in the agroecosystem in the past several years. They have caused many severe threats to non-target organisms, including humans. Therefore, the complete removal of toxic compounds is gaining wide attention to protect the ecosystem and the diversity of living organisms. Several methods, such as physical, chemical and biological, are applied to degrade compounds, but as compared to other methods, biological methods are considered more efficient, fast, eco-friendly and less expensive. In particular, employing microbial species and their purified enzymes makes the degradation of toxic pollutants more accessible and converts them into non-toxic products by several metabolic pathways. The digestive tract of insects is usually known as a superior organ that provides a nutrient-rich environment to hundreds of microbial species that perform a pivotal role in various physiological and ecological functions. There is a direct relationship between pesticides and insect pests: pesticides reduce the growth of insect species and alter the phyla located in the gut microbiome. In comparison, the insect gut microbiota tries to degrade toxic compounds by changing their toxicity, increasing the production and regulation of a diverse range of enzymes. These enzymes breakdown into their derivatives, and microbial species utilize them as a sole source of carbon, sulfur and energy. The resistance of pesticides (carbamates, pyrethroids, organophosphates, organochlorines, and neonicotinoids) in insect species is developed by metabolic mechanisms, regulation of enzymes and the expression of various microbial detoxifying genes in insect guts. This review summarizes the toxic effects of agrochemicals on humans, animals, birds and beneficial arthropods. It explores the preferential role of insect gut microbial species in

the degradation process and the resistance mechanism of several pesticides in insect species. Additionally, various metabolic pathways have been systematically discussed to better understand the degradation of xenobiotics by insect gut microbial species.

KEYWORDS

symbiotic microbes, enzymes, pesticides, non-target organisms, metabolic pathways

Introduction

In modern agriculture, for the management of various kinds of pests and the production of high-yield crops to meet the food availability for human beings, pesticides are extensively applied all over the world (Giambò et al., 2021). Pesticides are chemicals that control different pests such as rodents, arthropods, weeds and microbial pathogens (Huang and Chen, 2022). Pest management strategy is a vigorous arms race: on the one hand, farmers, pesticide inventors, agribusiness men, and researchers throughout the world struggle for the protection of crops and their higher production (Damalas and Koutroubas, 2018). While on the other hand, insects and other microbial pathogens follow their biological metabolism and drive to live and reproduce their generations (Pietri and Liang, 2018). Due to the repetitive application of pesticides with higher concentrations, insects and other pathogens fail to control them and develop cross-resistance (Daisley et al., 2018; Gressel, 2018). However, insect resistance against insecticides produces severely threaten non-target living organisms and contaminates the ecosystem (Khalid et al., 2021). Various studies have reported that pesticides' toxic residues are abundantly present in soil, sediments, and water bodies (Mulla, Ameen et al., 2020).

These hazardous compounds and their toxic metabolite residues significantly affect the climate and living organisms such as soil biota, fish, birds, mammals, plants and human beings (Lee et al., 2021; Pujar et al., 2022). In addition, their toxic residues ruin organisms' behavior, reproduction cycles and metabolism mechanisms, which can permanently alter the interrelated ecosystem (Zhao et al., 2019). These toxic compounds are degraded into simpler or less toxic substances using various methods such as chemical reactions, physical methods, photodegradation and biodegradation. Compared to other techniques, biological methods are less expensive, environment-friendly, more effective and easier to adapt to remove emerging pollutants (Hao et al., 2018).

Microbial species have been extensively applied for the biodegradation of environmental pollutants, including agrochemicals (Chen et al., 2015). To date, researchers throughout the world have screened millions of microbial species (bacteria, fungi, yeasts, algae, etc.) from the soil, sewage sludge, wastewater and other contaminated sites (Yang L. et al., 2021). Investigation of pure cultures of microbial species has revealed that toxic molecules are transformed into various metabolites (Ahlawat et al., 2020). Nevertheless, due to considering prominent features of insect gut microbial species like high resistance to pesticides and purification of novel suitable enzymes, various researchers have isolated a diverse number of microbial species for the biological treatment of wastewater and clean-up of the contaminated environment (Skidmore and Hansen, 2017).

Gut microbial species play a pivotal role in the detoxification, mineralization, and catabolism of organic molecules employed in pest control as determined by degradation or histochemical mechanisms (Berasategui et al., 2016). They are also considered superior organs for producing pheromones, synthesizing vitamins, and different enzymes to prevent pathogens (Ozdal et al., 2016). The gut of insects and other arthropods provides a rich nutrient medium for developing microbial species that can produce some essential enzymes and contribute significantly to insect physiology (Ramakrishnan et al., 2019; **Figure 1**). Insect gut microflora provides a prominent environment for transforming genes, mutant traits, and conjugative plasmids, which can adapt to harsh environmental conditions and perform smoothly in biodegradation processes (Xia et al., 2018).

More importantly, microbial species isolated from this source are rarely indigenous to the polluted environment. Hence, their use in bioaugmentation and biodegradation enhances their efficiency to remove environmental pollutants (Kadri et al., 2018). To keep in mind these critical points, insect associated-microbial species, especially bacteria, are more vigorous and beneficial because they are interrelated with the application of active ingredients (Blanton and Peterson, 2020). The coordination between symbiotic microbial species and resistance to pesticides in arthropods would provide new opportunities for managing pests and isolating efficient microbial species to protect the agroecosystem (Li et al., 2018).

This review investigates the resistance mechanisms of different pesticides in insect pathogens and the transforming mechanisms of their parent toxic compounds into less toxic intermediates by the isolation of gut microflora. Additionally, microbial species interlinked with insects and involved in the detoxification of pesticides will be essential in the designing of future novel ingredients to ensure their long-term efficiency. Therefore, investigating the linkages between environmental

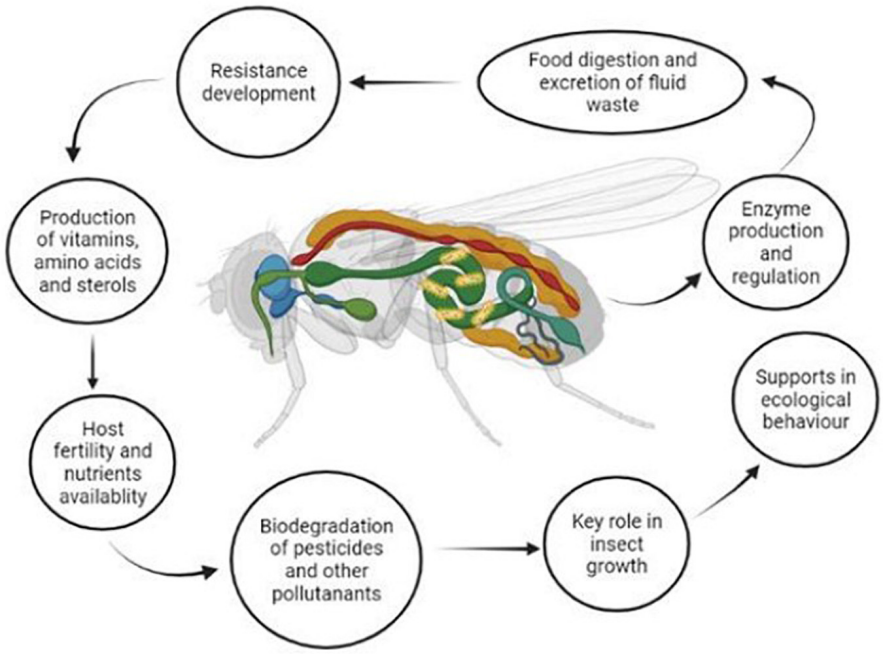

FIGURE 1
Role of insect gut microbiota in insect physiology.

contaminants and gut microflora is of great significance. This literature review will be beneficial and guider to reveal the possible impacts of gut microflora on the fate of organic pollutants and provide a more comprehensive insight into the mineralization, transformation, and biodegradation of pesticides and other emerging pollutants from the environment.

Fate of pesticides in the environment

Throughout the world, an extensive range of pesticides like insecticides, herbicides, rodenticides, fungicides, nematicides, molluscicides, rodenticides, bactericides, repellents, insect growth regulators and disinfectants have been generated for the management of specific target pests in agriculture, aquaculture, horticulture and households (van de Merwe et al., 2018). Currently, more than 3.5 million tons of pesticides are used throughout the world, out of which 47.5% are herbicides, 29.5% are insecticides, 17.5% are fungicides, and 5.5% are other types of pesticides (Sharma et al., 2019). Since its inception 50 years ago, China has grown to be the world's largest producer and consumer of pesticides. The other major pesticide-consuming countries are the United States, Argentina, Thailand, Brazil, Italy, France, Canada, Japan, and India (Olisah et al., 2019). These agrochemicals are extensively introduced into modern agriculture and urban ecosystems during their production, transportation, improper storage and unwise applications, which cause severe environmental threats (Ahmad et al., 2021). Local governments and environmental protection agencies regulate the production of pesticides and their applications. But the ecological management and risk assessment rules and regulations are generally restricted to formulating agrochemicals and their active ingredients and additives (Pokhrel et al., 2018). According to a combined statement of WHO and UNEP, approximately 200,000 people worldwide die, and roughly three million are affected yearly by pesticide residues (Meftaul et al., 2020). Another study revealed that the majority of cases, nearly 95% of them are reported from developing countries (Yadav et al., 2015). Agrochemicals severely effect the ecosystem through toxic residues at the application sites, such as agricultural farms, lawns and parks (Wu et al., 2007). However, these compounds pose severe threats to aquatic organisms by leaching down into the groundwater and through surface runoff into lakes, rivers, and other water bodies (Ahmad et al., 2022a). Furthermore, when pesticides are applied to crops, horticulture areas, home lawns and school parks, many people including, children and women, animals, beneficial arthropods, birds and wildlife creatures, are seriously affected (Masud et al., 2018).

Due to unwise applications of pesticides with higher concentrations, their toxic residues have been frequently revealed in the urban air, dust, soil and water bodies than in those of rural areas, predominantly due to primary, secondary and re-emissions of the parent compound and their toxic derivatives (Ren et al., 2018). Agronomic crops and other

ornamental plants can easily absorb these chemicals from contaminated sites and transfer them to their vegetative and reproductive parts (Kim et al., 2017). When farmers apply higher concentrations of pesticides to protect their crops from pests and diseases, their residues are entered into food commodities (Dai et al., 2010). However, various researchers are working to investigate toxic pesticide residues in fruits and vegetables growing in agricultural, rural and urban areas in developing and developed countries (Pietrzak et al., 2020; Barbieri et al., 2021; Zamule et al., 2021). Incorporating pesticide residues into daily food consumption is a foremost safety issue for consumers worldwide (Hasan et al., 2017). The excessive use of pesticides deliberately affects flora, fauna and the ecosystem (Arunkumar et al., 2017). We briefly discuss the risk of pesticides to humans' health and other non-target living organisms in the following sections.

Human health

The labors working in pesticide formulation industries, agriculture areas, and assassinators for managing household pests are generally affected by direct or indirect pesticide exposure. There are higher chances of risk for people working in the pesticide manufacturing industries at the time of formulation, packaging and production because they handle crude materials and other hazardous solvents (Gangemi et al., 2016; Nicolopoulou-Stamati et al., 2016). Various kinds of health disorders such as cancer, diabetes problems, respiratory issues, neurological disorders, reproductive syndromes and oxidative stress are produced due to direct or indirect exposure and handling of pesticides or their toxic active ingredients in foodstuffs (Carles et al., 2017; Grewal, 2017; Rani et al., 2021). Some studies have revealed that due to continuous risk assessment of highly toxic compounds, including pesticides such as lung cancer, breast cancer, leukemia and multiple myeloma have occurred in human beings (Han et al., 2018; Ruiz et al., 2018; Huang et al., 2019; Jaacks et al., 2019). Meng et al. (2016) carried out a study to investigate agrochemical exposure in indoor dust and blood samples. Results of this study revealed asthma is positively interlinked with exposure to alpha-hexachlorocyclohexane in humans.

In another study to evaluate pesticide exposure and its effects on human health, Tanner et al. (2009) carried out a study. They discovered that Parkinson's disease and 2-4 D herbicides are closely associated with their cause. Yan et al. (2016) reported that pesticides and Alzheimer's disease are closely interlinked, and a meta-analysis proved that pesticide exposure is hazardous for the brain and eyes. Recently, Shah et al. (2020) carried out a study investigating the effect of organochlorine pesticides such as β-hexachlorocyclohexane, dichlorodiphenyldichloroethylene and dieldrin on human epithelial ovary cells for the risk prediction of ovarian cancer. The findings of this study revealed that organochlorine pesticides highly affect human health and stimulate the measurement of reactive oxygen species (ROS), pro-inflammatory response and DNA damage in human epithelial ovary cells. Besides this, DDT organochlorine pesticide caused DNA damage, genetic instability, micronucleus formation and the sister chromatid exchange in humans (Yáñez et al., 2004; Savant et al., 2018). Cassidy et al. (2005) studied the relationship between women's breast cancer and heptachlor pesticide. The results of this study revealed that women's breast cancer risk was positively correlated with the level of heptachlor epoxide. Richardson et al. (2014) investigated the effect of dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE) on human health and found that these both pesticides were responsible for causing Alzheimer's disease. In another study, due to high exposure to organochlorine pesticides their toxic effects on human health were examined, and it was found that these pesticides cause Parkinson's disease (Freire and Koifman, 2012).

Impacts on water bodies

Imprudent application of pesticides in farming could pollute surface water *via* draining, runoff, leaching and drift. Polluted surface water harms non-target organisms, including humans and animals (Chrustek et al., 2018; Lee and Choi, 2020). Surface water is considered a major drinking water source in developing nations such as Pakistan, India, Bangladesh, Nepal, and Sri Lanka (Mojiri et al., 2020; Teklu et al., 2022). The residues of various pesticides from the groups of organochlorines, organophosphate, carbamates, neonicotinoids, and pyrethroids are found in the rivers of California (Anderson et al., 2018). Besides this, several other European countries also investigated pesticide residues and noticed that 76 types of pesticide residues are present in European soil. Furthermore, it was revealed that 83% of soil contained one type of residue, and 58% of soil contained two, three or more types of residues. The highest concentrations of glyphosate and its derivatives were detected frequently. The presence of pesticide residues in the surface water and rivers all over the world causes critical threats to aquatic organisms (Mitchell et al., 2017; Tian et al., 2018; Dias et al., 2020).

A study was conducted to evaluate the exposure of organochlorine and pyrethroid pesticide residues in surface water, fish, sediments and aquatic weeds in the southern region. Results of this study revealed that residues of organochlorine pesticides were identified in surface water, sediments, fish muscle, gills, liver, and aquatic weeds at a concentration of 0.001–34.44 μg/L, 0.01–16.72 μg/Kg, 0.01–26.05 μg/Kg, 0.01–40.56 μg/Kg, 0.01–65.14 μg/Kg, 0.01–5.53 μg/Kg, respectively. This study further explained that organochlorine pesticides such as eldrin, dieldrin, endosulfan, endrin, and heptachlor were the prominent pesticides identified with the above level of

maximum residue limit set by the World Health Organization in surface water, sediments and fish (Arisekar et al., 2019). In another study, residues of pesticides in the Guayas River at 181 places were investigated using the solid phase extraction method. Results of this study explained that 26 types of pesticide residues in fresh water at 108 sampling sites (60%) were detected with higher concentrations. The major types of pesticides found in river water are cadusafos, butachlor, and pendimethalin at 62, 21, and 21, with concentrations of 0.081, 2.006, and 0.557 μg/L, respectively. Finally, this study also demonstrated that all detected pesticides in river water are frequently found in agriculture and horticulture crops such as rice and banana, with higher concentrations due to irregular application methods like aerial spraying. Finally, their residues are transferred to the rice field and river water. This study also suggested that precaution measures such as legal regulations and awareness campaigns for farmers and local industries are highly recommended to control environmental contamination and prevent the accumulation of pesticide residues in aquatic and terrestrial systems (Deknock et al., 2019).

Recently, for the investigation of highly used herbicide residues such as atrazine, acetochlor, alachlor, hexazinone, metolachlor, simazine, terbuthylazine, trifluralin, and phenoxy acids (MCPA and 2,4-D) in two type of fishes (*Clarias gariepinus*, *Oreochromis mossambicus*) an experimental study was conducted. This study showed that all herbicides' residues were found in analyzed samples with a total concentration ranging from 42.3 to 238 ng/g in *Clarias gariepinus* and 72.2–291 ng/g in *Oreochromis mossambicus*. The most dominant herbicides which are found in fish tissues, gills and liver are phenoxy acid herbicides, acetochlor, atrazine and terbuthylazine with the ranges of 17.6 ± 12 ng/g, 28.9 ± 16 ng/g, 15.4 ± 5.8 ng/g, 12.7 ± 7.1 ng/g, 12.4 ± 12 ng/g, respectively (Tyohemba et al., 2021).

Threats to beneficial arthropods

Insect pollinators and predators play a pivotal role in developing many crops, as insect pollinators increase the yield and predators protect crops from pest infestation (Ihara et al., 2017). But due to excessive use of agrochemicals and unselective treatment in the modern agriculture system, their diversity and abundance are severely affected (Joshi et al., 2020). It has been reported that only 1% of pesticides reach the target site, whereas the remaining amount accumulates in the environment and contaminates it (Goergen et al., 2016; Fine et al., 2017; Stein et al., 2017). Beneficial arthropods are directly linked with pesticide exposure at the time of application or immediately after applying pesticides. The droplets of toxic residues could inlet on their cuticle by ingestion and influence their growth and mating behavior (Sgolastra et al., 2017).

Abraham et al. (2018) investigated the effects of glyphosate herbicides on beneficial insects (*Apis mellifera, Hypotrigona ruspolii*) under laboratory conditions. The bees were treated with the recommended concentration, a two-fold higher recommended concentration, and distilled water for control. The impact of glyphosate herbicide was compared with the lambda cyhalothrin. The herbicide was sprayed on plants as well as the bees were treated with herbicide-sprayed filter paper. Results of this study revealed that a more significant number of bees died after contact with the herbicide in both ways. This study concluded that spraying glyphosate herbicide was very dangerous for beneficial insects by contacting or spraying on fresh plants with more than the recommended dose. Another study investigated the effects of widely used neonicotinoids (acetamiprid, imidacloprid, thiamethoxam, and thiacloprid) on spiders. All the neonicotinoids with recommended doses were applied in field conditions, and short-term exposure was evaluated on spiders. Results of this study revealed that after 1 h, imidacloprid showed more critical effects and revealed partial acute lethality (15–32%). Acetamiprid showed strong sublethal effects, particularly when employed dorsally on *Philodromus cespitum*. After 1 day of application of thiacloprid and acetamiprid, *Linyphiidae* species were paralyzed or finally caused death, especially in males (Øezáè et al., 2019).

A study was conducted to reveal the toxicity of imidacloprid, thiamethoxam and sulfoxaflor on the aphid (*Aphis gossypii*). The impact of these pesticides on natural enemies of aphids, especially parasitoids (*Aphidius colemani*), was investigated with low lethal, median lethal and sublethal concentrations. This study showed that the median lethal concentration caused maximum mortality of parasitoids compared to sulfoxaflor, while imidacloprid had the least negligible impact on the diversity of parasitoids (Ricupero et al., 2020). To study the effects of more neonicotinoid pesticides such as imidacloprid, thiamethoxam, clothianidin and dinotefuran on the parasitoid larvae (*Coccinella septempunctata*) by the application of lethal and median lethal doses using the direct contact method. This study indicated that neonicotinoid pesticides are hazardous for the survival of larvae. The median lethal dose highly impacts the emergence of larval instars, pupal emergence and weight. Finally, this study concluded that pesticide application is hazardous for the survival of beneficial insects, primarily involved in integrated pest management services (Wu et al., 2021).

Toxic effects on plants and animals

Every day, increasing environmental pollution affects many living organisms and has always been considered a critical challenge in the scientific community (Niroumand et al., 2016). The high accumulation of pesticides in agricultural soils and their cumulative behavior and toxicity pose severe threats to

beneficial plants (Ferrando and Matamoros, 2020). It is well known that the accumulation of pesticides affects the behavior of soil microbial species and enzymes and is absorbed by plants, which further transfers it to non-target organisms through the food chain process (Sayed et al., 2020). The adaptation of medicinal plants to cure various diseases has been practiced for several centuries and even today plays a pivotal role in primary health care as a therapeutic agent in several developing nations (Reinholds et al., 2017; Kumar et al., 2018).

The application of herbal medicines to treat various illnesses has increased significantly in the past few decades due to their prominent features such as minimum side effects compared to synthetic drugs, inexpensive and excellent viability (Ammar et al., 2020). Besides their numerous benefits, toxic pesticide residues could be more dangerous and cause many diseases in humans and other living organisms (Righi et al., 2018). Recently, Luo et al. (2021) have studied the accumulation of pesticide residuals in various medicinal plants, which are frequently used throughout the world for the welfare of humanity. Results of this study explained that in 1771 samples, 88% of pesticide residues were detected. Terrifyingly, 59% of pesticide residues are beyond the European Pharmacopoeia (EP) limit and 43% are confined to 35 types of banned pesticides worldwide. Additionally, this study demonstrated that eight pesticide residues were five hundred times higher than the default maximum residue limit set by the environmental protection agency.

In another study, Li R. -X. et al. (2020) investigated the presence of various pesticide residues in herbal plants using the Quick Easy Cheap Effective Rugged Safe (QuEChERS) extraction method. All residues were detected through Ultra-Performance Liquid Chromatography and Gas Chromatography-Mass spectrometry analysis. This method was applied to 39 real samples of *Ophiopogon japonicus*, *Polygonatum odoratum,* and *Paeonia suffruticosa* obtained from different locations, and the results of this study revealed that in 92.3% of samples, residues of pesticides were detected. This study showed that 26% pesticide residues are frequently detected in three traditional Chinese medicine plants. In addition, tebuconazole and paclobutrazol residue levels were considerably higher in nine samples compared to the maximum residue limit.

The primary way of transformation of pesticides in the general population is the consumption of food commodities that might be polluted with toxic residues of pesticides (Nagy et al., 2020). However, their residues can ultimately be inserted into animals' digestive tracts *via* various pathways and affect their physical conditions (Altun et al., 2017; Yuan et al., 2019). Recently, Nerozzi et al. (2020) investigated the effects of glyphosate and its most famous formulation Roundup, on animal health and reproductive functions. In this study, the pig was chosen as a model animal. The commercial semen of pigs was treated with glyphosate and Roundup formulation at 0–360 μg/mL concentrations and incubated at 38°C for 3 h. The consequences of this study indicated that the application of high concentrations of glyphosate significantly reduced sperm viability, motility, mitochondrial activity and acrosome integrity. While on the other side, by treating lower concentrations (5–100 μg/mL) of Roundup formulation, all the disorders were observed after 1 h of incubation. Finally, this study concluded that pesticides active ingredients and inert materials negatively affect animals and the human reproductive system. Jarrell et al. (2020) also reported that glyphosate-based herbicides are more dangerous for animals' health and cause severe diseases such as the reproductive system, altering the regulation of enzymes, disrupting serum levels and activity, and loss of fertility.

An investigation was carried out to evaluate deltamethrin and ivermectin residues on local sheep milk and meat. A total of eighty samples (40 each for milk and meat) were obtained from different places, and detection of pesticide residues was observed by performing High-Performance Liquid Chromatography. Results of this study indicated that 92.5 of milk samples and 90% of meat samples were polluted with toxic deltamethrin residues. More alarmingly, this study highlighted that all samples were contaminated with ivermectin residues above the maximum residue limit set by the World Health Organization (WHO) and Food and Agriculture Organization (FAO) (Mani and Al Araji, 2022).

However, to remove various environmental pollutants, protect the diversity of living organisms, and save crops from pests, effective, eco-friendly, less expensive, and more applicable methods are urgently required.

System biology-based approaches for the pesticides degradation in agroecosystem

In order to gain a better knowledge of plants and microbes, researchers are using system biology technologies (Bhatt et al., 2016). Numerous details on the interactions between microbes, plants, humans and other non-target organisms by pesticides in nature have been fabricated because of advances in the fields of genomics and proteomics (Bhandari et al., 2021). Recently, a biological system-based approach was carried out to remove atrazine residues from the contaminated environment and to understand the complex biological network with different cellular systems modeling and simulation of atrazine were performed. The findings of this study revealed that two functional enzymes from bacteria (chlorohydrolase and monooxygenase) actively performed and completely degraded atrazine from the environment. To learn more about the biochemistry and physiology of atrazine in various cellular networks, additional analysis and simulations of the utilized model were performed. Atrazine degradation's 289 nodes and

300 edges were verified by topological analysis (Bhatt et al., 2020). Insecticides containing pyrethroids are frequently used to control pests in homes and agricultural crops (Bhatt et al., 2022b). The complete removal of various pyrethroid pesticides was achieved using a system biological based approach and a simulated model. Results of this study explain that the toxic metabolites of pyrethroids severely affect non-target organisms, especially beneficial arthropods, microorganisms and human health. In addition, this investigation actively contributed to analyzing the toxicity and removal of other emerging pollutants from the agroecosystem (Bhatt et al., 2021a). In another study, a potential bacterial strain, *Bacillus subtilis* 1D, was isolated from a polluted agriculture field and investigated their degradation efficiency to degrade cypermethrin from the environment. The findings of this study showed that bacterial stain efficiently degraded 95% of cypermethrin within 15 days and converted it into various metabolites. In addition, laccase and esterase enzymes were identified from bacterial strain and observed that both enzymes more rapidly degraded cypermethrin as compared to free cells (Gangola et al., 2018).

A biological molecular model and a purified methyl transferase enzyme were adopted to degrade residues of methyl halide from a polluted environment. This study explained that the enzymes played a crucial role in the remediation of methyl halide. In addition, this model demonstrates that a volatile poisonous substance impacts the earth's environmental layers and life systems (Bhatt et al., 2019). A potential *Bacillus* sp. FA3 was isolated from the contaminated environment and examined their degradation efficiency using the Box-Behnken design to degrade fipronil from the soil and water systems. Results of this study revealed that at optimum conditions (temperature of 32°C, pH 7, and rotational speed of 110 rpm), the bacterial strain performed efficiently and degraded 76% of fipronil within 15 days. Finally, this study concluded that *Bacillus* sp. FA3 was a superior candidate for removing fipronil from the wastewater and soil system and could be helpful for large-scale treatment (Bhatt et al., 2021b).

Diversification of symbiotic microbiota and their role in insect physiology

A diverse range of symbiotic microbial species have been produced within the insect gut and have contributed a very significant role in the regulation of insect metabolism, enhanced food digestion, increased excretion of waste fluids, protecting the host from enemies, developing resistance against toxins and degrading them into their intermediates (Smith et al., 2017; Heys et al., 2018; Tokuda et al., 2018; Hauffe and Barelli, 2019; **Figure 1**). The identification and characterization of insect gut microbial species are investigated mainly by culture-dependent or culture-independent techniques (Bourguignon et al., 2018; Bruno et al., 2019). However, the culture-dependent method usually produces biased results. It relies on various parameters and techniques, while in the culture-independent method, a lot of omics and molecular approaches are applied, such as 16S rRNA and BLAST analysis, which provide a better and more comprehensive picture of the microbial communities located in insect guts (Eski et al., 2018; Erlandson et al., 2019; Erb and Kliebenstein, 2020). The application of high throughput and next-generation sequencing provides new insights into obtaining microbial ecology (Harishankar et al., 2013). It reveals that the diversity of microbial species by using independent culture methods identified a higher number of microbial communities than traditional culture-based and conventional molecular methods (Bhatt et al., 2021c,a, 2022a; Mishra et al., 2021; Ahmad et al., 2022a). Therefore, a comprehensive evaluation of microbial communities within a host species plays a vital role in understanding insect physiology and their interactions with insect hosts (Armitage et al., 2022).

A comprehensive investigation was carried out to evaluate insect symbiotic microbial species and their significant roles in 305 insect samples belonging to 218 insect species in 21 taxonomic orders. Using an independent culture method and adopting 16S rRNA analysis, 454 pyrosequencing were performed, and 174,374 sequence reads were gained. This study's results indicated a total of 9301 bacterial operational taxonomic units (OTUs) at a distance level of 3% from all samples, with an average of 84.3% ($\pm$ 97.7) OTUs per sample. In addition, this study suggested that gut microbial species were dominated by *Proteobacteria, Wolbachia,* and *Firmicutes* with a ratio of 62.1, 14.1, and 20.7%, respectively. Finally, this study concluded that these bacterial communities could help in food digestion, the development of larval stages and enhanced the insect immune system (Yun et al., 2014). The findings of another study showed that the hindgut of subterranean termites contained a 90% population of bacteria and archaea (Hongoh, 2010). The diversity of bacterial species in the digestive tract of fruit flies (*Drosophila melanogaster*) was studied using 454 pyrosequencing of 16S rRNA gene amplicons. Results of this investigation explained that 5 OTUs enriched the sequence reads, and $\leq$97% of that sequence identity could be related to *Acetobacter pomorum*, *Acetobacter tropicalis*, *Lactobacillus brevis*, *Lactobacillus fructivorans,* and *Lactobacillus plantarum* (Wong et al., 2011).

In another study, using an independent culture technique and adopting molecular approaches such as denaturing gradient gel electrophoreses and 16S rRNA analysis, a high diversity of genus *Gammaproteobacteria* were identified in the gut of the locust *Schistocerca gregaria.* The results of this study suggested that this diversity of bacterial species engaged with a defensive mechanism and enhanced it against external pathogens and toxic chemicals (Dillon et al., 2010). Recently, Xue et al. (2021) investigated the diversity of gut microbial species in various life

TABLE 1 Pesticide resistance cases in various insects mediated by gut microbial species.

Name of pesticide	Insect common name	Insect scientific name	Gut microbiota	References
Prothiofos	Diamondback moth	*Plutella xylostella*	*Pseudomonas* sp., *Stenotrophomonas* sp., *Acinetobacter* sp., and *Serratia marcescens.*	Indiragandhi et al., 2007
Tebuconazole	Brown planthopper	*Nilaparvata lugens*	*Acinetobacter* sp.	Song et al., 2021
DDT	Diamondback moth	*Plutella xylostella*	*Bacillus thuringiensis* and *Saccharopolyspora spinosa*	Sarfraz and Keddie, 2005
Imidacloprid	Honeybee Fruit fly Fruit fly Whitefly Bed bug	*Apis mellifera* *Bactrocera tau* *Drosophila melanogaster* *Bemisia tabaci* *Cimex hemipterus*	*Bifidobacterium* sp., *Lactobacillus* sp., *Klebstella oxytoca, Pantoea agglomerans, Staphylococcus* sp. *Lactobacillus* sp., *Rickettsia* sp., *Frischella* sp. *Wolbachia* sp., *Yersinia* sp., *Bacillus* sp., and *Acetobacter* sp.	Kontsedalov et al., 2008; Prabhakar et al., 2008; Chmiel et al., 2019; Rouzé et al., 2019; Alberoni et al., 2021; Soh and Veera Singham, 2022
Atrazine	Jewel wasp	*Nasonia vitripennis*	*Serratia marcescens* and *Pseudomonas protegens*	Wang G. -H. et al., 2020
Chlorpyriphos	Diamondback moth	*Plutella xylostella*	*Enterobacteriales* sp., *Vibrionales* sp., *Pseudomonadales* sp., *Xanthomonadales* sp., and *Lactobacillales* sp.	Xia et al., 2013
Fipronil	Diamondback moth Honeybee	*Plutella xylostella* *Apis mellifera*	*Enterobacteriales* sp., *Vibrionales* sp., *Pseudomonadales* sp., *Xanthomonadales* sp., *Lactobacillales* sp., *Bifidobacterium* sp., *Alphaproteobacteria* sp., *Gammaproteobacteria* sp., and *Lactobacillus* sp.	Xia et al., 2013; Rouzé et al., 2019; Paris et al., 2020
Pyraclostrobin	Honeybee	*Apis mellifera*	*Gilliamella* sp. and *Lactobacillus* sp.	DeGrandi-Hoffman et al., 2017
Abamectin	Parasitic wasps	*Eretmocerus mundus, Eretmocerus eremicus,* and *Encarsia formosa*	*Arthrobacter* sp.	Fernández et al., 2019
Thiamethoxam	Whitefly Honeybee	*Bemisia tabaci* *Apis mellifera*	*Delftia* sp., *Rickettsia* sp., *Bifidobacterium* sp., *Lactobacillus* sp. *Alphaproteobacteria* sp., and *Gammaproteobacteria* sp.	Kontsedalov et al., 2008; Xie et al., 2012; Rouzé et al., 2019; Paris et al., 2020
Deltamethrin	Diamondback moth Mosquitos Cotton aphid	*Plutella xylostella* *Anopheles albimanus* *Aphis gossypii*	*Enterococcus mundtii, Carnobacterium maltaromaticum, Bacillus* sp., *Buchner* sp., *Pseudomonas* sp., *Pantoea agglomerans* and *Pseudomonas fragi*	Li et al., 2017; Dada et al., 2019; Shang et al., 2021b
Coumaphos	Honeybee	*Apis mellifera*	*Bifidobacterium* sp. and *Lactobacillus* sp.	Rouzé et al., 2019
Malathion	Fruit fly	*Bactrocera tau*	*Klebstella oxytoca, Pantoea agglomerans,* and *Staphylococcus* sp.	Prabhakar et al., 2008
Cypermethrin	Tobacco cutworm or cotton leafworm	*Spodoptera litura*	*Clostridium botulinum, Clostridium butyricum,* and *Pseudomonas putida*	Karthi et al., 2020
Phoxim	Silkworm	*Bombyx mori*	*Enterobacter cloacae, Staphylococcus* sp., *Methylobacterium* sp., and *Aurantimonadaceae* sp.	Li F. et al., 2020
Beta-cypermethrin	Cockroach	*Blattella germanica*	*Lactobacillus* sp., *Metarhizium anisopliae, Parabacteroides* sp., *Lachnoclostridium* sp., and *Tyzzerella* sp.	Zhang and Yang, 2019; Zhang J. et al., 2022
Carboxamide	Honeybee	*Apis mellifera*	*Alphaproteobacteria* sp. and *Gammaproteobacteria* sp.	Paris et al., 2020
Phosphine	Red flour beetle	*Tribolium castaneum*	*Bacillus subtilis, Staphylococcus* sp., *saprophyticus* sp., *Enterobacter* sp., *Lysinibacillus fusiformis, Klebsiella pneumonia,* and *Achromobacter* sp.	Gowda et al., 2021
Trichlorphon	Oriental fruit fly	*Bactrocera dorsalis*	*Citrobacter freundii*	Guo et al., 2017
Endosulfan	Fruit fly	*Bactrocera tau*	*Klebstella oxytoca, Pantoea agglomerans,* and *Staphylococcus* sp.	Prabhakar et al., 2008
Temephos	Asian malaria mosquito	*Anopheles stephensi*	*Pseudomonas* sp., *Aeromonas* sp., *Exiguobacterium* sp., and *Microbacterium* sp.	Soltani et al., 2017
Permethrin	Mosquitos African malaria mosquito	*Anopheles albimanus* *Anopheles gambiae*	*Pantoea agglomerans* and *Pseudomonas fragi.* *Sphingobacterium, Lysinibacillus* and *Streptococcus*	Dada et al., 2019 Omoke et al., 2021
Alphacypermethrin	Mosquitos	*Anopheles albimanus*	*Pantoea agglomerans* and *Pseudomonas fragi*	Dada et al., 2019
Thiacloprid	Honeybee	*Apis mellifera*	*Enterococcus faecalis, Snodgrassella alvi, Bartonella apis,*	Dickel et al., 2018;

(Continued)

TABLE 1 (Continued)

Name of pesticide	Insect common name	Insect scientific name	Gut microbiota	References
			Frischella perrara, Lactobacillus kunkeei, Frischella sp., *Bifidobacterium asteroids,* and *Gilliamella apicola*	Alberoni et al., 2021; Cuesta-Maté et al., 2021
Fenitrothion	Bean bug	*Riptortus pedestris*	*Burkholderia* sp.	Itoh et al., 2018
Pyriproxyfen	Silkworm Whitefly	*Bombyx mori* *Bemisia tabaci*	*Burkholderia* sp., *Rhizobia* sp., *Rickettsia* sp., *Caulobacter* sp., *Sphingobacteria* sp., and *Enterobacteria* sp.	Kontsedalov et al., 2008; Lu et al., 2022
Chlorpyriphos	Diamondback moth	*Plutella xylostella*	*Enterococcus* sp., *Enterobacter* sp., and *Serratia* sp.	Gurr et al., 2018
Acetamiprid	Honeybee Whitefly	*Apis mellifera* *Bemisia tabaci*	*Snodgrassella alvi, Bartonella apis, Frischella perrara, Lactobacillus kunkeei, Bifidobacterium asteroids, Gilliamella apicola,* and *Rickettsia* sp.	Kontsedalov et al., 2008; Cuesta-Maté et al., 2021
Spinosyns	Diamondback moth	*Plutella xylostella*	*Bacillus thuringiensis* and *Saccharopolyspora spinosa*	Sarfraz and Keddie, 2005
Pendimethalin	Ground beetle	*Pterostichus melas*	*Enterobacter* sp., *Pseudomonas* sp., *Pantoea* sp., and *Paracoccus* sp.	Giglio et al., 2021
Sulfoxaflor	Cotton aphid	*Aphis gossypii*	*Buchner* sp. and *Arsenophonus* sp.	Shang et al., 2021b
Avermectin	Gypsy moth	*Lymantria dispar asiatica*	*Weissella* sp., *Lactobacillus* sp., *Pseudomonas* sp., *Candida* sp., *Tausonia* sp., *Chaetomium* sp., *Diutina* sp., and *Alternaria* sp.	Zeng et al., 2020
Buprofezin	Small brown planthopper	*Laodelphax striatellus*	*Wolbachia* sp. and *Rickettsia* sp.	Li et al., 2018
Boscalid	Honeybee	*Apis mellifera*	*Gilliamella* sp. and *Lactobacillus* sp.	DeGrandi-Hoffman et al., 2017
Carbaryl	Fall armyworm	*Spodoptera frugiperda*	*Bacillus thuringiensis* and *Varimorpha necatrix*	Fuxa and Richter, 1990
Methyl parathion	Fall armyworm	*Spodoptera frugiperda*	*Bacillus thuringiensis* and *Varimorpha necatrix*	Fuxa and Richter, 1990
Spiromesifen	Whitefly	*Bemisia tabaci*	*Rickettsia* sp.	Kontsedalov et al., 2008
Glyphosate	Colorado potato beetle	*Leptinotarsa decemlineata*	*Agrobacterium* sp., *Ochrobactrum* sp., *Rhodobacter* sp., *Rhizobium* sp., and *Acidovorax* sp.	Gómez-Gallego et al., 2020
Guadipyr	Silkworm	*Bombyx mori*	*Pseudomonas* sp. and *Curvibacter* sp.	Hou et al., 2021
Lufenuron	Formosan subterranean termite	*Coptotermes formosanus*	*Pseudomonas aeruginosa, Serratia marcescens,* and *Bacillus thuringiensis*	Wang et al., 2013
Fenitrothion	Bed bug	*Cimex hemipterus*	*Wolbachia* sp., *Yersinia* sp., and *Bacillus* sp.	Soh and Veera Singham, 2022
Spiromesifen	Whitefly	*Bemisia tabaci*	*Rickettsia* sp.	Kontsedalov et al., 2008

stages of *Adelphocoris suturalis* by adopting the independent culture technique. Results of this study explained that the gut of the first and second instar was highly accomplished with the diversity of bacterial species. This study demonstrated that in the phylum, Proteobacteria and Firmicutes were dominant with a ratio of 87.06 and 9.43%, respectively, while at the genus level, *Erwinia* (28.98%), *Staphylococcus* (5.69%), and *Acinetobacter* (4.54%) were dominant bacteria. Finally, this study concluded that the diversity of bacterial species could be applied for biological control.

Functions of insect gut microbiota

The insect gut is divided into three primary regions: the anterior midgut or foregut, the posterior midgut, and the hindgut (Wang G. -H. et al., 2020). The anterior midgut and hindgut arise from the embryonic epithelium. They are sheltered from pathogens by an exoskeleton of chitin and integument glycoproteins, while the posterior midgut is mainly used for absorption and digestion (He et al., 2018). Additionally, the hindgut of insects serves as an extension of the body cavity and is used to collect dietary waste (Siddiqui et al., 2022). However, it offers an appropriate environment that stimulates the proliferation and diversification of insect gut microbiomes (Bruno et al., 2019). Many studies have reported that insect gut microbiota plays a significant role in developing symbiotic insect interactions facilitated by secondary metabolites (Shang et al., 2021a). Besides this, they also play an essential role in the detoxification of pesticides, providing a natural defense system, nutrient availability, development of resistance against toxins and pathogens, breakdown of food, and suitable for proper growth of insects (Jang and Kikuchi, 2020; Jing et al., 2020; Mogren and Shikano, 2021; Tilottama et al., 2021).

High benefits and more prominent features of insect gut microbial species provide new insights into the development of

beneficial arthropods, which are often used as biocontrol agents to solve environmental problems and further applications for the welfare of humans (Samoilova et al., 2016; Borrelli et al., 2017; Liao et al., 2017). However, considering the superior features of insect gut microbiota, this review mainly focuses on their potential applications for the detoxification of pesticides and their toxic metabolites for the cleanup of the environment (Wang S. et al., 2020).

Development of resistance against pesticides

Pesticides have been applied to manage pests and diseases since the start of agriculture for the production and protection of crops. However, the unwise use of pesticides accumulates in the ecosystem and contaminates plants, air, water and soil (Lewis et al., 2016). The storage of pesticides in plants can develop resistance or tolerance against various pests (Ramakrishnan et al., 2019). A lot of studies have demonstrated that resistance is also developed due to reduction of toxicity of a compound, the introduction of a new pesticide group, target site mutation or over expression, pre-date or wrong selection of pesticide, repetition of the same chemical, environmental changes, and degradation of parent compounds into their metabolites by insect gut microbiota and their detoxifying enzymes (Naik et al., 2018; Hawkins et al., 2019; Matsuda et al., 2020; **Table 1**).

Insect digestive systems have a robust defensive system mainly equipped with various microbial species such as bacteria, fungi, archaea, and protozoa (Chen et al., 2021). In a recent study, the isolation of various microbial species in the digestive tract of worker honeybees (*Apis mellifera*). Results of this study demonstrated that nine species of bacteria from various genera were isolated; five belonged to *Snodgrassella alvi*, *Gilliamella apicola*, two species were from *Lactobacillus*, and one from *Bifidobacterium* (Douglas, 2018). Various microbial communities allow insects to tolerate or reject toxic compounds through various metabolic processes and develop a peritrophic medium composed of chitin microfibrils and a protein-carbohydrate medium (Kamalakkannan et al., 2017; Rumbos et al., 2018). This peritrophic medium plays a pivotal role in the development of resistance against chemicals due to some prominent features such as releasing digestive enzymes, availability of nutrients and providing protection to epithelial cells from external microbes and toxins through a semipermeable membrane (Puri et al., 2022; Siddiqui et al., 2022). These physiological obstacles between the lumen and epithelium actively contribute to the defense mechanism and minimize the activation of pesticides on the host rather than reducing microbial load in the gut (Chen et al., 2022; **Figure 2**).

Recently, a laboratory experiment was conducted to investigate organophosphate pesticide resistance in a serious rice pest, *Cletus punctiger* by the gut symbiont. Results of this study demonstrated that a rice bug effectively degraded

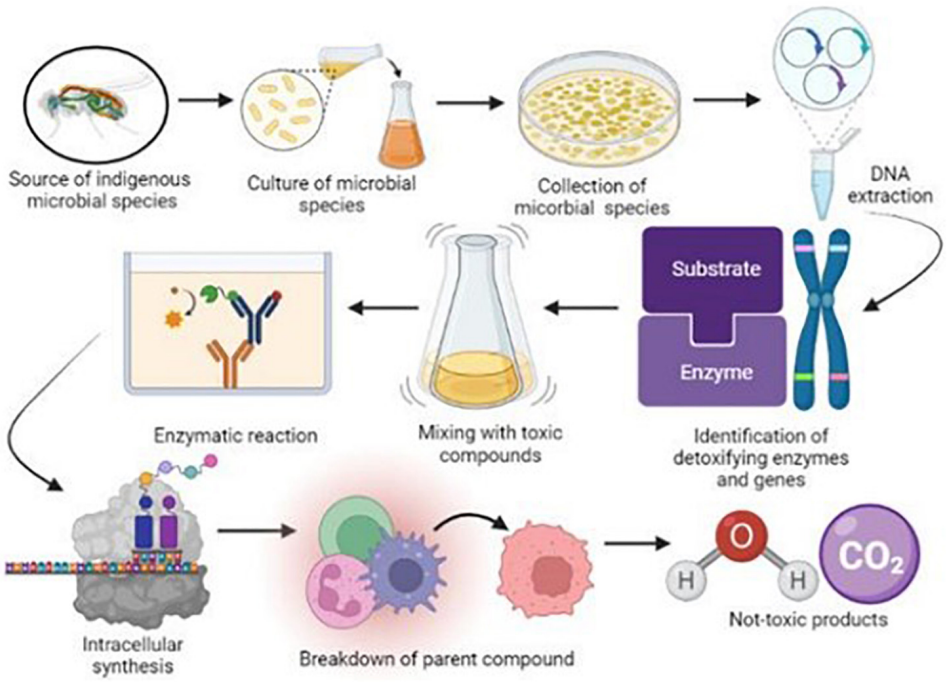

FIGURE 2
Schematic diagram of isolation of insect gut microbial species and their functions in biodegradation of environmental pollutants.

organophosphate fenitrothion by *Burkholderia* bacterial specie *via* oral infection and stayed it in the midgut part of the rice bug. The degradation of fenitrothion by the isolating bacterial species from the midgut revealed that gut microbiomes are highly capable of degrading pesticides in insects, and insect gut symbiosis plays a significant role in the development of resistance against fenitrothion in the host rice bug (Ishigami et al., 2021). In another study, the resistance of stored grain products against phosphine fumigation was studied. Four major stored grain pests (*Rhyzopertha dominica*, *Sitophilus granaries, Tribolium castaneum,* and *Trogoderma granarium*) were reared under laboratory conditions for up to seven generations. Results of this study indicated that insect gut symbiosis develops resistance in all pests. Regarding their level of resistance, *Rhyzopertha dominica* was highly resistant followed by *Tribolium castaneum, Trogoderma granarium,* and *Sitophilus granaries.* Although this study concluded that phosphine tablets are excessively applied to manage stored products and are considered very efficient against various stored grain pests, the development of resistance may lead to a serious failure of their applications (Wakil et al., 2021).

An investigation was carried out to study the role of gut microbiota in developing resistance against various insecticides in the laboratory and open field conditions in the larvae of *Spodoptera frugiperda*. The insect pests were collected from various corn fields in five Brazilian states. In a metagenomic experiment and 16S rRNA analysis, the isolation of bacterial species from insect gut in the selective medium was achieved. The maximum growth of microbial species in insecticides was observed, and it was found that all microbes utilized it as a sole source of carbon and energy. This study indicated that bacteria isolated from field larvae grew better and degraded insecticides more efficiently than those collected from laboratory-selected strains. However, this study concluded that due to the high efficiency and diversity of insect gut microbes in the field, larval insects are more capable of degrading pesticides and showed high resistance (Gomes et al., 2020).

A study was conducted to evaluate the resistance of chlorpyriphos in diamondback moths (*Plutella xylostella*) by insect gut microbiota. In this investigation, three bacterial species from insect guts such as *Enterococcus* sp., *Enterobacter* sp., and *Serratia* sp. were isolated and examined for their role in detoxifying chlorpyriphos and developing resistance in diamondback moths. Results of this study indicated that *Enterococcus* sp. increased resistance against the most widely used insecticide, chlorpyriphos. At the same time, *Serratia* sp. reduced resistance in the diamondback moth and for *Enterobacter* sp. no effect was observed. In addition, this study explained that *Enterococcus* sp., vitamin C and acetylsalicylic acid increased the regulation of antimicrobial peptides, which played a crucial role in the development of insecticide resistance (Xia et al., 2018). In another study, Wang et al. (2021) explained that insect gut microbial species play a significant role in insecticide deltamethrin resistance in *Aedes albopictus*. Additionally, experimental results indicated that by full-length 16S rRNA analysis, two bacterial species were collected from insect guts such as *Serratia oryzae* and *Acinetobacter junii* and investigated their growth in six kinds of growth media in biotic and abiotic conditions. Further, they observed that both symbiotic bacteria are mainly facultative in an anaerobic environment. Moreover, this study explained that insect symbiotic bacterial species actively promoted insect resistance against insect pesticides.

Molecular mechanism of resistance against pesticides by insect gut microbiota

To identify the complete profile and total biodiversity of microbial communities in insect gut microbiota and polluted sites, modern molecular biological approaches including clone libraries, probes, reverse sample genome probing, fluorescence *in situ* hybridization, community profiling or DNA fingerprinting, next-generation sequencing and pyrosequencing provide a more significant explanation as compared to the conventional biological tools (Ahmad et al., 2022b). Various functional parts of an insect's gut microbes, such as enzymes and genes, are responsible for developing pesticide resistance in insects (Bhatt et al., 2022b). Metagenomic analysis was performed to identify major microbial species in the gut of a honeybee (*Apis mellifera*) and their functional roles in developing resistance. This study's results revealed that insect gut microbe gene contents (*Gilliamella apicola*) are related to various host-dependent symbiotic functions. Moreover, as evidenced by the case of pectin breakdown by *G. apicola*, genetic variations are related to functional variations. The glycoside hydrolase and polysaccharide lyase enzyme families discovered in the honeybee metagenome are depicted with their respective cleavage sites on the schematic of the pectin molecule (Engel and Moran, 2013). In another study, an investigation of gut microbial species from three diamondback moth larvae was carried out to study prothiofos resistance. Findings from 16S rRNA showed that the bacterial community from the prothiofos-resistant larval gut was more diversified. In addition, the secretion of chitinase enzymes from the population of insect gut bacteria significantly contributed to host antagonism against entomopathogens and nutrition (Indiragandhi et al., 2007). Pesticide-resistance cockroach species such as German cockroaches, American cockroaches, and Oriental cockroaches are rich sources of insect gut microbiota and play a crucial role in insect physiology (Zhang X. C. et al., 2022). For example, the effect of beta-cypermethrin resistance development in cockroaches (*Blattella germanica*) by the gut microbial population and their genetic association with host growth was investigated. Results of 16S rRNA gene sequencing and

metagenomics indicated that *Lactobacillus* spp. were abundantly present in the foregut and midgut of cockroaches. In addition, carbohydrate-active enzymes actively contribute to developing resistance, insect growth, and fitness (Zhang and Yang, 2019). However, modern molecular biological tools efficiently describe the microbial interaction with the host and external pathogens. Moreover, in the future, these approaches could be applied to using and managing environmental bioprocesses through knowledge-based control.

Role of gut microbiota for biodegradation of pesticides

In many insect species, resistance to pesticides has been confirmed. It has been found that they are very beneficial for degrading toxic compounds due to their digesting abilities (Schmidt and Engel, 2021). The degradation of pesticides depends on various factors such as microbial remediation and the chemical hydrolysis process, which are additionally correlated with many physiological properties such as pH, temperature, organic matter, and moisture content (Bhatt et al., 2022b). However, the insect gut provides a favorable environment for developing diverse microbial communities. Hence, they efficiently deliver many promising facilities to their host (Shan et al., 2021). Symbiotic microbial species isolated from insect gut can perform in extreme environmental conditions to degrade pesticides and other emerging pollutants (Francis and Aneesh, 2022; **Figure 3**).

Recently, Wang et al. (2022) investigated the degradation of various pesticides by isolating microbial species from stored grain pests and studied the resistance mechanism. In this experiment, from multiple locations, adults of different stored grain pests (*Sitophilus oryzae*, *Cryptolestes ferrugineus*, and *Rhyzopertha dominica*) were collected and isolated as five bacterial species. Results of this study indicated that all screened bacterial species could degrade deltamethrin, malathion, and pirimiphos-methyl efficiently and use their residues as a source of carbon and energy, which are favorable for their growth. Additionally, this study revealed that when bacterial species are treated with 0.5–10 mg/kg of malathion, pirimiphos-methyl, and 0.3–0.75 mg/kg of deltamethrin, gnotobiotic reinoculation and their survival rates in the host are significantly increased, which implies that development of insecticide resistance highly depends on concentration rate. Moreover, this study also explained that the *in vitro* biodegradation of pesticides through gut bacteria was not entirely consistent with their *in vivo* operation in host pesticide resistance, which suggested that instead of direct degradation of pesticides, other physiological and morphological processes are also responsible for pesticide tolerance or resistance.

In another study, insects of Orthoptera and Dermaptera were collected from various sites. Fourteen bacterial species were isolated for the biodegradation of deltamethrin from a

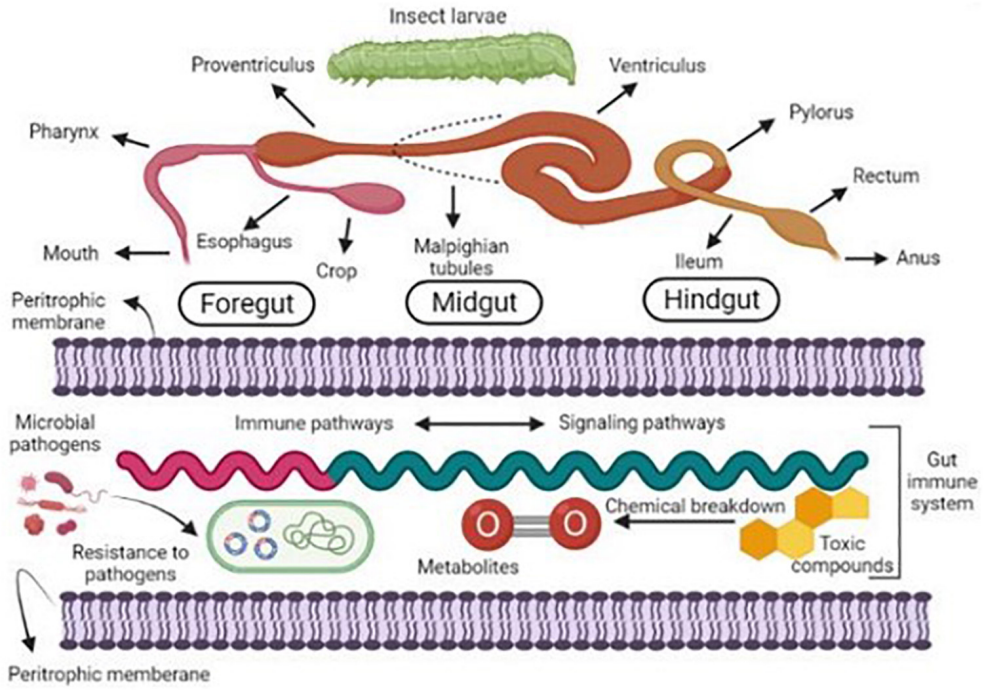

FIGURE 3
Graphical representation of gut microbes' development of resistance against pesticides.

polluted environment. All the bacterial species were analyzed by 16SRNA and identified as *Poecilimon tauricola*, *Locusta migratoria*, *Gryllus bimaculatus*, *Forficula Auricularia, Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Bacillus atrophaeus*, *Acinetobacter lwoffii*, *Rhodococcus coprophilus*, *Brevundimonas vesicularis*, *Pseudomonas syringae*, *Yersinia frederiksenii*, *Bacillus licheniformis*, *Enterobacter intermedius,* and *Serratia marcescen.* In addition, this study explained that eleven of them were gram-negative bacteria, and three were gram-positive bacteria, potentially deleting deltamethrin up to 100 mg/L. This study concluded that insecticide-tolerated gut microbiota is enriched with nutrients and is considered a powerful tool for the remediation of various kinds of pollutants from the ecosystem (Özdal and Algur, 2020). An indigenous rod-shaped gram-negative bacterium was isolated from the digestive tract of a grasshopper (*Poecilimon tauricola*) for the potential biodegradation of α-endosulfan and α-cypermethrin. By morphological, physiological, and 16S rRNA sequence analysis, the bacterial strain was characterized as *Acinetobacter schindler* and named B7. This study demonstrated that when the bacterial strain was treated with 100 mg/L of both pesticides in a glucose-mediated non-sulfur medium, significant growth of the bacterial strain was observed. Additionally, this study showed that within 10 days, the bacterial strain was capable of degrading 67.31 and 68.4% of α-endosulfan and α-cypermethrin, respectively (Ozdal and Algur, 2022).

Organophosphates are an influential group of pesticides that are excessively applied to control insect pest infestations across several agricultural and horticultural crops. The residues of this group are very toxic to the environment and spread many diseases to non-target organisms. Therefore, it is essential to remove their residues from the ecosystem by a potential degradation method. Recently, a study was conducted on the effective biodegradation of organophosphate pesticides, chlorpyriphos and polyethylene, by isolating insect gut microbial species. This study isolated four potential bacterial species: *Bacillus licheniformis*, *Pseudomonas cereus*, *Pseudomonas putida*, and *Bacillus subtilis,* from the gut of the citrus mealybug *(Planococcus citri).* Results of this study revealed that all symbiotic bacterial species utilized chlorpyriphos and polyethylene as a sole source of carbon and energy and enhanced their growth and enzymatic activity. Findings of the degradation experiment showed that after the treatment of 45 days, a satisfactory reduction of polyethylene weight was noticed, and scanning electron microscope analysis suggested that a biofilm formation around the polyethylene sheet by bacterial isolates was also observed. While in the case of chlorpyriphos, results indicated that after 21 days, significant degradation was observed in soil and water. In addition, this study revealed that *Pseudomonas cereus* and *Pseudomonas putida* have more potential to degrade both pollutants in diverse environmental conditions (Ibrahim et al., 2021).

To degrade clothianidin residues in an open environment, seven bacterial species such as *Edwardsiella* sp., two *Serratia* sp., *Rahnella* sp., *Pantoea* sp., *Hafnia* sp., and *Enterobacter* sp. were isolated from the digestive tract of the honeybee. To examine the growth of all bacterial strains, they were treated with various concentrations of clothianidin as a sole source of carbon and energy. They found that all bacterial strains provide satisfactory growth up to 10 ppb of clothianidin. Results of the degradation experiment showed that within 3 days, all endogenous bacterial strains noticed complete degradation of clothianidin (El Khoury et al., 2022).

For the biodegradation of multi-pesticides such as chlorpyriphos, cypermethrin, malathion, quinalphos, and triazophos, 13 indigenous microbial species were isolated from the gut of the cotton bollworm (*Helicoverpa armigera*) and tested for their degradation efficiency. After physiochemical, morphological and 16S rRNA sequence analysis, all bacterial strains were identified as *Bacillus pumillis* CL1, *Enterococcus casseliflavus* CL2, *Bacillus subtilis* CL3, *Rhodococcus* sp. CL4, *Pseudomonas* sp. CL5, *Staphylococcus* sp. CL6, *Pseudomonas aeruginosa* CL7, *Proteus vulgaris* HL1, *Cellulosimicrobium cellulans* HL2, *Klebsiella oxytoca* HL3, *Bacillus subtilis* HL4, *Stenotrophomonas maltophilla* HL5, and *Pseudomonas* sp. HL6. Results of this study indicated that strains CL2 and CL4 provided more rapid growth in the presence of malathion and chlorpyriphos in a mineral salt medium. Gas chromatography and mass spectrometry analysis revealed that strain CL4 has the potential to degrade 44% of chlorpyriphos and strain CL2 was capable to degrade 26% of chlorpyriphos and 57.1% of malathion in mineral salt medium (Madhusudhan et al., 2021). In another study, a symbiotic gut bacterium, *Citrobacter* sp. (CF-BD), was isolated from the digestive tract of tephritid fruit flies (*Bactrocera dorsalis*) and investigated their degradation efficiency against trichlorphon insecticide. Results of this study showed that insect gut microbiota plays a vital role in developing resistance against toxin chemicals and efficiently degrade trichlorphon into metabolites. This study also explains that various hydrolase genes were identified in the bacterial isolate CF-BD. In the presence of trichlorphon, the maximum gene expression was observed, and it was found that these critical genes play a crucial role in developing resistance in tephritid fruit flies (Cheng et al., 2017).

Screening of gut microbial enzymes for biodegradation of pesticides

In the insect body system, various potential natural enzymes are linked with different biological processes and play a vital role in toxic detoxifying substances in target sites (Lin et al., 2015; Naik et al., 2018; Bhandari et al., 2021). Based on some previous reports, it is found that resistance or

TABLE 2 Functions of insect enzymes in detoxification of pesticides and insect physiology.

Insect common name	Insect scientific name	Name of pesticide	Name of enzyme	Functions	Reference
Spongy moth	*Lymantria dispar*	Methidathion	Superoxide dismutase, catalase, glutathione peroxidase	Develop defense mechanism and protect from oxidative stress	Aslanturk et al., 2011
Fall armyworm	*Spodoptera frugiperda*	Organophosphate insecticides	Alkaline phosphatase, esterase, glutathione S-transferase, aminopeptidase, and proteinase	Resistance, detoxification of pesticides	Zhu et al., 2015
Parasitic wasps	*Eretmocerus mundus, Eretmocerus eremicus* and *Encarsia formosa*	Abamectin	Esterases	Resistance, support to gut microbes, play key role in insect biology, ecology and behavior	Fernández et al., 2019
Colombian mosquito	*Aedes aegypti*	Pyrethroid insecticides	Esterases and oxidases	Resistance and mutation development	Granada et al., 2021
Whitefly	*Bemisia tabaci*	Neonicotinoid insecticides	Cytochrome P450	Insecticide resistance, support to symbiotic bacteria	Barman et al., 2021
Honeybee	*Apis mellifera*	Flumethrin	Catalase	Resistance, increased immunity to pathogens and improvement of detoxification genes (*GST, Hymenoptaecin, Defensin1, Catalase, GAPDH*)	Yu et al., 2021
Greater Wax Moth	*Galleria mellonella*	Malathion	Esterase and glutathione S-transferase	Resistance, detoxification of malathion and development of complex biological products	Serebrov et al., 2006
Yellow fever mosquito	*Aedes aegypti*	Permethrin	Cytochrome P450 monooxygenases	Insecticide resistance, perform multiple biological functions and metabolize pesticide	Somwang et al., 2011; David et al., 2014
Yellow fever mosquito	*Aedes aegypti*	Deltamethrin	Cytochrome P450	Metabolic resistance	Faucon et al., 2015
Diamondback moth	*Plutella xylostella*	Fenvalerate, fipronil, flufenoxuron and monocrotophos	Hydrolases, transferases and oxygenase's	Detoxification of pesticides and resistance development	Mohan and Gujar, 2003
Yellow fever mosquito	*Aedes aegypti*	Glyphosate and alpha pyrene	Cytochrome P450 monooxygenases, glutathione S-transferases and carboxy/cholinesterase	Resistance, improvement of detoxification genes and development of biological products	Riaz et al., 2009
Brown planthopper	*Nilaparvata lugens*	Acephate, thiamethoxam and buprofezin	Esterases, glutathione S-transferases and mixed-function oxidases	Resistance	Malathi et al., 2017
Housefly	*Musca domestica*	Diazinon	Cytochrome P450	Resistance and role in insect biology, ecology, and behavior	Cariño et al., 1994
African malaria mosquito	*Anopheles gambiae*	Bendiocarb	Cytochrome P450	Resistance and detoxification of pesticide	Edi et al., 2014
Boisduval	*Tetranychus cinnabarinus*	Abamectin and fenpropathrin	Carboxylesterases, mixed function oxidase, glutathione S-transferases, and hydrolases,	Resistance	Lin et al., 2009
Green peach aphid	*Myzus persicae*	Neonicotinoid insecticides	Cytochrome P450	Resistance and improve detoxification genes	Puinean et al., 2010
Yellow fever mosquito	*Aedes aegypti*	Organophosphate, carbamate and some pyrethroid insecticides	α and β Esterases, mixed-function oxidases, glutathione-S-transferase, acetylcholinesterase, and insensitive acetylcholinesterase	Resistance, support to gut microbes, play key role in insect biology, ecology, and behavior	Flores et al., 2006

(Continued)

TABLE 2 (Continued)

Insect common name	Insect scientific name	Name of pesticide	Name of enzyme	Functions	Reference
Yellow fever mosquito	*Aedes aegypti*	DDT and deltamethrin	Glutathione S-transferase and dehydrochlorinase	Resistance and detoxification of pesticides	Lumjuan et al., 2011
Annual bluegrass weevil	*Listronotus maculicollis*	Bifenthrin	Cytochrome P450 monooxygenases, glutathione S-transferases, and carboxylesterases	Detoxification, resistance and development of biological products	Ramoutar et al., 2009
Yellow fever mosquito	*Aedes aegypti*	Permethrin, temephos and atrazine	Cytochrome P450 monooxygenases	Resistance	Poupardin et al., 2008
Australian sheep blowfly	*Lucilia cuprina*	Organophosphate insecticides	Carboxylesterases and acetylcholinesterase	Resistance, detoxification of insecticides and provide protection from external pathogens	Jackson et al., 2013
Green peach aphid	*Myzus persicae*	Imidacloprid, acetamiprid and cyhalothrin	Acetylcholinesterase, carboxylesterase, glutathione-S-transferase, and mixed-function oxidase, superoxide dismutase, catalase, peroxidase, amylase	Food digestion, resistance development, breakdown of pesticide compounds and provide protection from external pathogens	Cai et al., 2021
Cotton bollworm	*Helicoverpa armigera*	Esfenvalerate, indoxacarb, emamectin benzoate and chlorantraniliprole	P450 enzymes	Resistance and detoxification of pesticides	Wang et al., 2018
Australian cotton bollworm	*Helicoverpa armigera*	Fenvalerate	Cytochrome P450 monooxygenase and carboxylesterases	Resistance and provide protection from external pathogens	Joußen et al., 2012
Red spider mite	*Tetranychus urticae*	Abamectin	Cytochrome P450	Resistance	Riga et al., 2014
Asian malaria mosquito	*Anopheles stephensi*	Pyrethroid and organophosphate insecticides	Cytochrome P450s, esterase's, glutathione S-transferases and acetylcholine esterase	Resistance and detoxification of pesticides	Safi et al., 2017
Migratory locust	*Locusta migratoria*	Carbaryl, malathion, and deltamethrin	Cytochrome P450 monooxygenases	Resistance	Guo et al., 2012
Oriental fruit fly	*Bactrocera dorsalis*	Fenitrothion	Acetylcholinesterase	Resistance and support to detoxification genes	Hsu et al., 2006
African malaria mosquito	*Anopheles gambiae*	Deltamethrin	Cytochrome P450 enzymes	Resistance	Yahouédo et al., 2017
White-backed planthopper	*Sogatella furcifera*	Imidacloprid, deltamethrin and triazhophos	Cytochrome P450 enzymes	Detoxification of pesticides and development of resistance	Zhou et al., 2018
Bed bug	*Cimex lectularius*	Deltamethrin	Cytochrome monooxygenase, esterase's, glutathione S-transferase, and carboxylesterase	Resistance	Gonzalez-Morales and Romero, 2019
Cowpea aphid	*Aphis craccivora*	Thiamethoxam	Glutathione S-transferase and mixed function oxidases	Resistance	Abdallah et al., 2016
Small brown planthopper	*Laodelphax striatellus*	Chlorpyriphos and dichlorvos	Alkaline phosphatase, carboxylesterase, acetylcholinesterase, acid phosphatase, glutathione S-transferase and cytochrome P450 monooxygenase	Resistance and detoxification of pesticides	Wang et al., 2010

tolerance of pesticides is interlinked with these biochemical processes which are held in the insect body and sensitivity of pesticides which are further degraded by metabolic enzymes such as hydrolase, esterase, laccase, acetylcholinesterase, carboxylesterase, glutathione S-transferase, cytochrome P450 and many more (Ismail, 2020; Clark et al., 2021; Yang Y. -X. et al., 2021; Ahmad et al., 2022b; Siddiqui et al., 2022). Insect pests' resistance against different pesticides by insect gut microbial enzymes was reported (**Table 2**). In the midgut of the tobacco budworm (*Heliothis virescens*), many essential

enzymes were purified, such as 58 proteinases, four cadherins, 13 aminopeptidases, and five alkaline phosphatases. Other putative detoxification enzymes include 20 cytochrome P450 oxidases, 11 glutathione S-transferases, nine esterase's, and 15 cytochrome oxidases. These enzymes contributed to insect physiology and reduced the toxicity of pesticides (Zhu et al., 2011).

An investigation was conducted to understand the enzymatic molecular mechanism for biodegradation of chlorpyriphos, glyphosate, phoxim, and esfenvalerate. In this study, 263 bacterial colonies were isolated from the gut of a cricket (*Teleogryllus occipitalis*), cultured individually, and examined for their degradation efficiency. Based on morphological, physiological, and 16S rRNA analysis and found that 55 bacteria species showed a high resemblance to 28 genera. Among these 55 bacterial species, 18 have the potential to degrade 50%, and six were able to degrade 70% of chlorpyriphos at an initial concentration of 400 mg/L within 1 day of incubation in a mineral salt medium. In addition, purification of extracellular hydrolase enzymes was studied in these isolates and found that free cells and hydrolase enzymes play a crucial role in the degradation of chlorpyriphos, glyphosate, phoxim, and esfenvalerate. A carboxyl esterase enzyme was purified from the mosquito gut bacteria (*Escherichia coli*) and studied for its effectiveness in degrading malathion. The results of the degradation experiment revealed that these carboxylesterase enzymes could efficiently degrade more than 80% of malathion. This study concluded that due to their rapid degradation ability, superior stability, and high activity, these enzymes could further degrade other organophosphate pesticides that contaminate the environment (Zhang et al., 2004).

In another study, a study was carried out on the biodegradation of organophosphate and pyrethroid pesticides from the contaminated environment to evaluate the resistance mechanism in insect pests (*Helicoverpa armigera*). In this study, a yeast (*Pichia pastoris* HaGST-8) was isolated from the insect gut and purified glutathione-S-transferase enzymes to detoxify chlorpyriphos dichlorvos and cypermethrin up to a concentration of 2–15 mg/L. Results of this study revealed that these enzymes have the potential to degrade all organophosphate pesticides completely and cypermethrin partially (53%) in an aqueous solution. Moreover, this study suggested that isolated yeast provides satisfactory growth in all pesticides at higher concentrations (200–400 mg/L) and concluded that these purified enzymes could be further utilized to degrade other pesticide groups such as organophosphates, carbamates, pyrethroids, organochlorines, and organophosphates from food, soil, and water resources (Labade et al., 2018). Two potential enzymes, such as cytochrome P450 monooxygenase and esterase, were purified from an insect gut (*Aedes aegypti*) bacterium and characterized for their efficiency in degrading propoxur and naled insecticides. Results of this study indicated that both types of enzymes play

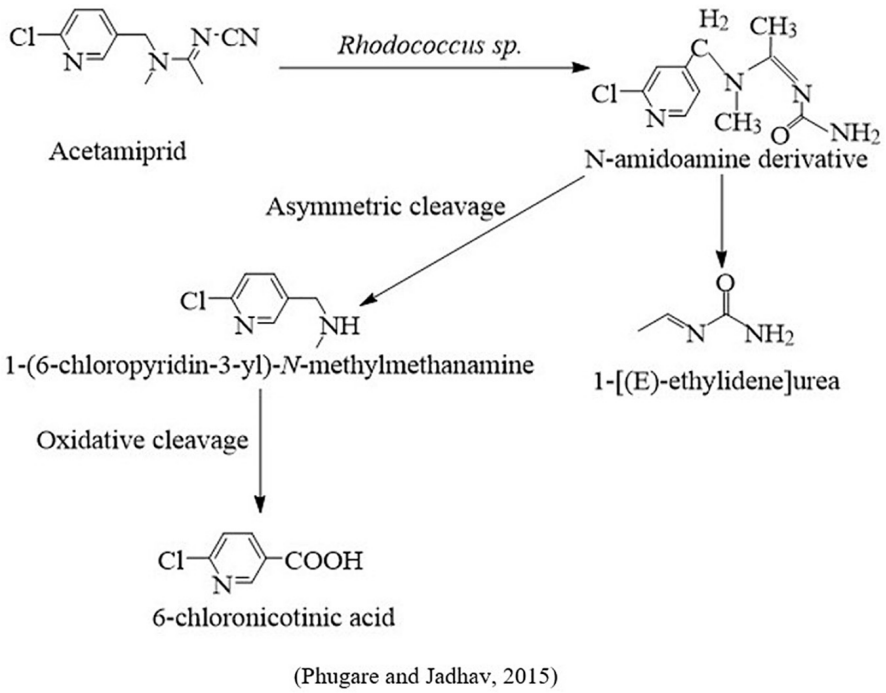

FIGURE 4
Microbial metabolic pathway of acetamiprid degradation by insect gut microbiota.

a crucial role in the degradation of pesticides and are further metabolized into non-toxic substances. This study concluded that insect gut symbiotic bacteria and their associated enzymes reduced the toxicity of pesticides, enhanced resistance, and played an essential role in the digestion of food (Scates et al., 2019).

The potential and purification of three enzymes such as hydrolases, transferases, and oxygenase's, were purified from the insect (*Plutella xylostella*) gut bacterium (*Bacillus thuringiensis var. kurstaki* HD-1) and investigated their role in the development of resistance and degradation of insecticides such as fenvalerate, fipronil, and flufenoxuron. This study demonstrated that all detoxifying enzymes were responsible for developing resistance against the diamondback moth insecticides and metabolizing them into less toxic substances (Mohan and Gujar, 2003). However, it has been proven that a diverse number of prokaryotic and eukaryotic microbial species in the insect gut and their associated enzymes actively contribute to the degradation of various pesticides and metabolize them into less toxic substances. These less toxic substances were further utilized by microbiota as a sole source of carbon, sulfur, and energy and play a key role in insect physiology (Mohammadi et al., 2021).

Microbial metabolic pathways

The high application of pesticides to control various pests has produced long-term hazardous residual pollution in the ecosystem (Shahid et al., 2021). A lot of insect pest species depend on insect gut microbial species to attain nutrients, in defense mechanisms, exploit novel food resources and undergo metabolization of parent compounds into their intermediates (Chen et al., 2020). Various microbial species break down parent compounds into their metabolites, which pose the same or higher toxicity than the parent compound and disturb the environmental equilibrium.

An acetamiprid-degrading bacterium that provides the highest growth on a mineral salt medium was isolated and, based on morphological, physiological, 16S rRNA, and BLAST analysis, identified as *Rhodococcus* sp. BCH2. This study showed that the acetamiprid was rapidly metabolized into its three metabolites, such as *N*-amidoamide derivative, 1-(6-chloropyridin-3yl)-*N*-methylmethanamine, and 6-chloronicotinic acid. Additionally, toxicological effects of the parent compound and their metabolites on silkworm (*Bombax mori*) concerning genotoxicity, antioxidant enzymes, lipid peroxidation, and protein oxidation were also investigated. This study suggests that the parental molecule has more hazardous effects on insect physiology than its derivatives (Phugare and Jadhav, 2015; **Figure 4**). In another study, five intestinal bacterial species were screened to understand the chlorpyriphos biodegradation mechanism. After characterization of physiological and morphological properties, bacterial species were identified as *Lactobacillus lactis*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Escherichia coli,* and *Enterococcus faecalis.* Plate assay findings

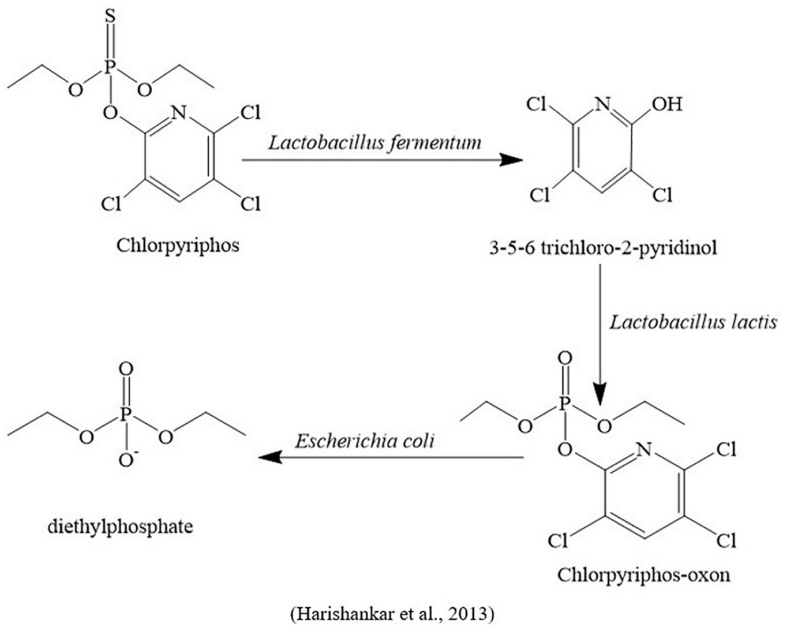

FIGURE 5
Microbial metabolic pathway of chlorpyriphos degradation by insect gut microbiota.

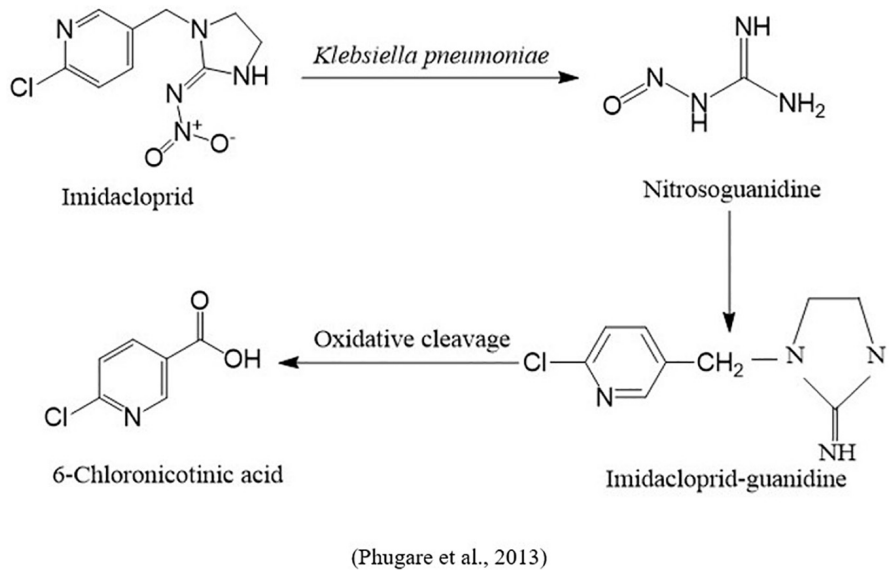

FIGURE 6
Microbial metabolic pathway of imidacloprid degradation by insect gut microbiota.

indicated that three of them (*Lactobacillus fermentum, Escherichia coli,* and *Lactobacillus lactis*) could provide maximum growth even using higher dosages (>1400 μg/L), while the other two bacterial species (*Lactobacillus plantarum* and *Enterococcus faecalis*) were able to grow using less concentrations (100 and 400 μg/L), respectively. Based on growth parameters, the best three bacterial species were investigated to degrade chlorpyriphos and found that *L. fermentum* was able to degrade 70% of chlorpyriphos and generate one metabolite named 3,5,6-trichloro-2-pyridinol, *L. lactis* degraded 61% of chlorpyriphos into chlorpyrifos oxon, and *E. coli* provided less chlorpyriphos degradation (16%) and breakdown in chlorpyrifos oxon and diethyl phosphate (**Figure 5**).

Resistance to pesticides in insect pest species is increased *via* the metabolism pathway and regulation of various genes and enzymes and is considered a significant problem throughout the globe (Kalsi and Palli, 2017). A study was carried out to examine the resistance of imidacloprid through the regulation of genes in vinegar flies (*Drosophila melanogaster*) and evaluate how imidacloprid metabolites are generated and affect vinegar flies. These questions have been addressed by coupling the genetic tools of gene overexpression and CRISPR gene knock-out with the mass spectrometric technique, the Twin-Ion Method. In this study, *the Cyp6g1* gene, responsible for developing resistance against different insecticides, including imidacloprid, was identified. It found that gut microbes living in vinegar flies were responsible for generating oxidative and nitro-reduced metabolites, which were further interconnected with overexpression of the gene *Cyp6g1*. Additionally, this study revealed that imidacloprid was metabolized into toxic metabolites that were not further degraded into less harmful products and were excreted relatively hardly (Fusetto et al., 2017). In another study, the biodegradation of imidacloprid by the strain *Klebsiella pneumoniae* BCH1 and the effect of its toxic metabolites on silkworm (*Bombyx mori*) were studied. The strain was able to degrade 78% of imidacloprid within a week and produced three metabolites: nitrosoguanidine, imidacloprid guanidine, and 6-chloronicotinic acid by using gas chromatography and mass spectrometry. The toxicity of imidacloprid and its metabolites revealed that they enhanced oxidative stress, lipid peroxidation, protein oxidation, DNA damage, and changed the activity of antioxidant enzymatic status (Phugare et al., 2013; **Figure 6**).

Conclusion and future perspectives

The applications of synthetic pesticides are heavily applied throughout the globe to develop insect pests' infestation strategies. Their toxic residues are accumulated in the agroecosystem, which causes severe threats to animals, humans, birds, and other non-target organisms. Investigating microbial species and their associated enzymes in insect pests' digestive tracts is essential in agricultural research. Recent advances in independent culture methods such as next-generation sequencing, BLAST analysis, and 16S rRNA analysis have

provided new insights into understanding the extensive range of symbiotic microbial communities and their functions with insects. Symbiotic microbial species are very beneficial for the regulation of insect physiology and contribute to a very significant role, such as in insect fitness, by providing amino acids, vitamins, lactic acids, and sterols, enhanced immunity system, food digestion, excretion of waste fluids, host fertility, increased resistance to toxins and external pathogens; and degradation of pesticides and allelochemicals into less toxic products by the production of different hydrolytic enzymes. The biodegradation of pesticides by isolating indigenous insect symbiotic microbial species and their associated catabolic enzymes, genes, and proteins has become an excellent option to clean up the contaminated environment.

Additionally, insects can swiftly obtain novel metabolic activities and colonize new ecological niches through symbiotic interactions with microbiota that have previously fully developed well-tuned metabolic pathways and converted toxic compounds into derivatives. However, the various characteristics of symbiotic microbial species and their associated enzymes that work mutually with insect guts as a superior biocontrol agent are yet to be ascertained. Several omic approaches to predict hidden microbial communities, database approaches for their identification, and systematic biomolecular tools are urgently required to discover unknown features of insect gut microbial species. In the coming days, the relevant discoveries will immensely provide new myriads to explore the proliferation and diversification of insect gut microbial communities and, on the other hand, develop several industrial applications and environmentally friendly technologies for generating wealth, such as the production of biofuels.

Author contributions

YL conceived the project and contributed to revise the manuscript. SJ prepared the original draft. SA prepared the tables and figures and revised all the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Highly efficient degradation of cypermethrin by a co-culture of *Rhodococcus* sp. JQ-L and *Comamonas* sp. A-3

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Cypermethrin is an important synthetic pyrethroid pesticide that widely used to control pests in agriculture. However, extensive use has caused its residue and the metabolite 3-phenoxybenzoic acid (3-PBA) to seriously pollute the environments and agricultural products. In this study, a highly efficient cypermethrin-degrading bacterial consortium was acclimated from long-term pyrethroid-contaminated soil. Two strains, designated JQ-L and A-3, were screened from the consortium, and identified as *Rhodococcus* sp. and *Comamonas* sp., respectively. Strain JQ-L transformed 100 mg/L of cypermethrin to 3-PBA within 60 h of incubation; however, 3-PBA could not be further degraded by the strain. Strain A-3 utilized 3-PBA as sole carbon for growth, and completely degraded 100 mg/L of 3-PBA within 15 h of incubation. Co-culture of JQ-L and A-3 completely degraded 100 mg/L of cypermethrin within 24 h of incubation. Furthermore, a complete catabolic pathway of cypermethrin and the metabolite 3-PBA by the co-culture was proposed. This study provided a promising strategy for efficient elimination of cypermethrin residue-contaminated environments and agricultural products.

KEYWORDS

cypermethrin-degrading, *Rhodococcus* sp., *Comamonas* sp., catabolic pathway, 3-Phenoxybenzoic acid

Introduction

Cypermethrin [cyano-(3-phenoxyphenyl)methyl (1R,3S)-3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate] is an important synthetic pyrethroid pesticide (Tallur et al., 2008; Lin et al., 2011; Chen et al., 2012a; Chen et al., 2014; Zhan et al., 2020). Due to its broad spectrum and high efficiency, cypermethrin was widely used to control various pests in cotton, rice, corn, soybean, fruit trees

and vegetables (Dorman and Beasley, 1991; Katsuda, 1999). In recent years, the usage of cypermethrin has rapidly increased and become a dominant pesticide worldwide with the restrictions of highly toxic organophosphorus and organochlorine pesticides. However, extensive usage of cypermethrin has resulted in frequent detection of its residues in the environments and agricultural products, which accumulated in human or mammal bodies through food chain (Schettgen et al., 2002; Kuivila et al., 2012; Łozowicka et al., 2012; McCoy et al., 2012). Although cypermethrin generally has lower toxicity to human or mammalian than organophosphorus and organochlorine pesticides, prolonged exposure to high concentrations of cypermethrin might cause endocrine disruption, spleen damage, and carcinogenesis (Wolansky and Harrill, 2008; Dewailly et al., 2014; Sun et al., 2014; Ye et al., 2017; Zepeda-Arce et al., 2017; Chrustek et al., 2018; Navarrete-Meneses and Pérez-Vera, 2019; Gammon et al., 2019). Furthermore, cypermethrin residue in the environments had strong acute toxicity to some non-target organisms, such as aquatic invertebrate, fish and bee (Stratton and Corke, 1982; Smith and Stratton, 1986; Jin et al., 2011). Therefore, it is urgent to develop effective technology to eliminate cypermethrin residue from the environments and agricultural products.

Biodegradation is an ideal technology for removal of cypermethrin residue because of its high efficiency, environmental friendliness and low cost (Guo et al., 2009; Lin et al., 2011; Chen et al., 2012a; Ruan et al., 2013). To date, a lot of microorganisms that were able to degrade cypermethrin have been isolated, such as *Bacillus licheniformis* B-1 (Liu et al., 2014; Zhao et al., 2016), *Bacillus cereus* ZH-3 (Chen et al., 2012b), *Sphingobium wenxiniae* JZ-1 (Guo et al., 2009; Wang et al., 2009), *Trichoderma viride* and *Aspergillus niger* (Saikia and Gopal, 2004; Zhao et al., 2016; Zhan et al., 2020). However, most of these microorganisms possessed a relatively low cypermethrin-degrading efficiency and could not completely mineralized cypermethrin due to their lack of a complete catabolic enzymes system. E.g., *Bacillus licheniformis* B-1, *Bacillus cereus* ZH-3 and *Acinetobacter junii* LH-1-1 transformed cypermethrin to 3-phenoxybenzoic acid (3-PBA), but could not further degrade 3-PBA (Chen et al., 2012b; Liu et al., 2014). 3-PBA is an endocrine disruptor due to its antiestrogenic activity and is persistent in environment; furthermore, 3-PBA is hydrophilic and therefore easier to migrate in the environment than cypermethrin (Halden et al., 1999; Han et al., 2008; Chen et al., 2011; Chen et al., 2012b; Sun et al., 2014). Therefore, simultaneous elimination of cypermethrin and the metabolite 3-PBA are crucial to environmental and human health. Co-culture of strains capable of degrading cypermethrin and 3-PBA, respectively, is an effective method to completely eliminate cypermethrin residue (Huang et al., 2018; Zhan et al., 2020). In previous reports, Liu et al found that co-culture of cypermethrin-degrading strain *Bacillus licheniformis* B-1 and 3-PBA-degrading strain *Sphingomonas* sp. SC-1 or *Aspergillus oryzae* M-4 significantly improved the degradation efficiency of cypermethrin when compared with culture of *Bacillus licheniformis* B-1 alone (Liu et al., 2014; Zhao et al., 2016; Zhao et al., 2019), and Chen et al. (2012b) found that co-culture of cypermethrin-degrading strain *Bacillus cereus* ZH-3 and 3-PBA-degrading strain *Streptomyces aureus* HP-S-01 completely mineralized cypermethrin within 72 h of incubation.

In this study, we screened two strains JQ-L and A-3 from a highly efficient cypermethrin-degrading bacterial consortium. Strain JQ-L was identified as *Rhodococcus* sp. and could transform cypermethrin to 3-PBA, while strain A-3 was identified as *Comamonas* sp. and could completely degrade 3-PBA. Co-culture of JQ-L and A-3 efficiently and completely degraded cypermethrin within 24 h of incubation. Furthermore, a complete catabolic pathway of cypermethrin by co-culture of JQ-L and A-3 was also proposed in this study.

Materials and methods

Chemicals and media

Cypermethrin (97%) was purchased from Jiangsu Yangnong Chemical Group Co., Ltd, 3-PBA (98%) and chromatographic grade acetonitrile were purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). All other chemicals and solvents used in this study were analytical grade. Cypermethrin was dissolved in methanol as stock solutions (24 mM), and sterilized by membrane filtration (0.22 μm). The R2A medium consisted of 0.25 g/L tryptone, 0.5 g/L yeast extract and 0.5 g/L casein acid hydrolyzate. The Luria-Bertani (LB) medium consisted of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl, pH was adjusted to 7.0. Mineral salt medium (MSM) consisted of 1.0 g/L NH_4Cl, 1.0 g/L NaCl, 1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4 and 0.2 g/L $MgSO_4{\cdot}7H_2O$, pH 7.0. For solid medium, 1.5% of agar was added. All medium was autoclaved at 121°C for 30 min.

Enrichment, isolation, and identification of cypermethrin-degrading strain

The soil sample was collected from the long-term pyrethroid-contaminated site near Jiangsu Yangnong Chemical Group Co., Ltd (119° 15′ E, 32° 26′ N) in September 2020. Soil sample (10 g) was added to a 250 mL Erlenmeyer flask containing 90 mL of MSM supplemented with 0.24 mM of cypermethrin. The flask was then incubated at 30°C and 180 rpm on a rotary shaker. At certain intervals, the degradation of cypermethrin was detected using ultraviolet-visible spectrophotometer (UV/VIS) or Gas chromatography

(GC) as described below. When approximately 60–70% of the added cypermethrin was degraded, 10 mL of the enrichment culture was transferred into 90 mL fresh medium. The transfer was repeated for 10 times until the enriched consortium acquired highly efficient cypermethrin-degrading ability. Then, the last round enrichment culture was serially diluted and spread on R2A plates. After incubated at 30°C for 4–6 days, colonies with different morphologies were selected and purified by the streak plate method. The abilities of the isolates to degrade cypermethrin or 3-PBA were detected by GC or high-performance liquid chromatography (HPLC), respectively, as described below.

The acquired isolates were characterized and identified by morphological, physiological and biochemical characteristics as well as 16S rRNA gene sequence analysis (Zhang et al., 2012). The phylogenetic tree was constructed by the neighbor-joining method using the MEGA software (version 6.0) with Kimura's two-parameter calculation model. The topology of the phylogenetic tree was assessed by bootstrap analysis of 1,000 replications.

Inoculum preparation

The isolates were stored in 15% glycerol at −80°C. Before each experiment, the isolates were thawed and grown individually in 250 mL Erlenmeyer flasks containing 100 mL of LB medium. Each strain was harvested in the late-exponential growth phase by centrifuging (5,000 rpm, 5 min) and washed twice with sterile water, and finally resuspended in MSM, the cell density was adjusted to $OD_{600\ nm}$ of 2.0.

Biodegradation of cypermethrin and 3-phenoxybenzoic acid by pure isolates

To investigate the abilities to degrade cypermethrin or 3-PBA by the isolates, cells were transferred (2% inoculation amount) into 250-mL Erlenmeyer flasks containing 100 mL MSM supplemented with 0.24 mM cypermethrin (and 1 g/L glucose) or 3-PBA. The Erlenmeyer flasks were incubated at 180 rpm and 30°C on a rotary shaker. At certain intervals, an Erlenmeyer flask was removed from the shaker and the cultures were used for growth and chemical analysis. The growth of the strain was determined by measuring the optical density (OD) at 600 nm, the residual cypermethrin concentration was analyzed by UV/VIS (qualitative detection) or GC (quantitative detection), and the 3-PBA concentration was analyzed by UV/VIS or HPLC as described below. All experiments were conducted in triplicates and the results were averages from three independent experiments.

Effect of pH on the degradation of cypermethrin or 3-PBA was tested in MSM, the pH was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 by citrate/Na_2HPO_4 buffer (pH 4.0–5.0), Na_2HPO_4/NaH_2PO_4 buffer (pH 6.0–8.0) and glycine/NaOH buffer (pH 9.0–10.0). Effect of temperature was evaluated at 16, 25, 30, 37, and 42°C, respectively. Effect of salinity (w/v, NaCl concentration) was measured at NaCl concentration (w/v) of 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0%, respectively. Effect of cypermethrin or 3-PBA concentrations was evaluated at 0, 10, 20, 50, 100, 150, and 200 mg/L.

Chemical analysis

For cypermethrin detection, the culture was extracted with an equal amount of dichloromethane, the organic layer was dried and redissolved in *n*-hexane. Qualitative detection was carried out using a UV/VIS spectrophotometer (UV-2450, Shimadzu, Japan) at wavelengths from 200 to 340 nm. Quantitative detection was carried out on GC. The GC conditions were as follows: electron capture detector, HP-5 capillary column (30.0 mm × 0.530 mm × 1.5 μm), injector/interface temperature of 240°C, oven temperature of 260°C, detector temperature of 300°C, and N_2 carrier gas at 1 mL/min. The column temperature was programmed from 150 to 260°C with a rate of 25°C/min. For 3-PBA detection, the sample was dissolved in methanol and analyzed by HPLC using an UltiMate 3000 titanium system (Thermo Fisher Scientific) equipped with a C_{18} reversed-phase column (4.6 mm × 250 mm × 5 μm) (Thermo Fisher Scientific, MA, United States). The mobile phase consisted of acetonitrile, water, acetic acid (50: 49: 1 [vol/vol/vol]) at a flow rate of 0.8 mL/min and a column temperature of 40°C. A VWD-3100 single-wavelength detector was used to monitor the UV absorption with the detection wavelengths being 240 nm, the injection volume is 20 μL. To detect the catabolic metabolites of cypermethrin or 3-PBA, the sample was dissolved in methanol and analyzed by LC-TOF-MS as described by Liu et al. (2021).

Genome sequencing and genes analysis

Genomic DNA was extracted from the cell pellets of A-3 using a Bacteria DNA Kit (OMEGA). The genome of A-3 was sequenced using the Illumina NovaSeq 6000 by Biozeron Biotechnology Co., Ltd. (Shanghai, China). Gene prediction and annotation were performed by BLAST analysis in the UniProtKB/Swiss-Prot, non-redundant protein (NR), KEGG, and COG databases of the National Center for Biotechnology Information (NCBI) and the Rapid Annotation Subsystem Technology (RAST).

Data availability

The accession numbers of the 16S rRNA gene sequences of strain JQ-L and A-3 were OP2048080 and OP2048079, respectively; the accession number of the genome of A-3 was JANHNY000000000; the accession numbers of *pobA* and *pobB* were OP219508 and OP219509, respectively; the accession numbers of *proA1* and *proA2* were OP219511 and OP219509, respectively; the accession numbers of *dmpLMNOPQR* were OP219514–OP219520, respectively; and the accession number of *catA2* was OP219513.

Results

Enrichment and screening of cypermethrin-degrading strains

In this study, we used cypermethrin as the sole carbon source to enrich cypermethrin-degrading bacteria. It took 7 days to degrade approximately 70% of the added 0.24 mM of cypermethrin for the first round of enrichment. With the progress of acclimation, the ability of the bacterial consortium to degrade cypermethrin was more and more strong. After ten rounds of transfer, the enriched consortium acquired highly efficient cypermethrin-degrading ability. As shown in **Supplementary Figure 1**, cypermethrin has two characteristic absorption peaks (240 nm and 280 nm) in the UV region (200 to 340 nm). After incubation for 12 h, the two peaks almost completely disappeared, and no new peak was generated. The results indicated that the added cypermethrin was completely degraded by the consortium, and no aromatic metabolite was accumulated.

From the enriched consortium, approximately 20 colonies with different morphologies were isolated and purified. Then, their abilities to degrade cypermethrin were qualitatively determined using UV scanning. The result showed that only one strain, designated as JQ-L, was able to change the UV scanning spectrum of the culture. As showed in **Supplementary Figure 2**, after treated by strain JQ-L for 60 h, the peak at 280 nm decreased, and a new peak at 300 nm was generated. However, the generated peak did not decrease with prolonged incubation. The result indicated that strain JQ-L transformed cypermethrin to a metabolite which could not be further degraded by strain JQ-L.

Since the enriched consortium could completely degrade cypermethrin, we speculate that there must be strains that could degrade the metabolite produced in the conversion of cypermethrin by JQ-L in the consortium. Therefore, strain JQ-L was co-cultured with other isolated stains to degrade cypermethrin, respectively. UV scanning result indicated that when strain JQ-L was co-cultured with strain A-3, the peaks at 240 nm and 280 nm decreased, and no new peak was accumulated (**Supplementary Figure 2**). The results indicated that strain A-3 could completely degrade the metabolite generated during cypermethrin transformation by stain JQ-L.

Identification of strains JQ-L and A-3

Colonies of strain JQ-L grown on LB agar for 5 days were yellow, round, convex, dry with irregular margin, approximately 2–4 mm in diameter (**Supplementary Figure 3A**). Cells of strain JQ-L were aerobic, Gram-reaction-positive, non-sporeforming, non-motile and rod-shaped (0.5–0.6 μm wide and 1.5–2.0 μm long) (**Supplementary Figure 3C**). Grew at pH 6.0–9.0 (optimum, pH 7.0), at 15–37°C (optimum, 30°C) and with 0–3.0% (w/v) NaCl (optimum, 1% NaCl). Strain JQ-L utilized glucose, mannose and maltose, but not methanol, did not hydrolyze starch, urea and esculin, positive for the indole test, nitrate reduction and gelatin liquefaction, resistant to bacitracin, but sensitive to kanamycin, tetracycline, streptomycin, erythromycin, clindamycin and gentamicin.

The almost-complete 16S rRNA gene sequence of strain JQ-L was 1,485 bp. Strain JQ-L showed the highest identity with *Rhodococcus aetherivorans* 10bc321^T (99.9%), and shared 95.5% identities with *R. ruber* DSM43338^T and *R. electrodiphilus* JC435^T. Phylogenetic analysis based on the NJ tree showed that strain JQ-L belonged to the genus *Rhodococcus* and formed a subclade with *R. aetherivorans* 10bc321^T, *R. ruber* DSM43338^T and *R. electrodiphilus* JC435^T (**Figure 1A**). According to the phenotype and phylogenetic analysis of 16S rDNA sequence, strain JQ-L was preliminarily identified as *Rhodococcus* sp.

Colonies of strain A-3 grown on LB agar for 5 days were pale yellow, round, convex, moist with smooth edge, approximately 1.5–3 mm in diameter (**Supplementary Figure 3B**). Cells of strain A-1 were aerobic, Gram-reaction-negative, non-spore forming, and rod-shaped (0.7–0.8 μm wide and 2.0–2.3 μm long), flagellum was observed (**Supplementary Figure 3D**). Grew at pH 5.0–9.0 (optimum, pH 7.0), at 15–37°C (optimum, 30°C) and with 0–3.0% (w/v) NaCl (optimum, 1% NaCl). Negative for indole test and gelatin liquefaction and positive for nitrate reduction, resistant to kanamycin, streptomycin and penicillin, but sensitive to gentamicin, tetracycline and chloramphenicol. Strain A-3 degraded and utilized 3-PBA, 4-PBA, catechol, phenol and protocatechuate, but not methanol, 2-PBA or diphenyl ether.

The 16S rRNA gene sequence (1,521 bp) of strain A-3 shared 100% identity with *Comamonas thiooxydans* DSM 17888^T. In the phylogenetic tree, strain A-3 was clustered in the genus *Comamonas* and formed a clade with *C. thiooxydans* DSM 17888^T and *C. testosteroni* ATCC 11996^T (**Figure 1B**). Thus, based on the above analysis, strain A-3 was preliminarily identified as *Comamonas* sp.

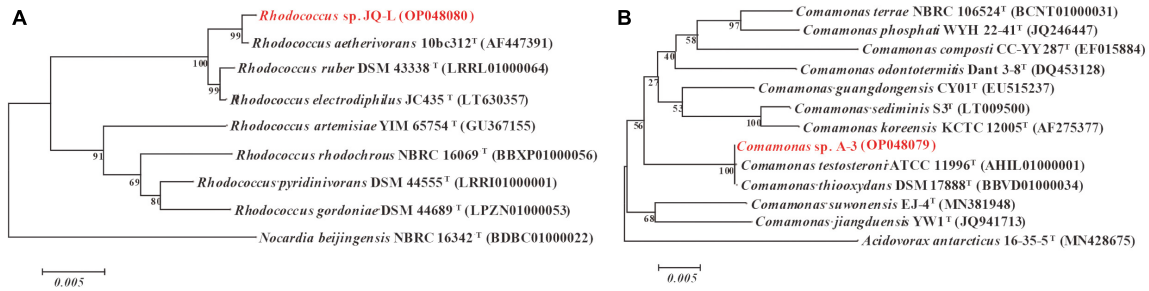

FIGURE 1
Phylogenetic analysis of strains JQ-L **(A)** and A-3 **(B)** with closely related type strains by neighbor-joining method based on 16S rRNA gene sequences. Bootstrap values (%) are indicated at the nodes, the scale bar represents 0.005 substitutions per site. The GenBank database accession numbers were shown in parentheses.

Degradation of cypermethrin by strain JQ-L

The degradation of cypermethrin by strain JQ-L was shown in Figure 2. The results showed that strain JQ-L degraded 79.8% and almost 100% of the added 0.24 mM of cypermethrin within 36 and 60 h of incubations, respectively. Meanwhile, a metabolite with retention time of 4.50 min in HPLC was generated, the retention time of the metabolite was consistent with that of standard 3-PBA (Supplementary Figure 4), MS/MS analysis indicated that the molecular ion peak $[M + H]^+$ of this metabolite was 214.0 m/z with fragment ion peaks at 169.1 m/z (Supplementary Figure 5) which was equal to the theoretical molecular mass of 3-PBA. Thus, the metabolite was identified as 3-PBA. During the degradation, the accumulation of 3-PBA continuously increased, its concentration increased to 0.22 mM within 60 h of incubation, and the produced 3-PBA did not decrease within prolonged incubation. These results indicated that strain JQ-L degraded cypermethrin to 3-PBA, and the generated 3-PBA could not be further degraded by strain JQ-L. The optimal conditions for cypermethrin degradation by JQ-L were 30°C, pH 7.0 and 1% NaCl (Supplementary Figure 6). Furthermore, strain JQ-L could also degrade permethrin, fenpropathrin, and deltamethrin, but not bifenthrin (Supplementary Figure 7).

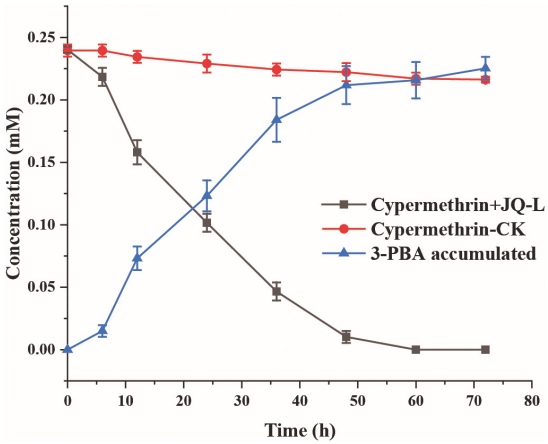

FIGURE 2
Degradation of cypermethrin by strain JQ-L. CK, 100 mg/L cypermethrin without inoculation; JQ-L, inoculation with strain JQ-L.

To investigate whether the accumulation of 3-PBA affected the cypermethrin-degrading efficiency by strain JQ-L, different concentrations of 3-PBA were added to the culture medium, the growth of strain JQ-L and the degradation of cypermethrin were measured. The results in Figure 3 showed that the addition of 3-PBA significantly inhibited the growth of JQ-L and the degradation efficiency of cypermethrin, and the greater the amount added, the higher the degree of inhibition. When the added 3-PBA reached 100 mg/L, the growth of JQ-L and the cypermethrin degradation were inhibited by approximately 80%. The results indicated that 3-PBA had obvious toxic effect on the growth of JQ-L and cypermethrin degradation.

Degradation of cypermethrin and 3-phenoxybenzoic acid by strain A-3

The abilities of strain A-3 to degrade cypermethrin and 3-PBA were investigated. Cypermethrin could not be degraded by stain A-3 (data not shown), and 50–200 mg/L of cypermethrin in the medium had no significant effect on the growth of strain A-3 (Supplementary Figure 8). Figure 4A showed the UV scanning analysis of 3-PBA degradation in MSM by strain A-3. The results showed that the characteristic absorption peaks of 3-PBA at 260 and 300 nm completely disappeared after 48 h of incubation, and no new peak was produced, indicating that strain A-3 completely degraded 3-PBA. Figure 4B showed that strain A-3 completely degraded 0.47 mM of 3-PBA within 15 h of incubation. At the same time, its $OD_{600\ nm}$ increased from 0.01 to 0.26, indicating that strain A-3 rapidly degraded 3-PBA

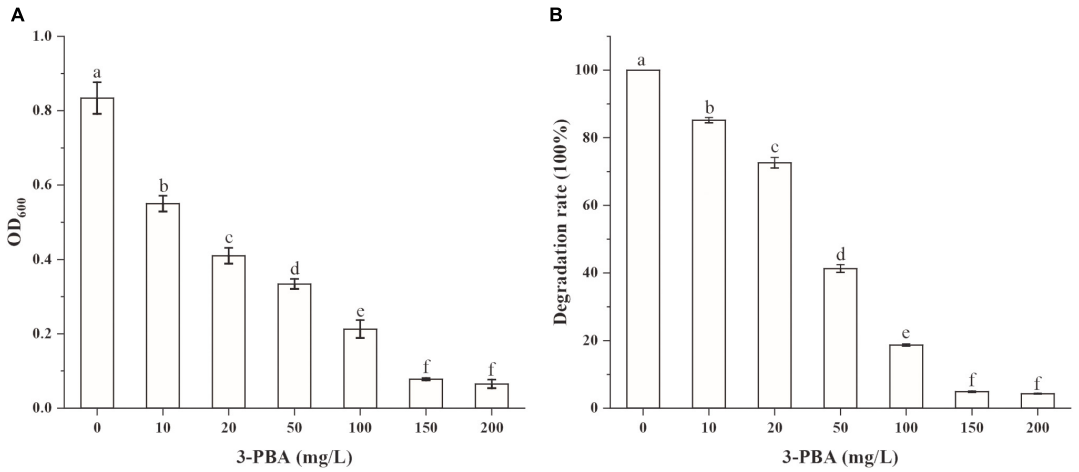

FIGURE 3
The effect of 3-PBA on the growth of strain JQ-L **(A)** and cypermethrin degradation by strain JQ-L **(B)**. Different lowercase letters indicate significant difference under $P < 0.05$.

and utilized it as the sole carbon source to grow. The optimal conditions for 3-PBA degradation by A-3 were 30°C, pH 7.0 and 1% NaCl (**Supplementary Figure 9**).

Degradation of cypermethrin by co-culture of strains JQ-L and A-3

The degradation of cypermethrin by the co-culture of strains JQ-L and A-3 was studied. **Figure 5** showed that co-culture of JQ-L and A-3 completely degraded 0.24 mM of cypermethrin within 24 h of incubation, while it took approximately 60 h to degraded the same amount of cypermethrin when only JQ-L was inoculated. This result indicated that co-culture of JQ-L and A-3 significantly accelerated the degradation rate of cypermethrin. The possible reason was that A-3 rapidly degraded the generated 3-PBA, thus relieved the toxic effect of 3-PBA on JQ-L.

Identification of intermediate of 3-phenoxybenzoic acid degradation by *Comamonas* sp. A-3

The intermediates of 3-PBA degradation by strain A-3 were detected by MS/MS. As shown in **Figure 6**, three compounds were detected. Compound I showed a molecular-ion peak at m/z 213.05, which was consistent with that of 3-PBA; and its two characteristic fragment ion peaks were also fit to those of 3-PBA (**Figure 6A**). The molecular-ion peak of compound II (93.03) was fit to that of phenol, and the fragment ion peak (65.03) of compound II was also consistent with that of phenol (**Figure 6B**). Thus, based on the above analysis, compounds I and II were identified as 3-PBA and phenol, respectively. Compound III showed a molecular-ion peak at m/z 203.02 (**Figure 6C**), however, its fragment ion peak was not detected.

Analysis of 3-phenoxybenzoic acid degradation related genes in the genome of A-3

To further investigate the catabolic pathway of 3-PBA in strain A-3, the genome of stain A-3 was sequenced. The genome size of A-3 was 5,511,770 bp, and 5578 putative ORFs were predicted. The genes that possibly involved in catabolism of 3-PBA and other aromatic compounds were predicted by bioinformatics analysis. The results in **Table 1** showed that a 3-PBA dioxygenase gene operon *pobAB*, a protocatechuate 4,5-dioxygenase gene operon *proA1A2*, a phenol catabolic gene operon *dmpRKLMNOP*, and a catechol 2,3-dioxygenase gene *catA2* was predicted in the genome of A-3.

Discussion

Cypermethrin is a refractory and toxic pesticide that extensively used to control pests in agriculture (Dorman and Beasley, 1991; Katsuda, 1999). At present, a lot of cypermethrin-degrading bacteria have been isolated; however, they usually could only transform cypermethrin to 3-PBA due to lack of downstream catabolic pathway. Furthermore, 3-PBA was toxic and had a feedback inhibition effect, resulting in these bacteria strains often showing relatively low cypermethrin-degrading efficiencies (Chen et al., 2011; Chen et al., 2012a; Liu et al., 2014; Zhao et al., 2016). On the contrary, bacterial consortium has the advantages of species diversity and complete

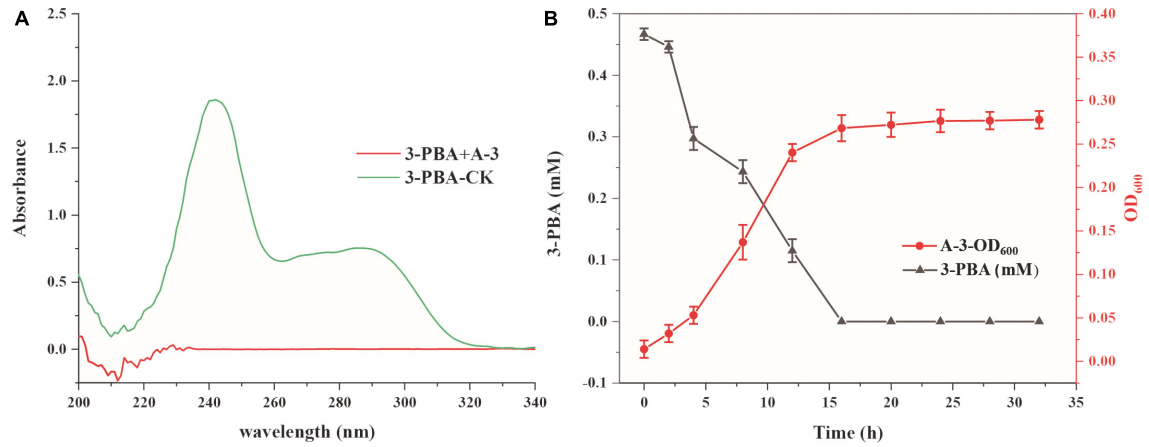

FIGURE 4
Degradation of 3-PBA by stain A-3. **(A)** UV scanning detection of 3-PBA degradation by strain A-3; **(B)** time course curve of 3-PBA degradation and growth of A-3.

metabolic enzyme system, thus could efficiently and completely mineralized complex refractory compounds (McCoy et al., 2012; Huang et al., 2018). However, the disadvantage of consortium is that it is difficult to culture the consortium in a large-scale and maintain its degradation ability for a long time. Therefore, it is necessary to simplify the consortium to improve the feasibility and reduce the cost. Previous studies have shown that co-culture of cypermethrin-degrading strain and 3-PBA degrading strain could effectively and completely degrade cypermethrin. E.g., compared with strain B-1 alone, the half-life ($t1/2$) of cypermethrin by co-culture of B-1 and 3-PBA-degrading strain SC-1 was shortened from 84.53 to 38.54 h, and approximately 75% of the added 0.24 mM of cypermethrin was degraded by the co-culture within 72 h of incubation (Liu et al., 2014).

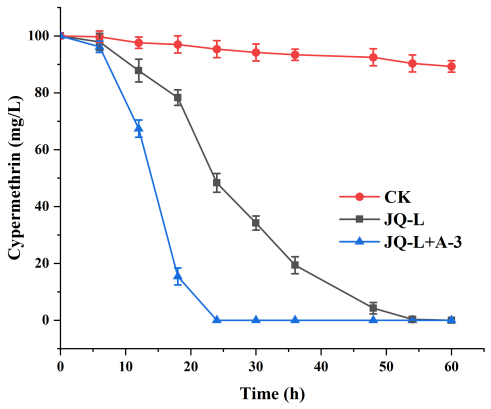

FIGURE 5
Time course curve of cypermethrin degradation by co-culture of JQ-L and A-3.

Chen et al reported that co-culture of *Bacillus cereus* ZH-3 and 3-PBA-degrading strain *Streptomyces aureus* HP-S-01 increased the cypermethrin-degrading efficiency by two times when compared with *Bacillus cereus* ZH-3 alone, the co-culture completely mineralized cypermethrin within 72 h of incubation (Chen et al., 2012b). In this study, a bacterial consortium that capable of efficiently and completely degrading cypermethrin was acclimated using the soil near a pesticide factory. Two strains JQ-L and A-3 were screened from the consortium and identified as *Rhodococcus* sp. and *Comamonas* sp., respectively. The roles of the two strains in the catabolism of cypermethrin were studied. *Rhodococcus* sp. JQ-L transformed cypermethrin to 3-PBA, however, the strain could not further degrade 3-PBA, resulting in the accumulation of 3-PBA in the culture medium, which seriously inhibited the growth and degradation ability of JQ-L. Strain A-3 could not degrade cypermethrin, however, it completely and efficiently degraded and utilized 3-PBA as sole carbon for growth. Co-culture of JQ-L and A-3 could completely degrade cypermethrin. Compared with JQ-L alone, co-culture of JQ-L and A-3 significantly reduced the total degradation time of cypermethrin from 60 to 24 h. Furthermore, in previous reports (Chen et al., 2012b, Liu et al., 2014; Zhan et al., 2020; Zhao et al., 2021), it took 72 h or more for co-culture of cypermethrin-degrading strain and 3-PBA-degrading strain to completely degrade 0.24 mM of cypermethrin, while in this study, it took only 24 h for co-culture of JQ-L and A-3 to completely degrade the same amount of cypermethrin. The possible reasons for the high degradation efficiency of co-culture of JQ-L and A3 were that the two strains were isolated from the same consortium, and their growth conditions were similar, so they could grow well at the some culture system; secondly, JQ-L had enzymes that converted cypermethrin to 3-PBA, while A-3 has enzymes that completely degraded 3-PBA, thus, co-culture of JQ-L and A3 possessed a complete catabolic enzymes system;

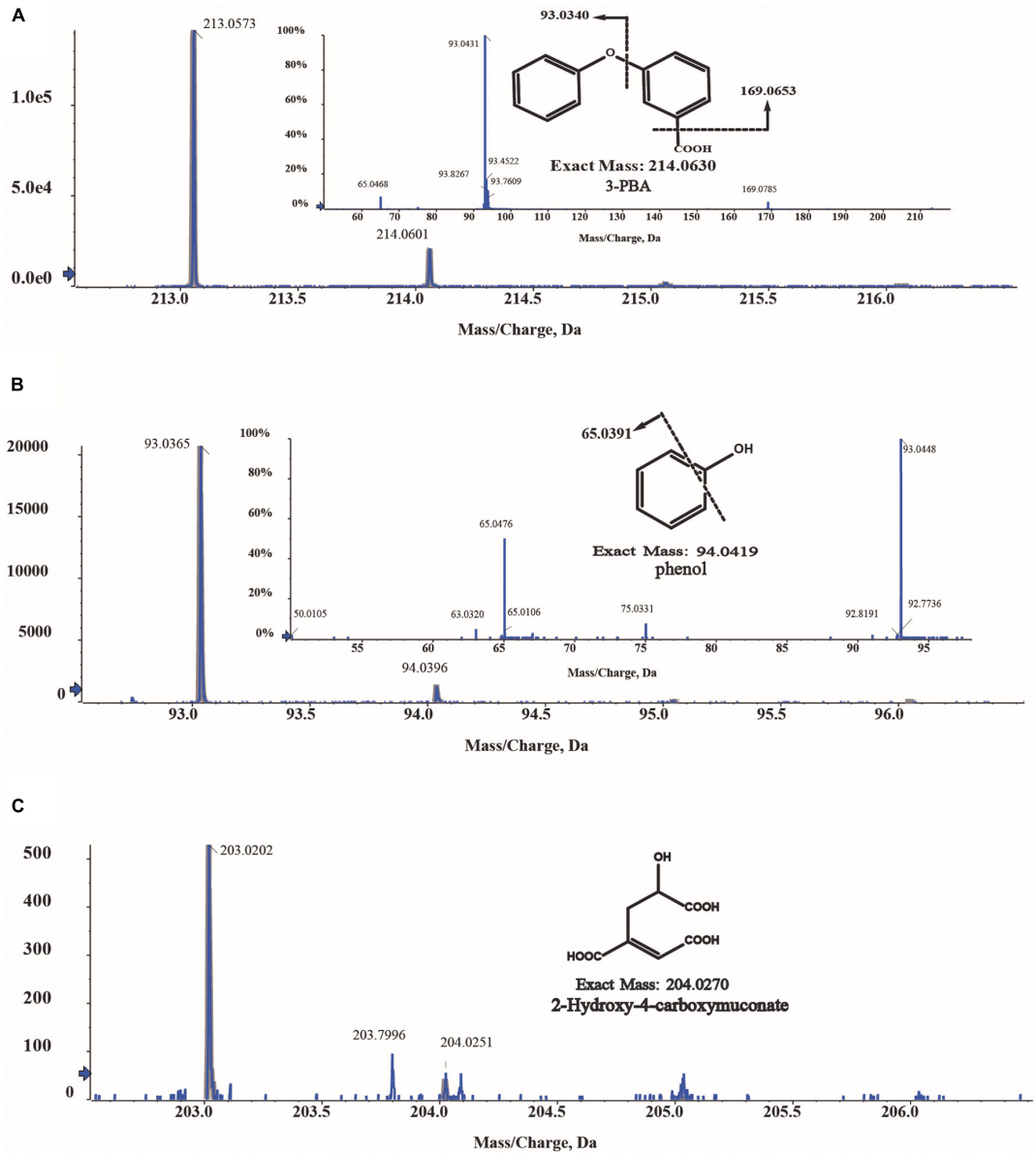

FIGURE 6
MS/MS analysis of the metabolites of 3-PBA degradation by strain A-3. **(A)** MS/MS analysis of compound I; **(B)** MS/MS analysis of compound II; **(C)** MS/MS analysis of compound III.

furthermore, when A3 was co-cultured with JQ-L, it degraded and removed the metabolite 3-PBA thus eliminated the toxicity and inhibition effect of 3-PBA on JQ-L, thereby significantly improving the efficiency of cypermethrin degradation by JQ-L.

3-Phenoxybenzoate is an important intermediate in the synthesis of most pyrethriods and is also the metabolite of their degradation by microorganisms (Huang et al., 2018). 3-PBA belongs to diphenyl ether compound (DE) in structure. DEs are important environmental contaminants, and are highly persistent in environments due to the presence of a diaryl ether linkage. Microbial degradation is one of the most important paths for the dissipation of DEs in the environment (Wang et al., 2014). So far, many 3-PBA-degrading strains were isolated, and the catabolic pathway of 3-PBA have been elucidated (Wang et al., 2014; Zhao et al., 2016; Huang et al., 2018). In all of these reported 3-PBA-degrading strains, the initial degradation step was the cleavage of the diaryl ether. Two 3-PBA cleavage patterns have been identified to date. In *Pseudomonas pseudoalcaligenes* POB310, 3-PBA was cleaved to protocatechuate and phenol under the catalysis of a two-component dioxygenase PobAB (Dehmel et al., 1995); while in *Sphingobium wenxiniae* JZ-1, 3-PBA was cleaved to

TABLE 1 Deduced function of each ORF that possibly involved in 3-PBA catabolism in the genome of A-3.

ORF, coding protein	Position, number of amino acid residues	Similar gene (accession number), source	Identity/coverage %
pobA, Phenoxybenzoate dioxygenase subunit alpha	Scaffold 27, 409	(Q52185.1), *Pseudomonas oleovorans*	97.3/99
pobB, Phenoxybenzoate dioxygenase subunit beta	Scaffold 27, 319	(Q52186.1), *Pseudomonas oleovorans*	99.4/99
proA1, Protocatechuate 4,5-dioxygenase alpha chain	Scaffold 11, 149	(P22635.1), *Sphingobium* sp. SYK-6	64.9/78
proA2, Protocatechuate 4,5-dioxygenase beta chain	Scaffold 11, 289	(P22636.1), *Sphingobium* sp. SYK-6	60.5/99
dmpL, Phenol hydroxylase, P1 oxygenase component DmpL	Scaffold 41, 330	(P19730.1), *Pseudomonas* sp. CF600	46.5/85
dmpM, Phenol hydroxylase, P2 regulatory component DmpM	Scaffold 41, 97	(P19731.1), *Pseudomonas* sp. CF600	47.7/87
dmpN, Phenol hydroxylase, P3 oxygenase component DmpN	Scaffold 41, 536	(P19732.1), *Pseudomonas* sp. CF600	63.8/94
dmpO, Phenol hydroxylase, P4 oxygenase component DmpO	Scaffold 41, 118	(P19733.1), *Pseudomonas* sp. CF600	45.4/99
dmpP, Phenol 2-monooxygenase, reductase component	Scaffold 41, 357	(P19734.3), *Pseudomonas* sp. CF600	57.5/99
dmpK, phenol hydroxylase component DmpK	Scaffold 41, 63	(P19729.1), *Pseudomonas* sp. CF600	36.4/85
dmpR, Positive regulator of phenol hydroxylase, DmpR	Scaffold 41, 563	(Q43965.1), Acinetobacter guillouiae	43.9/95
catA2, Catechol 2,3-dioxygenase	Scaffold 41, 314	(Q04285.1), *Pseudomonas putida*	41.3/99

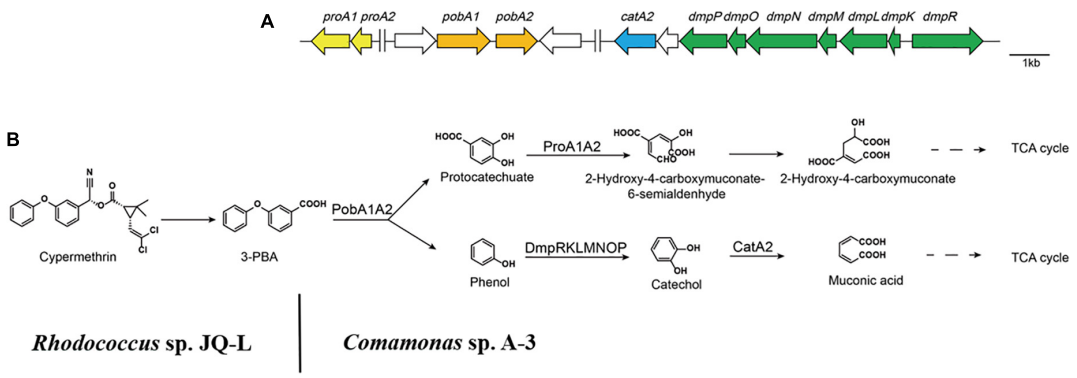

FIGURE 7
The catabolism of cypermethrin by co-culture of JQ-L and A-3. **(A)** Organization of genes possibly involved in the catabolic pathway of 3-PBA in *Comamonas* sp. A-3. Arrows indicate the size and transcriptional direction of each gene. **(B)** The proposed complete catabolic pathway of cypermethrin by co-culture of *Rhodococcus* sp. JQ-L and *Comamonas* sp. A-3.

3-hydroxybenzoate and catechol under the catalysis of a four-component dioxygenase PbaA1A2BC (Wang et al., 2014). In this study, we found that *Comamonas* sp. A-3 could degrade 3-PBA, catechol, phenol and protocatechuate, and phenol was identified as the metabolite of 3-PBA degradation by A-3. Analysis of A-3 genome indicated that 3-PBA dioxygenase gene *pobAB* but not *pbaA1A2BC* was presented in A-3 genome, indicating that 3-PBA was cleaved to protocatechuate and phenol in A-3 which was similar to that of *P. pseudoalcaligenes* POB310 (Wang et al., 2014). Furthermore, A-3 genome contained two gene operons *proA1A2*, *dmpRKLMNOP* and a gene *catA2*. Operon *proA1A2* encoded a two-component 4,5-dioxygenase that catalyzed the ring cleavage of protocatechuate (Fuchs et al., 2011), in operon *dmpRKLMNOP*, *dmpR* encoded a regulatory protein, while *dmpL*, *dmpM*, *dmpN*, d*mpO*, and *dmpP* encoded a multiple component phenol monooxygenase

catalyzing the hydroxylation of phenol to catechol (Powlowski et al., 1997), and *catA2* encoded a catechol 2,3-dioxygenase, which catalyzed the ring cleavage of catechol to muconic acid (Fuchs et al., 2011). Thus, *proA1A2*, *dmpRKLMNOP*, and *catA2* together with *pobAB* constituted a complete catabolic pathway of 3-PBA in strain A-3. Based on the substrate spectrum, metabolite identification, and bioinformatics analysis, a complete catabolic pathway of cypermethrin by co-culture of JQ-L and A-3 was proposed in **Figure 7**, *Rhodococcus* sp. JQ-L transformed cypermethrin to 3-PBA; then in *Comamonas* sp. A-3, 3-PBA was cleaved to protocatechuate and phenol, phenol was hydroxylated to catechol, which was cleaved to muconic acid, and protocatechuate was cleaved to 2-hydroxy-4-carboxymuconate 6-semialdehyde. In the MS/MS analysis of the metabolites of 3-PBA degradation by strain A-3 (**Figure 6C**), the molecular-ion of compound III was 203.02, which was consistent with that of 2-hydroxy-4-carboxymuconate, the dehydrogenation product of 2-hydroxy-4-carboxymuconate 6-semialdehyde, thus, we inferred that compound III was 2-hydroxy-4-carboxymuconate.

In conclusion, in this study, two bacterial strains *Rhodococcus* sp. JQ-L and *Comamonas* sp. A-3 were screened from a highly efficient 3-PBA-degrading consortium. *Rhodococcus* sp. JQ-L could transform cypermethrin to 3-PBA, which could not be further degraded by JQ-L but was toxic to JQ-L. *Comamonas* sp. A3 could not degrade cypermethrin, but completely degrade 3-PBA. Co-culture of JQ-L and A3 could efficiently and completely degrade cypermethrin and the toxic metabolite 3-PBA. Thus, the two strains have good application potential in the bioremediation of cypermethrin residue-contaminated environments and agriculture products.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary material**.

Author contributions

JH, KZ, and CY conceived the presented idea, contributed to the writing, and prepared the figures and tables. LW, YD, and YY participated in revising the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1003820/full#supplementary-material

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Insights into the recent advances in nano-bioremediation of pesticides from the contaminated soil

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In the present scenario, the uncontrolled and irrational use of pesticides is affecting the environment, agriculture and livelihood worldwide. The excessive application of pesticides for better production of crops and to maintain sufficient food production is leading to cause many serious environmental issues such as soil pollution, water pollution and also affecting the food chain. The efficient management of pesticide use and remediation of pesticide-contaminated soil is one of the most significant challenges to overcome. The efficiency of the current methods of biodegradation of pesticides using different microbes and enzymes depends on the various physical and chemical conditions of the soil and they have certain limitations. Hence, a novel strategy is the need of the hour to safeguard the ecosystem from the serious environmental hazard. In recent years, the application of nanomaterials has drawn attention in many areas due to their unique properties of small size and increased surface area. Nanotechnology is considered to be a promising and effective technology in various bioremediation processes and provides many significant benefits for improving the environmental technologies using nanomaterials with efficient performance. The present article focuses on and discusses the role, application and importance of nano-bioremediation of pesticides and toxic pollutants to explore the potential of nanomaterials in the bioremediation of hazardous compounds from the environment.

KEYWORDS

pesticides, bioremediation, nano-bioremediation, microbial degradation, environment

Introduction

The use of pesticides increased in Indian agriculture with the green revolution between years 1967 and 1972. The use of pesticides played an important role in increasing crop production, but on the other hand, it has raised several serious issues related to human and animal health. Pesticides have been used for protection from pests but the fact is that only 1% of pesticides used could target the pests and the remaining cause contamination of soil, water and air (Sun et al., 2018; Figure 1).

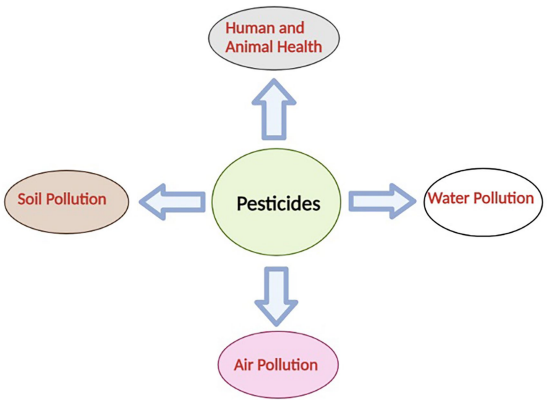

FIGURE 1
Schematic representation of the effects of pesticides on environment.

Pesticides used for crop protection not only integrate into the food chain but also affect the soil health by affecting the soil microbiome and its enzyme activity (Degeronimo, 2015). Moreover, ~40% of applied pesticides are converted into transformed products, which may remain in the soil for a longer period, even for a decade (Erinle et al., 2016). These transformed products contaminated groundwater *via* leaching (Robinson and Piatt, 2019). Pesticide residues entered into the food chain affect human health by targeting several organs for example they affect the endocrine system like the thyroid gland (Huang H.S. et al., 2017), cause neurological disorders (Schmidt et al., 2017), have direct cytotoxic effects (Erdoğmuş et al., 2015) and even may increase the rate of mutations in human beings (Teodoro et al., 2019).

Chlorpyrifos (Pesticide of the organophosphate group) has been reported to cause a reduction in children's intelligence quotients (Sun et al., 2018). Pesticide residues also affect animal health and productivity. In human beings, pesticides cause health problems while in animals also, various disease conditions like cancer, immunosuppression (Nicolopoulou-Stamati et al., 2016), birth defects, hepatic and nephrotoxicity (Choudhary et al., 2018) have been reported in the farm as well as in wild animals. As pesticides target the endocrine system, several reproductive and fertility problems have been encountered in farm animals (Marlatt et al., 2022). Pesticides not only affect the female reproductive system (Ramakrishna et al., 2022) but also are detrimental to the male reproductive system, causing toxicity to the sperm plasma membrane (Torres-Badia et al., 2022). Pesticides have deleterious effects on the biodiversity of invertebrates, mainly insects have been observed in many countries in the last few decades (Vogel, 2017). Due to the indiscriminate use of pesticides, agricultural land used by winter migrating birds is reduced and resulting in a significant reduction in the bird population. Some pesticides like imidacloprid reduced the reproductive capacity and the survival rate of birds like the white crowned sparrow (*Zonotrichia leucophrys*; Eng et al., 2017). Adverse effects of pesticides like neonicotinoids were observed in bees, bumble bees and other useful insects (Phelps et al., 2020). Even the bats were affected due to an omnivorous diet and exposed to pesticide residual contamination (Oliveira et al., 2020). Due to a reduction in the population of bees, birds and beneficial insects, pesticides have made a tremendous economic impact on the environment (Ali et al., 2021) and according to a few estimates, this loss may be 100 times more than the money spent on the conservation of the global environment and biodiversity (Organization for Economic Co-operation and Development (OECD), 2019). Around 8,000 species of insects are at the risk of extinction and in some countries like Germany, the insect population has been reduced by 75% in the last three decades (Hallmann et al., 2017). Pesticides not only affected the natural habitats and biodiversity of places where they were used, but as an effect of wind and evaporation, they could reach the atmosphere and contaminate the sites which were located far away (De Neri, 2020). Various studies have concluded that the use of pesticides for a longer period may affect soil health by interacting with the microflora, microfauna, and macrofauna of the soil (Dahiya et al., 2022).

Keeping these facts in mind, essential measures should be taken for the remediation of pesticides from soil and the environment. In this article, we have focused and discussed the significant.

The objective of this review article is to highlight the importance, impact and significant applications of the nanomaterials in the bioremediation process, for the effective remediation of toxic pollutants such as pesticides and heavy metals from the environment.

Techniques of remediation

The soil has its capacity for degradation of compounds used in the pesticides up to a certain extent but a high concentration of these compounds is toxic for the soil microflora involved in the bioremediation and therefore needs necessary interventions in this area (Cheng et al., 2016a). Several techniques based on physical, chemical and physicochemical principles have been used for the bioremediation of the soil (Baldissarelli et al., 2019).

Physiochemical processes of bioremediation

Advance oxidative process

Technique of advanced oxidative process has been used as a pretreatment or treatment technique. This technique works on the principle of oxidation of polluting compounds into water, carbon dioxide and inert compounds. Ambient temperature and pressure are the pre-requisites for this technique (Cheng et al., 2016b). Though this technique has shown promising potential, most of the studies have been conducted in laboratory conditions and need

further modification for scale-up in field conditions. Economic aspects of the application of this technique are also yet to be studied (Morillo and Villaverde, 2017). There are several variations of an advanced oxidative process like Fenton's reaction, photocatalysis, plasma oxidation and ozonization, which have been tested for soil bioremediation.

Fenton process

This process is based on the oxidation of iron ions (Fe^{2+}) in a medium containing hydrogen peroxide to produce a reactive hydroxyl radical. Hydroxyl radicals further oxidize the organic pollutants into less harmful compounds. Apart from hydrogen peroxide, some other reagents like permanganate (MnO_4^-), persulfate ($S_2O_8^-$) and ozone have also been used but each reagent exhibited its own merits and demerits (Cao et al., 2013; Cheng et al., 2016a). This technique has a few advantages as it may be used *in situ* (onsite) or *ex situ* (offsite) as well as it is environment friendly and easy to operate (Cao et al., 2013) but the application of this technique reduces the pH of the soil (pH < 4), which may affect the soil microbiome. Fenton's method has mainly used an aqueous media for the treatment of groundwater or wastewater (Rosas et al., 2014). For the degradation of chlorinated pesticides like DDT (Dichlorodiphenyltrichloroethane), Fenton's method was used in combination with other oxidative systems like zero valence ion, Ethylene diamine tetraacetic acid, air (ZVI/EDTA/Air; Cao et al., 2013) with the amino ZVI/Air system (Zhou et al., 2014) and with trisodium citrate (Vicente et al., 2012). Though the variety and efficiency of Fenton's reaction to degrade contaminants and pollutant have shown promising potential but the studies using this technique for mass soil contaminants treatment is still the same (Baldissarelli et al., 2019).

Heterogeneous photocatalysis

In this technique, metal oxides like titanate and zinc oxide are based as photosensitizers in the photo induce process. As they have semi filled valence band structure, they cause displacement of electrons from hydroxyl radicals of pollutant compounds (Santos et al., 2015). The efficacy of this technique depends on several factors like soil morphology, surface, pH, particle size, soil depth and light intensity (Castro et al., 2016). Though the reuse of these metal oxides is laborious yet this technique has been demonstrated by some workers in their studies (Sharma et al., 2015).

Plasma oxidation and ozonation

Plasma oxidation is a technique of production of electrons by providing energy and space for reactive molecules. This technique may be used for the oxidation of various compounds like hydrocarbons and pesticides (Cheng et al., 2016a) but the requirement of a high energy source is a limitation of this technology. Therefore, some modifications were made to this technique and with low energy consumption; technique was used for remediation of non-miscible liquid pesticides from the soil in a short reaction time (Aggelopoulos et al., 2015). This technique has been used for the remediation of various pollutants like Pentachlorophenol (Barjasteh et al., 2021), pentanitrophenol and glyphosate (Wang et al., 2014).

Soil washing

The technique of soil washing was used as a physical method, chemical method, or a combination of the physical and chemical methods for the treatment of organic and inorganic contaminants (Usman et al., 2022). This technique was found more effective in soil having high permeability means containing a good ratio of sand and gravel (>50%; Morillo and Villaverde, 2017). Though the soil washing technique was used effectively, its application resulted in the production of wash solution containing a high concentration of diverse pollutants and xenobiotics. Soil washing was tested in a combination with a few other techniques like Fenton oxidation to increase the efficacy of soil washing. A combination of soil washing (Sodium dodecyl sulfate as surfactant) and electrolysis was tested to remove residual pesticides (Atrazine; dos Santos et al., 2015), β methyl cyclodextrin (MCD) in combination with sunflower oil was used to remove the organochlorine pesticides from the soil (Ye et al., 2014).

Chemical extraction-solvent extraction

The technique is based on the extraction of soil contaminants by using supercritical fluid. Extraction using a solvent system like methanol has high solvency and recovery. In this method, carbon dioxide is passed through contaminated soil and assist solubilization of toxic compounds in a solvent system like methanol (Bielská et al., 2013). The solvent system is having high solvency, and may recover a wide variety of pollutants. Though the efficacy of removal of pollutants depends on several factors like the type of extraction, and soil properties (pH, organic content, etc.). In a selective extraction system, several organic compounds were extracted using carbon dioxide as an extraction fluid (Bielská et al., 2013). In an alternative method, Forero-Mendieta et al. (2012) used a combination of carbon dioxide and methanol as a solvent and co-solvent system and remove 31 pesticides like Iprodione, tetradifon, and acephate with an efficacy of more than 70%. Supercritical fluid extraction has been used in combination with dispersive liquid–liquid micro extraction (DLLM) to detect organophosphorus pesticides like Thionazin, Sulfotep, and disulfoton in the soil. The pesticide removal was reached up to 95% with supercritical CO_2 under the conditions of 150 bar, 60°C, 10 min of static extraction and 30 min of dynamic extraction

(Naeeni et al., 2011). Veterinary pharmaceutical products and fungicides were also detected and recovered from the soil by using the solvent extraction technique (Chitescu et al., 2012). A combination of soil washing techniques with solvent extraction methods could remove DDT from the soil up to 94% (Mao et al., 2013).

Electrokinetics

Electrokinetics, electrokinetic soil processing, or electromigration involves the application of a continuous and low-intensity current between the electrodes in the soil. Electric current causes electrolysis of soil, water and makes acidic solutions close to the anode. One of the acid fronts moves from the anode to the cathode, which results in the desorption of soil contaminants (Gomes et al., 2014). This technique results in the movement of soil pollutants and their concentration in a small area (Bocos et al., 2015). This method causes minimum disturbance in the soil environment and is economically feasible. This technique involves two processes; electromigration causes the removal of polar contaminants (ions) while electroosmosis removes non-polar contaminants (dos Santos et al., 2016). This technique is mainly used in fine granulometry soils, which have low hydraulic conductivity and large specific surface area (Morillo and Villaverde, 2017). The pesticides like Molinate and Bentazone were removed from the soil by the process of Electrokinetics, molinate was removed as catholyte while bentazone was found to be present on both of the electrodes (Ribeiro et al., 2011) but for the removal of the compounds like Pentachlorophenol (PCP), electrokinetics was not found to be very efficient technique and required techniques like permeable reactive barriers for efficient removal (Li et al., 2011). Electrokinetics could efficiently remove the commonly used pesticides 2, 4 D (2,4-dichlorophenoxyacetic acid) from the soil and reduced the concentration of 2,4 D in soil up to 80% within 60 days of treatment (Risco et al., 2015). Different configurations of electrodes have been tested to increase the efficiency of electrokinetics among which the formation of one anode and six cathodes was found more efficient than one cathode and six anodes (Risco et al., 2016). Solar power energies and wind energies were used as an alternative to conventional electric energy sources for the electrode to reduce the cost of the operation (Souza et al., 2016).

Among all the physiochemical processes described advanced oxidation process especially the Fenton technique may be considered to be the best method as it may be used for the bioremediation of a wide range of contaminants including many organic contaminants, it can be used in the area of contaminants or out of it. It is environmentally friendly and requires a shorter treatment time. It can be operated easily with low operation costs (Baldissarelli et al., 2019). But even Fenton oxidation suffers from a few limitations as it causes a reduction in soil pH, oxidizes harmless organic material of the soil and immobilization Fenton technique of inorganic reactive species on the treatment wall (Cheng et al., 2016a). Alternative techniques such as biodegradation, and using microorganisms have been used for effective decontamination of soil and water.

Chemistry and classification of pesticides

Pesticides can be classified by several means depending on the origin of the Pesticide, chemical properties, and pest controlling capacities. Based on their origin, pesticides may be classified into two groups; biopesticides and chemical pesticides. Biopesticides may further be divided into three subgroups, i.e., microbial, biochemical and plant-incorporated protectants. Chemical pesticides can be classified based on their nature (organic and inorganic) and based on ionization properties (ionic and non-ionic). In organic and inorganic based classification, inorganic pesticides are mainly mineral derivatives while organic pesticides may be divided further into four groups; synthetic, plant based, animal based and microorganism based. Synthetic organic pesticides are of three types; organophosphates (like chlorpyrifos), organochlorine (like Lindane) and carbamates (like carbaryl). Plant-based organic pesticides can be divided into two subgroups; synthetic (like Allethrins) and natural (like Nicotine). Animal-based organic pesticides can be divided into two groups; synthetic (Fish oil) and natural (dried blood). Microorganism-based organic pesticides are of three types; bacterial (*Bacillus thuringiensis*), fungal (*Pseudozyma flocculus*) and viral (*Baculovirus*; Giri et al., 2021). Based on the classification of their ionization properties, pesticides can be divided into two groups; ionic and non-ionic pesticides. Ionic pesticides are divided into four groups, cationic (like Chlormequat, Diquat), basic (like Atrazine, Cyanazine), acidic (like Laxynil, Fenae) and miscellaneous (like Cacodylic acid, Terbacil). Non-ionic pesticides can be classified into several subgroups, chlorinated HCs (like DDT, Lindane), organophosphates (Ethion, Methyl Parathion), Dinitroanilines (like Oryzalin, Nitralin), Carbanilate (like Chlorpropham, Swep, Barban), Benzonitrile (like Dichlobenil), Ester (eg methyl ester of Chloramine), Acetamides (like CCDA), Carbothioate (like Molinate), Thiocarbamates (Metam and Ferbam), Anilides (Alachlor, Propanil), Urea (cycluron) and Methyl carbamates (carbaryl dichromate, Terbutol; Giri et al., 2021).

Bioremediation of pesticides

The term bioremediation deals with the methods of degradation of pesticides by using the metabolic capacities of microbes. In this process, natural or genetically modified microbes utilize pesticides for their metabolic activities and convert them into environmentally benign metabolites (Karimi et al., 2022). The process of bioremediation can be classified into two groups, i.e., Bio-stimulation and Bio-augmentation.

Biostimulation

In the process of biostimulation, microbial activity is enhanced by the addition of vitamins, substrate, oxygen and other nutrients. The addition of stimulatory nutrients results in swift depletion of the available stock of inorganic nutrient and result in pesticide degradation (Giri et al., 2021). To stimulate the process of biostimulation by microbes, water soluble nutrients like sodium nitrate, potassium nitrate, and potassium hydrogen phosphate have been added to the fertilizer (Adams et al., 2015). The ratio of Nitrogen: Phosphorous is maintained between 1:5 and 1:10 for 1%–5% N by weight of pesticide for the degradation of pesticides but if the site has been contaminated with the different types of pesticides. This ratio may not be sufficient enough for the biostimulation process. Fernandes et al. (2018) used *Pseudomonas* species for the degradation of atrazine in the soil at very high concentrations. For biostimulation, citrate at 4.8 mg/g of soil was used. The addition of citrate stimulated the bioremediation process and the efficiency of removal of atrazine was found to be 79.00 to 87%.

Bio-augmentation

Bio-augmentation involves the addition of an exogenous micro population with the specific remediation efficacy into a polluted site. The native microbes are added to the contaminated site may be on site or off site to eliminate hazardous compounds. The process of bio-augmentation has been used for the degradation of a wide range of pollutants like NH_3, H_2S, organic compounds etc. from the soil and water (Hassan et al., 2022). The pre-grown microbial culture enhances the microbial population at the contaminated site and reduces the clean-up time and cost of the operation (Giri et al., 2021). Wang et al. (2013) removed atrazine from highly contaminated soil (atrazine concentration 400 mg/kg soil) by using Arthrobacter sp-based bio-augmentation. The process displayed up to 90% removal of atrazine from the soil. *Bacillus cereus* was used for the removal of chlorpyrifos contamination in the soil and the average degradation was found to be 88% within 8 days of the treatment (Farhan et al., 2021). The strains of *Pseudomonas bacillus* subtilis were used in the bio-augmentation process and removed 95% of Chlorpyrifos from the soil within 15 days of treatment (Gangola et al., 2018).

Dichloro-diphenyl-trichloroethane (DDT) has been banned in many countries for its use as a pesticide in agriculture but still, in most parts of the world, the residues of DDT are present as contaminants in the soil and its detoxification is a challenging task. Some fungus like *Gloeophyllum* trabeum and *Daedalea dickinson* has been used in the degradation of pesticides from the soil (Mathur and Gehlot, 2021). A wide range of pesticides has been degraded by the process of bio-augmentation using microorganisms. Carbofuran was degraded using *Syncephalastrum racemosum* and the rate of degradation was around 75% after inoculation of culture in the soil (de Sousa Lira and Orlanda, 2020). Cypermethrin was degraded using several bacterial strains from *Bacillus, Pseudomonas, Streptomyces*, etc. and by different fungi such as *Aspergillus niger*, *Aspergillus terricola*, *Trichoderma viride* (Maqbool et al., 2016; Bhatt et al., 2019a). Lindane and Parathion were degraded by a bio-augmentation process using *Paenibacillus dendritiformis* and *Serratia marcescens*, respectively, (Jaiswal et al., 2022). Various microorganisms, which degrade different pesticides, are enlisted (Table 1).

Factors influencing the bioremediation process

Bioremediation of pesticides in the soil is a complex process, which involves several interdependent interactions within the soil, soil to air, soil to water, and characteristics of the pesticides. The rate of bioremediation depends upon interdependent physiochemical and biological processes, which are regulated by several factors.

TABLE 1 Microorganisms involved in degradation of different pesticides.

Microorganism	Pesticide	Source	References
Pseudomonas sp.	Cypermethrin	Soil	Tang et al. (2015)
Bacillus cereus	Cypermethrin	Soil	Narayanan et al. (2020)
Sphingomonas sp.	Allethrin	Wastewater	Bhatt et al. (2020)
Enterobacter sp.	Chlorpyrifos	Soil	Singh et al. (2004)
Sphingomonas sp.	Oxyfluorfen	Soil	Keum et al. (2008)
Burkholderia sp.	Fenitrothion	Soil	Hong et al. (2007)
Acinetobacter sp.	Chlorpyrifos	Soil	Amani et al. (2018)
Ochrobactrum sp.	Methyl parathion	Soil	Qiu et al. (2007)
Bacillus pumilus	Chlorpyrifos	Soil	Anwar et al. (2009)
Pseudomonas putida	Organophosphate	Soil	Li et al. (2016)
Burkholderia gladioli	Prophenofos	Soil	Malghani et al. (2009)
Bacillus aryabhattai	Chlorpyrifos	Soil	Pailan et al. (2015)
Bacillus subtilis FZUL-33	Acephate	Soil	Lin et al. (2016)
Aspergillus niger	Cypermethrin	Soil	Bhatt et al. (2020)
Trichoderma viridae	Cypermethrin	Soil	Maqbool et al. (2016)

Chemical structure of pesticides

The chemical structure of pesticides plays an important role in regulating the rate of bioremediation of pesticides. The pesticides having polar groups like C-OH, -COOH, etc. on the phenyl ring are more susceptible to microbial biodegradation in comparison to halogen or alkyl substituents. Even a minor alteration in a structural substituent may cause drastic changes in microbial susceptibility (Geed et al., 2016). In the process of bioremediation, oxidation and reduction of active functional groups result in their conversion to simple molecules like CO_2, H_2O, Nitrate, Phosphate and NH_3 (Sharma, 2020). Chlorinated hydrocarbons like DDT are more resistant to bioremediation as they have low solubility in water and high absorption affinity in soil. On the other hand, compounds like 2-4D and Carbofuran can be degraded from the soil by microorganisms in a few days within the same class of pesticides; a minor group substitution may change the susceptibility of pesticides to microbial degradation (Geed et al., 2017).

Pesticide concentration

Pesticide concentration in soil is another important factor in deciding the rate of degradation of pesticides in the soil. The biodegradation rate depends upon the residual concentration of pesticide in the soil and it follows pseudo first order kinetics (Zaranyika et al., 2020). The rate of biodegradation decreases proportionally with residual concentration of pesticides

$$d[P]/dt = -K[P]$$

where, d[P]/dt = pesticide concentration gradient with respect to time; K = biodegradation rate constant.

The half life of pesticides may vary from 10 days to 200 days. Pesticides like Inceptisol and Ultimo have half life ranging from 10.1 to 29.2 days while several pesticides like DDT, endosulfan, and atrazine have half life varying from 100 to 200 days. The residues of less biodegradable pesticides remain adsorbed on soil particles so these are not available for microbial degradation (Giri et al., 2021).

Soil types

Soil organic content, pH, the concentration of clay material and moisture contents are the important factors, which contribute to deciding the rate of degradation of pesticides in the soil (Rasool et al., 2022). Adsorption of pesticide residues with soil particles reduces the bioavailability of pesticides for the microbes and increases the half life of the residues (Giri et al., 2021). Water is one of the most important factors, which decides the motion and diffusion of pesticide molecules for microbial-assisted biodegradation. The rate of biodegradation of pesticides is directly proportional to soil moisture content and extremely low in dry soil (Chowdhury et al., 2008). Soil aeration and oxygen level also affect the rate of pesticide degradation as few pesticides like DDT, which is fairly stable in aerobic soil but degrades slowly in submerged soils (Raffa and Chiampo, 2021). Soil temperature produces a great impact on the stability of molecular conformation of the pesticides. It affects the solubility and rate of hydrolysis of pesticides in soil samples. The optimum soil temperature of microbial degradation ranges from 20°C to 40°C as in this range of temperature microbes has maximum activity (Singh et al., 2022).

The pH of the soil also affects the rate of bioremediation of pesticides. The degradation of the pesticides depends upon the activity of the enzymes produced by the microorganism. The enzymes have a very narrow range of pH for these activities. Most of the bacterial enzymes work at soil pH between 6.5 and 7.5. Apart from the activity of microbial enzymes produced by a microorganism, pH also influences the pesticide adsorption, biotic and abiotic degradation processes (Rasool et al., 2022). The degradation of pesticides also depends upon the chemical susceptibility toward hydrolysis by acidic or basic pH of the soil (Liu et al., 2015). Soil organic content also affects the rate of microbial degradation of pesticides. Organic contents increase the rate through co-metabolism of pesticides. The organic content of the soil also acts as a source of nutrients for the soil microbes. Therefore, increases the microbial population rapidly and results in an increased rate of microbial degradation of the pesticides. The rate of bacterial-mediated biodegradation of organochloride was increased with the addition of organic carbon sources in the soil (Krohn et al., 2021).

It has been deduced that a minimum of 1% of organic content should be present for effective microbial biodegradation of pesticides (Huang et al., 2018). In the case of pesticides, which are present in low concentrations in the soil, the co-metabolism of microbes has proven an effective measure for bioremediation. The organic content of the soil contains co-substrate, which facilitates co-metabolism of the microbes (Banwart et al., 2014).

Carriers in bioremediation

To increase the efficacy and rate of bioremediation methods of immobilization were introduced. These methods limited the mobility of microbes and their enzymes and immobilization also enhanced the viability of microbes and the catalytic functions of their enzymes. In the process of immobilization, the natural activity of microorganisms to form biofilm on the surface of various materials was explored. Immobilization not only increased the efficacy of the bioremediation but also reduced the cost of operation as it made multiple uses of biocatalysts possible. It provided a stable environment for microbes and reduced the genetic

mutations in a microorganism (Mehrotra et al., 2021). Mainly five techniques have been used in the process of immobilization; these were adsorption, binding on the surface, flocculation, entrapment and encapsulation.

Adsorption

In this process, microorganisms are adsorbed on the surface of water insoluble carriers by weak bonds. It is a simple and economic method but as there is a high probability of cells leaking from the carriers to the environment, this method is not recommended for the use in case of genetically modified microorganisms (Dzionek et al., 2016).

Binding on a surface

In this process, the surface of the carrier is washed with buffer, which makes the surface hydrophilic. The microbes and enzymes having a negative charge bind with the surface of the carrier. In another method of binding; covalent binding, a binding agent is required and carriers are chemically activated. This method is mainly used for the binding of enzymes because binding agents may affect the viability of microbes. As the covalent binding is very strong, the leaking of molecules (enzymes) is efficiently prevented by covalent bonding (Jiang et al., 2022).

Entrapment in the porous matrix

This method has been mainly used in microbial bioremediation. As a result of entrapment, microbial cells can move within the carrier and prevent the leaking of the cells in the environment but allow the exchange of nutrients and metabolites. In a heterogeneous carrier system, the population of microorganisms is physiologically diverse as the cells located near the surface have high metabolic activity. The entrapment method has several advantages as it is a non-toxic, economical and highly versatile method. It efficiently prevents microorganisms from environmental factors. The efficiency of the entrapment method depends on the selection of a suitable ratio of the size of pores and cell size (Mehrotra et al., 2021).

Encapsulation

In this method, immobilized particles are separated from the environment using a semi-permeable membrane. This method provides significant protection to microbes against external environmental conditions but the limited permeability of the membrane may affect the viability of the cells (Priyanka et al., 2022).

Materials used for bioremediation

The method to be used for the immobilization process should sustain a few properties. It should be non-toxic, economic, stable, insoluble and regenerative. Carriers to be used for adsorption and binding on the surface should have high porosity (Dzionek et al., 2016). Carriers can be classified into two groups; natural carriers and synthetic carriers. Each group can be divided into two subgroups; organic and inorganic. Natural organic carriers include alginates, chitosan, sawdust, charcoal, plant fibers, bagasse, rice husk, etc. These carriers contain many functional groups which stabilize biocatalysts (Cubitto and Gentili, 2015). Most of these materials are waste of the food industries so these are economic and biocompatible but they have a low resistance to the biodegradation and sensitivity to organic solvent (Paliwal et al., 2015). They have a very narrow pH range for their stability. Synthetic organic carriers like polypropylene, polystyrene, polyacrylonitrile, polyvinyl alcohol and polyvinyl chloride have several functional groups with diversified properties. The macromolecular structure of synthetic organic carriers may be regulated as per the desired order of functional groups in the chain. Moreover, their porosity, polarity and hydrophobic nature can also be modified. These are commercially available at economical prices. Inorganic carriers like magnetite, silica-based material, ceramics, porous glass and nanoparticles have high chemical, physical and biological resistance. The number of functional groups present on these carriers is very less and it prevents their sufficient bonding with microorganisms and catalysts. Inorganic carriers can be used in the formation of hybrid carriers by combining them with natural polymers or synthetic nanoparticles (Yunoki et al., 2014).

Nanotechnological interventions in bioremediation

Nanotechnology is a branch of science, which deals with synthesized particles, which are very small in size (<100 nm). In the last few years, nanotechnology has been used in various fields like medicine, textiles, optics, etc. The use of nanoparticles and application of nanotechnology in agriculture was started at the beginning of the 21st century (Fraceto et al., 2016) and more than 230 nano-products have been used in various agricultural operations (Rajput et al., 2022). Nanotechnology has been integrated with the bioremediation process and termed Nano-bioremediation. Nano-bioremediation targeted cleaning of the environment by accelerating bioremediation using nanoparticles (Bhatt et al., 2021). Nano-bioremediation is further subdivided into two subgroups, i.e., nano-phytoremediation of nanoparticles with phytoremediation and microbial nano-remediation (Singh et al., 2020; Kumari et al., 2022; Figure 2).

The basic principle of nano-bioremediation is the degradation of contaminants using a catalyst as nanoparticles. As the nanoparticles are very small in size, it allows them to interact more

deeply and have a large surface area as per unit mass, more numbers of nanoparticles can come into contact with the environment. This enhances the efficacy and the rate of bioremediation (Cecchin et al., 2017).

The process of bioremediation involves the use of living organisms for the remediation of pollutants. Once nanotechnology is integrated with the process of bioremediation, the interaction of nanoparticles with living organisms becomes the key factor in deciding the efficacy of nano-bioremediation. In a few cases, the interaction of nanoparticles and biotic components resulted in biocidal and proven harmful to the organisms involved in the bioremediation (Juárez-Maldonado et al., 2019). Therefore, evaluation of the interaction of nanoparticles and biotic components is the prerequisite of the nano-bioremediation process. The efficacy of nano-bioremediation can be influenced by several factors like size, shape, chemical nature of the nanoparticles, the physiological properties of the organism, pH and temperature of the soil, nature of the contaminant, etc. (Tan et al., 2018). These factors work directly or indirectly. Temperature, pH and media are crucial for the optimal development of biological organisms while the direct interaction of nanoparticles and organisms regulates various actions like dissolution, absorption and biotransformation (Kranjc and Drobne, 2019). Nano-bioremediation is a two-step process. In the first contaminants are broken down by nanoparticles to a conducive level for bioremediation and in the second step; pollutants are biodegraded (Cecchin et al., 2017).

Nano-phytoremediation of contaminated soil

Nano-phytoremediation is a method for remediation of pollutants, and contaminants by using synthesized nanoparticles from the plants. Plants are the natural detoxifiers for the soil as they absorb diverse types of compounds and detoxify them. Phytoremediation is a Greek word, which means restore/remedy through plants (Pandya et al., 2022) but phytoremediation has a few limitations as slow remediation time and plant waste. Nanotechnology has increased the efficacy of remediation of contaminated soil and water. Organic contaminants like atrazine, molinate and chlorpyrifos have been degraded with nano-sized zerovalent irons. Enzymes encapsulated in nanoparticles increased the efficiency of bioremediation significantly (Yadav et al., 2017).

There are several factors, which affect the efficacy of nano-bioremediation. These are the physical and chemical properties of compounds, their molecular weight, solubility in the water, soil environment (pH, temperature and percentage of organic matter) and characteristics of plants (Gulzar and Mazumder, 2022). Integration of nanoparticles and phytoremediation is the most important step in nano-phytoremediation. The studies indicate that the application of nanoparticles detoxified various organic, inorganic and metal pollutants from the soil. The use of nano-zerovalent iron, and magnetite nanoparticles rapidly degraded organic pollutants from the soil (Madhura et al., 2019). Nanoparticles of TiO_2 (n TiO_2) and PEI-copper nanoparticles

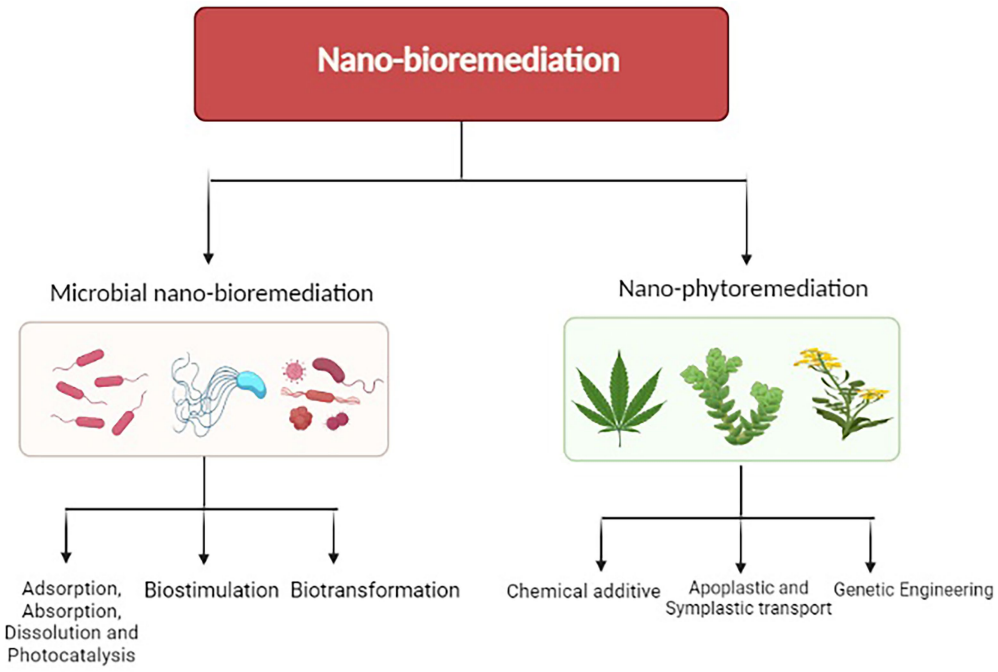

FIGURE 2
Nano-bioremediation: a promising strategy for the remediation of pesticides.

reduced the half lives of Phenanthrene and atrazine, respectively (Li et al., 2016; Kalidhasan et al., 2017). The technique of nano-phytoremediation worked for a wide range of soil pollutants ranging from heavy metals to organic compounds. The application of nanoparticles enhanced the uptake of pollutants by plants and also improved the stress tolerance capacity of the plants (Pillai and Kottekottil, 2016; Souri et al., 2017).

Important factors in the interaction of plants and nanoparticles

Though there are several factors, which affect the uptake of nanoparticles by plants like the type and chemical composition of nanomaterial, the size of nanoparticles plays the most crucial role in the uptake of the nanoparticles (Schwab et al., 2016). The nanoparticles can be transported into the plants in two ways; Apoplastic transport (transport through xylem vessels), Symplastic transport (transport between the cytoplasm and sieve plates; Horejs, 2022).

Apart from the size of nanoparticles, the soil temperature is also an important factor as it affects the growth substances and root lengths (Ahmadpour et al., 2012). The properties of the plants also affect the efficacy of nano-bioremediation. For achieving high efficiency, a plant should have fast growth, large biomass, a well-developed root system, high toxicity tolerance limit, high accumulation capacity, a non-consumable for animals and easy for genetic manipulation. Nanoparticles should be non-toxic for plants and should have the properties to enhance germination, root-shoot elongation, enhanced phoenzyme production, increased plant growth hormone and capabilities to bind with contaminants of the soil (Sajid et al., 2015). Nano-phytoremediation technology has been used with natural as well as genetically engineered plants. Nanoparticles enhanced plant growth and their efficacy in remediation of the soil contaminants. These particles increased the production of plant growth hormone and enhanced the uptake of soil pollutants by plants (Dimkpa et al., 2012; Liu et al., 2016) Nano-zerovalent iron nanoparticles were used with plants like *Alpinia calcarata* Roscoe, *Ocimum sanctum*, *Cymbopogon citratus* and with all three plants, these particles enhanced the remediation efficacy against Endosulfan (Pillai and Kottekottil, 2016). Similarly, sialic acid nanoparticles increased the phytoremediation efficacy of Isatis cappadocica Desv for Arsenic (Souri et al., 2017). Nanoparticles cause many physiological changes in the plants, which results in increased efficacy of phytoremediation but the effectiveness and safety of the nanoparticles are decided by several factors like chemical composition, size, shape, stability, concentration, and surface coating and reactivity of the nanoparticles. The efficacy of nanoparticles may vary from plant to plant also (Varma and Khanuja, 2017; Yang et al., 2017). Nano-phytoremediation technology has few limitations such as most of the experiments have been conducted at microcosm levels so extensive studies are required. Formation of aggregation is a common phenomenon with nanoparticles so studies to modify their surfaces to enhance the sustainability of nanoparticles are essentially required and in last the toxicity of various nanoparticles for the soil and the environment needs to be evaluated.

Microbial nano-bioremediation

It is the process in which nanoparticles are used with soil microbes to enhance biodegradation processes. Microorganisms can uptake the metal ions and reduce them. In this process, the metal ions are converted into nanoparticles. Microbial enzymes along with the metals form useful nanoparticles for nano-bioremediation (Pandey, 2018). Microbial nano-bioremediation is a two-phase process, which involves abiotic and biotic processes (Usman et al., 2020). In the first phase, nanoparticles enter the system and particles of pollutants undergo varieties of the processes like adsorption, absorption, dissolution and photocatalysis (Abebe et al., 2018). In the second phase, several biotic processes like biostimulation and biotransformation remove these particles from the system (Desiante et al., 2021). The second phase (biotic phase) plays a very important role in the bioremediation of pollutants. Microbial nano-bioremediation has been used for a variety of pollutants like inorganic and organic (Figure 3). Nanoparticles involved in the microbial degradation and enlisted in Table 2.

Microbial nano-bioremediation for heavy metals

Heavy metals are increasing in the environment and soil due to anthropogenic activities and disturbed biogeochemical cycles. Heavy metals like Pb, As, Cd, etc. not have any well-defined function in the biological system but they have a toxic effect on the biotic component of the environment even in the low concentration (Bist and Choudhary, 2022). In acidic soil with low nutrient levels, the toxicity of heavy metals increases (Liu et al., 2018). Heavy metals generate reactive oxygen, which damages the macromolecules (proteins and nucleic acids) of microorganisms and plants (Bist and Choudhary, 2022). Immobilization of heavy metal molecules is the most common technique used for their bioremediation (Suman et al., 2018). Nanoparticles including bio-organic nanoparticles (synthesized using biological organisms) have been used in the removal of heavy metals from the soil Bio-organic nanoparticles such as silver nanoparticles produced in *Morganella psychrotolerans* have been used for the remove of heavy metals (Arif et al., 2016; Enez et al., 2018). Iron oxide nanoparticles coated with polyvinyl pyrrolidone (PVP) are used with *Halomonas* sp. (gram-negative bacteria) for the bioremediation of lead and cadmium (Alabresm et al., 2018). Magnetic nanoparticles of Fe_3O_4 coated with phthalic acid treated with *S. aureus* were used for bioremediation of Cu, Ni and Pb. The

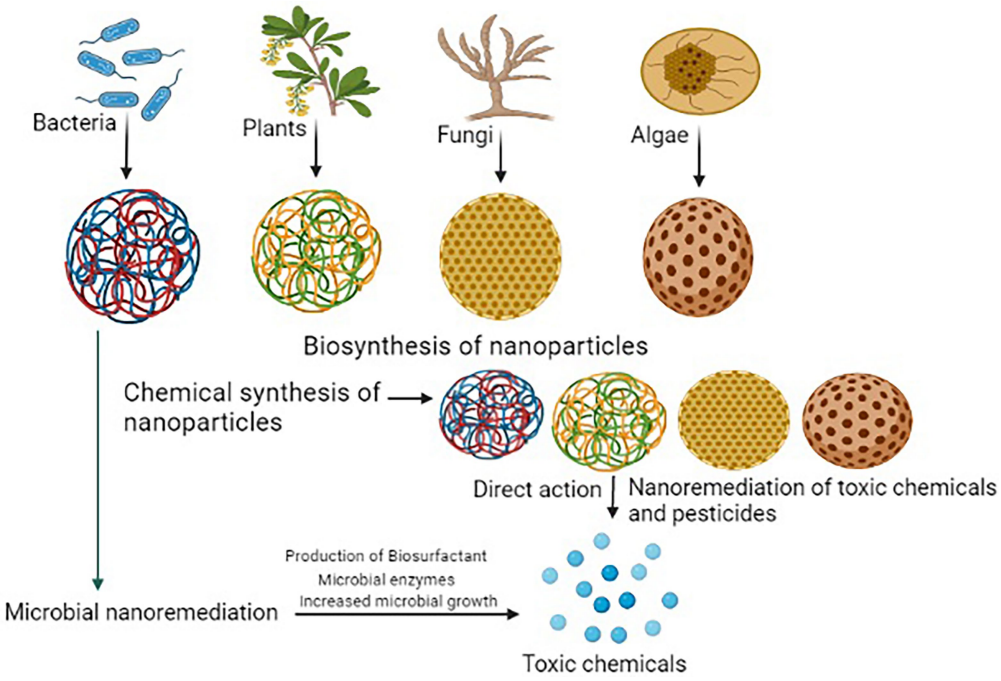

FIGURE 3
Nano-particles mediated removal of toxic pollutants from the soil.

efficiency of these particles was 83%–89% for Cu^{2+}, 99.4%–100% for Pb^{2+} and 92.6%–97.5% for Ni^{2+}. It was observed that the functional group present on the microbial surface and core of nanoparticles played an important role in the removal of heavy metals (Mahmoud et al., 2016). Heavy metals resistant bacteria *B. cereus* and *L. macroides* in combination with zinc oxide were used for the remediation of Cu, Cd, Cr and Pb. It was deduced that zinc oxide nanoparticles along with *B. cereus* could remove these metals efficiently (Baragaño et al., 2020). The strain of *B. cereus* ($XMCr^{-6}$) reduced Cr^{+6} to Cr^{+3}. The reduced Cr^{+3} exhibited affinity to the bacterial cell surface and by reacting with oxygen formed Chromium oxide nanoparticles as a byproduct (Laslo et al., 2022). Selenium nanoparticles in combination with probiotic bacteria (*L. casei*) were used for cadmium-contaminated land and water treatment. The efficiency of cadmium absorption was found 65% with *L. casei*, which was significantly higher than *L. casei* alone (43%–78%; Dong et al., 2013).

In the approach of bio-organic nanoparticle synthesis, heavy metals pollutants can be used by selective microbes followed by their removal from the environment and yielding value for the waste. In this approach, Enterococcus faecelis was used for the removal and recovery of lead. The lead nanoparticles were synthesized by bacteria in extracellular and intracellular modes. The size of these nanoparticles was ~10 nm. These particles exhibited high catalytic efficiency and reduced 5.0 μmol Cr^{+6} in 12 h (Cao et al., 2020).

In anaerobic sludge, tellurium nanoparticles were synthesized by supplementation with riboflavin in the presence of *Rhodobacter* capsulates using polluted tellurite Te^{4+} oxy anions present in the wastewater (Ramos-Ruiz et al., 2016). These findings indicate that nano-bioremediation may be effectively used for the remediation of heavy metals toxicity.

Nano-bioremediation of organic pollutants

Organic pollutants mainly persistent organic pollutants have negative impacts on human and environmental health. Therefore, the remediation of these compounds from the soil is essential. Magnetic nanoparticles in combination with *Rhodococcus engthropolis* caused the desulfurization of dibenzothiophene (DBT; Ansari et al., 2009). Bimetallic (Pd/nFe) nanoparticles were used in combination with *Sphingomonas wittichii* for bioremediation of NBR.2, 3, 7, 8-tetrachlorodibenzo-p-dioxin hydrocarbons (Bokare et al., 2012). The silica nanoparticles biofunctionalized with the lipid bilayer of *Pseudomonas* aeruginosa were used to clean up PAH(benzo[a] Pyrene). The membrane lipids of *Pseudomonas* played a role to enhance the sequestration of PAH (Wang et al., 2015). The grapheme oxide nanoparticles along with the laccase enzyme of Trametes versicolor were studied for biodegradation of PAH and were found to be effective in remediation of PAH (Patil et al., 2016). Alcaligenes faecelis in combination with iron oxide nanoparticles improved the degradation of hydrocarbon compounds of crude oil

TABLE 2 Role of different nanoparticles in microbial and pesticide degradation.

Nanoparticles	Function	References
Bacillus subtilis immobilized on Ferric oxide	Degradation of azo dyes (80%)	Nadi et al. (2019)
Calcium oxide	Degradation of Glyphosate	Cabrera-Penna and Rodríguez-P'aez (2021)
Copper oxide	Dye degradation and inhibit the growth of pathogenic bacteria	Nwanya et al. (2019)
DP-ZnO NPs	Degradation of Methylene blue and eosin yellow	Rambabu et al. (2021)
Ferric oxide	Degradation of Textile effluent	Fouda et al. (2021)
Halomonas immobilized with magnetic nanoparticles	Removal of Palladium	Cao et al. (2020)
Iron oxide	Removal of different heavy metals from wastewater	Mahanty et al. (2020)
Magnetite	Degradation of Phenazopyridine	Gholizadeh et al. (2021)
Manganese-Titanium oxide	Degradation of Acetaldehyde	Karafas et al. (2019)
MZnO/TiO_2-Fe_3O_4	Degradation of Chlorpyrifos	Saljooqi et al. (2020)
Silver oxide nanoparticles	Removal of methylene blue	Shah et al. (2019)

contamination (Oyewole et al., 2019). *Sphingomonas* strain NM05 has been used for the degradation of hexachlorocyclohexane (HCH). Once the strain was treated with Pd/FeO bimetallic nanoparticles, the degradation efficacy of the strain was enhanced ~2 folds (Singh R. et al., 2013). The peroskite ($LaFeO_3$) nanoparticles with proteobacteria were used for the degradation of organic contaminants in marine sediments (Hung et al., 2021). Nanoparticles not only enhanced the remediation efficacy of microbes but are also used for the improvement of soil health. Silicon nanoparticles have been reported to improve the soil microflora and biomass. These particles enhanced the growth of rhizospheric microbes (Rajput et al., 2022).

Algae mediated nano-bioremediation

Phyto-nanotechnology is an efficient, cost-effective and eco-friendly strategy, which is extensively used for the remediation of toxic compounds from the ecosystem (Gole et al., 2022). This technology involves the plant based synthesis of nanoparticles with almost no risk to the ecosystem and humans. Various types of metal nanoparticles like silver, palladium and gold have been synthesized with algae belonging to different groups such as Chlorophyceae, Cyanophyceae, Phaeophyceae and Rhodophyceae. Algae are the largest photoautotrophic group of microbes, having the potential to act as nano-machineries for the metallic nanoparticles. The fabrication of algae based nanoparticles is less time consuming process (Khanna et al., 2019). Algae have several properties like high potential of metal uptake, easy to handle and harvest, low cost, low toxicity, which make them suitable to serve as nano-factories (Sharma et al., 2015). Silver nanoparticles were produced using number of brown algae species such as *Cystophora moniliformis*, *Gelidiella acerosa* and *Padina pavonica* (Azizi et al., 2014) while other species like *Cystoseira baccata*, *Dictyota bartayresianna*, *Ecklonia cava*, and *Sargassum wightii* have been used in the fabrication of gold nanoparticles (AuNPs). Similarly *Phormidium valderianum* and *S. platensis* are also responsible for the AuNPs biosynthesis (Iravani et al., 2017). Algal species such as *Cylindrospermum stagnale*, *Spirulina platensis*, *Plectonema boryanum*, and *Microchaete diplosiphon* have been reported for the synthesis of AgNPs having varied morphologies (Husain et al., 2015). Various algae have been recognized for the remediation of toxic compounds and heavy metals from the wastewater (Goswami et al., 2021). Studies showed that the microalgae from Chlorellaceae family removed heavy metals such as lead, copper, and selenium from the wastewater (Oyebamiji et al., 2019). Microalgae have the ability to remove the toxic heavy metals from the acid mine drainage, which facilitate the inhibition of direct discharge of acid mines into the water bodies that may lead to the damage to aquatic habitat as well as create serious environmental pollution (Samal et al., 2020). The synthesis of nanoparticles and the algae mediated bioremediation belong to same process, which occur simultaneously (Dahoumane et al., 2016). Recently *Chlorella vulgaris*, a green microalgae was reported for the efficient removal of Au(I) and Au(III) complexes (He and Chen, 2014). The metal uptake potential of *Nannochloropsis oculata* was evaluated from the acid mine drainage. The result revealed that 99% of copper content was removed by *N. oculata* (Martínez-Macias et al., 2019). Furthermore, a microalga such as *Chlorella kessleri* was used for the removal of heavy metals from wastewater (Sultana et al., 2020). Studies have showed that microalgae have potential to remove heavy metals from the wastewater. However extensive research is required in this aspect to enhance the remediation efficiency and complete utilization of the biomass. Various studies have reported the wastewater treatment by the immobilization of microalgae biomass, which is considered as an effective technique for the remediation of the heavy metal (Cheng et al., 2019). The consortium of microalgae has attracted interest of the

researchers to remove heavy metals for the wastewater treatment. The heavy metals such as nickel, cadmium and lead have been removed from the wastewater using the consortia of microalgae (Abdel-Razek et al., 2019).

Fungi mediated nano-bioremediation

Fungi are the eukaryotic microorganisms, which include molds, yeasts, mildews and mushrooms (Duhan et al., 2017). Fungi act as biocatalysts and are used in bioremediation as they can survive in intense conditions as well as elevated concentration of heavy metals (Dixit et al., 2015). In green nanotechnology, nanoparticles are synthesized using fungi, which play a pivotal function in the removal of toxic compounds and organic pollutants (Singh et al., 2018). In recent times, the synthesis of metal nanoparticles from fungi has gained a big interest of researchers around the world (Sunny et al., 2022). There are several advantages of metal nanoparticles synthesized using fungi such as higher capacity of metal uptake, simple and low cost fabrication, tolerant against metals, high scalability, highly stable (Yadav et al., 2015).

Various fungus like *Fusarium*, *Verticillium*, *Penicillium*, and *Aspergillus* have been used as potential candidates for the metallic nanoparticles synthesis (Ovais et al., 2018). Studies have reported that metals like gold, silver, titanium, platinum, selenium, palladium and silica can be utilized for the fabrication of nanoparticles (Gholami-Shabani et al., 2016). Silver nanoparticles (AgNPs) synthesized from *F. oxysporum* are different in features from those synthesized with *Aspergillus fumigates* (Alves and Murray, 2022). Studies have reported the synthesis of AgNPs using *Coriolus versicolor* and *Trichoderma reesei* (Vahabi et al., 2011; Deniz et al., 2019). The gold nanoparticles (AuNPs) have been synthesized using *Cylindrocladium floridanum* fungus (Narayanan and Sakthivel, 2011). Platinum nanoparticles were synthesized using the *N. crassa* fungus and *Fusarium oxysporum* was used for fabrication of silica nanoparticles (Castro-Longoria et al., 2012). The selenium nanoparticles have been synthesized from *Mariannaea* sp. (Zhang et al., 2019). The green synthesis of the nanoparticles from different fungus has led to a significant application in the remediation of hazardous organic pollutants through the adsorption of heavy metals (Gaur et al., 2014). The kind of metal, environmental factors and fungal biomass affect the capacity of biosorption (Dhankhar and Hooda, 2011). Studies have showed the proficient adsorption, immobilization capacity and chelation activity of heavy metal ions by arbuscular mycorrhizal fungi (Upadhyaya et al., 2010). Various fungal species like *Allescheriella* sp., *Botryosphaeria rhodina* sp., *Stachybotrys* sp. exhibited the metal-binding capability (Benjamin et al., 2019). The synthesis of AgNPs from *Rhizopus oryzae* have several ecological uses like wastewater treatment (Zhang et al., 2014) and adsorption of pesticides (Das et al., 2012). Fungi like *Fusarium solani* have higher tolerance against few heavy metals like cadmium, nickel and lead, also have better capacity of nanoparticles synthesis (Rasha, 2017). The extremophilic fungi have significant ability and application in nano-bioremediation of heavy metals due to ability to survive in severe conditions, which makes them significant for the purpose of nano-bioremediation (Bahrulolum et al., 2021). The marine fungi were assessed for their potential of bioremediation as well as the ecological importance (Thatoi et al., 2013). The degradation of pentachlorophenol was observed by *Trichoderma harzianum* (Vacondio et al., 2015). The heavy metals like $CdCl_2$, $CuSO_4$, Pb and $ZnSO_4$ could not affect the growth of *Cryptococcus* sp., a psychrophilic fungus (Singh M.P. et al., 2013).

Nanoparticles in combination with white-rot fungi (WRF) have immense potential of bioremediation (He et al., 2017). Studies have reported the remediation of toxic contaminants from wastewater and good stability of WRF-magnetic nanoparticles due to proficient immobilization. Antibiotics like sulfonamide have been reported to be degraded by *Echinodontium taxodii*-Fe_3O_4 nanoparticles (Shi et al., 2015). The remediation of cadmium and 2,4-dichlorophenol was achieved through immobilization of *Phanerochaete chrysosporium* along with titanium oxide nanoparticles (Chen et al., 2013) *P. chrysosporium* along with Fe_2O_3 nanoparticles revealed degradation of phenols (Huang Z. et al., 2017). Likewise, selenium nanoparticles in combination with *Phanerochaete chrysosporium* reported effective remediation of zinc (Espinosa-Ortiz et al., 2016). Similarly *P. chrysosporium*, with silver nanoparticles showed enhanced removal of Cd^{2+} and 2,4-dichlorophenol (Zuo et al., 2015; Huang Z. et al., 2017). Therefore, it can be concluded that nanoparticles in combination with fungi resulted in an increased rate of remediation. Hence more studies and research should be conducted for development of such effective strategies using ecological microbiology and nanotechnology.

Conclusion

The various reports available on pesticide contamination of soil indicate that the level of pesticides in the soil is increasing day by day, which is affecting human, soil and environmental health. Hence pesticides should be used rationally, especially in the underdeveloped and developing countries where an efficient monitoring system is lacking. In case of real need, the preference should be given to the application of pesticides, which have short half life and high biodegradability. To improve soil health, crop rotation programs and the use of organic manure should be implemented more effectively. Though there are various techniques used for the remediation of soil pesticide contamination, most of these techniques have their limitations. The various experiments conducted with the integration of nanoparticles with the bioremediation process have shown promising potential but more extensive research and

experimentation are required in this area. Various nanoparticles studied have enhanced the efficacy of the bioremediation but the safety of the nanoparticles for the environment and the food chain is still a matter of concern. Therefore, extensive research is required in the area of the safety analysis of nanoparticles.

Author contributions

YS wrote the manuscript. MK conceptualized and reviewed manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Identification and environment-friendly biocontrol potential of five different bacteria against *Aphis punicae* and *Aphis illinoisensis* (Hemiptera: Aphididae)

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The current work is aimed at isolating and identifying new Entomopathogenic bacterium (EPB) strains associated with *Steinernema feltiae* and assessing the EPB's biocontrol potential on *Aphis punicae* and *Aphis illinoisensis* adults in the laboratory. From *S. feltiae*, five bacterial isolates were isolated and molecularly characterized. *Lysinibacillus xylanilyticus* strain TU-2, *Lysinibacillus xylanilyticus* strain BN-13, *Serratia liquefaciens* strain TU-6, *Stenotrophomonas tumulicola* strain T5916-2-1b, and *Pseudochrobactrum saccharolyticum* strain CCUG are the strains. Pathogenicity tests demonstrated that bacterial cells were more toxic against the two aphid species than bacterial cell-free supernatants. *S. tumulicola* strain T5916-2-1b cells and filtrate were reported to have the strongest potential to kill *A. punicae* and *A. illinoisensis* individuals within 6h after treatment, with 100% mortality of both insects 24 and 48h after treatment. Based on the results of the study, it looked like endogenous *Steinernema*-associated EPB could be used directly as a biocontrol agent for *A. punicae* and *A. illinoisensis*.

KEYWORDS

entomopathogenic bacteria, molecular identification, *Aphis punicae*, *Aphis illinoisensis*, biological control

Introduction

Since several years, entomopathogenic nematodes (EPNs) from the Steinernematidae and Heterorhabditidae families have been investigated as biocontrol agents. This is because they have a wide range of hosts and host-seeking behavior, as well as mass rearing and are easily applicable with low cost. They also exhibit long-term efficacy, they are compatible with most chemicals, they are safe for the environment (for non-target organisms such as humans), their use reduces the amount of pesticide residues in food, they increase the activity of other natural enemies and they increase biodiversity in managed ecosystems (Gaugler and Kaya, 1990).

Gram-negative bacteria from the Enterobacteriaceae family, *Photorhabdus* sp. and *Xenorhabdus* sp., live in symbiotic associations with EPNs from the *Heterorhabditis* and *Steinernema* genera, respectively (Boemare et al., 1993; Sajnaga and Kazimierczak, 2020). Infective juveniles (IJs) of steinernematid and heterorhabditid nematodes carry the symbiotic bacteria in their midguts, which inhabit in the soil of various ecological systems (Dillman et al., 2012). The nematodes aggressively seek for insect hosts, the symbiotic *Xenorhabdus* sp. and *Photorhabdus* sp. are released into the hemocoel after entering by the insect's mouth, anus, or spiracles, respectively (Salvadori et al., 2012). The symbiotic bacteria play a wide range of biological roles, the most important of which is to keep the pathobiome conditions in the polyxenic colonized insect cadaver and soil appropriately balanced for the EPN/EPB symbiotic complex (Ogier et al., 2020). The symbiotic bacteria then invade the insect's haemolymph, destroy tissues, and explore a variety of immunosuppressive factors, such as toxin complexes, hydrolytic enzymes, hemolysins, and antimicrobial substances that kill the insect host in less than 48 h (Fang et al., 2011; Shi et al., 2017). Finally, in the insect host, the symbiotic bacteria multiply fast, causing septicaemia a process for turning insect cadavers into a suitable food source for nematode development and reproduction. Multiple recent investigations, however, when entomopathogenic partners were injected into insects alone, the results put a question mark on this hypothesis, they were found to exhibit decreased virulence or to be nonvirulent (Bisch et al., 2015; Kim et al., 2017; McMullen et al., 2017). Similarly (Ogier et al., 2020), revealed that the association between *Steinernema* and *Xenorhabdus* was not monoxenic, and that several Proteobacteria were found in the bacterial population associated with laboratory-reared IJs from *Steinernema carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. weiseri*. They also observed that a dozen Proteobacteria species (*Pseudomonas*, *Stenotrophomonas*, *Alcaligenes*, *Achromobacter*, *Pseudochrobactrum*, *Ochrobactrum*, *Brevundimonas*, *Deftia*, and others) were found to be linked with the main symbiont (*Xenorhabdus nematophila*). Non-symbiotic bacteria are hypothesized to join IJ vectors *via* the cuticle or intercuticular region, where they eventually enter the insect haemocoel during IJ penetration.(Singh et al., 2014). In soil-dwelling *Caenorhabditis elegans* nematodes, *Pseudomonas*, *Ochrobactrum*, and *Stenotrophomonas* have been frequently identified (Dirksen et al., 2016). Likely, Proteobacteria are also the most common bacterial group found in plant root bacterial populations. (Hartman et al., 2017) and in soils covered with plants, such as the rhizosphere (Kumar et al., 2022).

Many insect pests affect the quality and yield of pomegranate and grapevine cultivation. The pomegranate aphid, *Aphis punicae* Passerini (Hemiptera: Aphididae), is a major pest attacks pomegranate crop around the world. Fruits, leaves, and inflorescences are consumed by both adults and nymphs. Pomegranate aphid infestation results in pale, curled leaves, slowed development, and dropped flowers, as well as the transmission of viral infections and the secretion of honey dew, which fungi survive in lowering crop quality and yield (Moawad and Al-Barty, 2011). *Aphis illinoisensis* (Shimer) is a grapevine pest that feeds on the lower surface of new leaves, young terminal shoots (Blackman and Eastop, 2011), and fruit clusters, causing some grape berries to fall off (Pfeiffer and Schultz, 1986). Pomegranate and grapevine aphids have been documented as invasive pests in southern European, North African, and Asian countries since the early 2000s (El-Gantiry et al., 2012; Salim et al., 2022). Unfortunately, aphids have a high reproductive potential, and using insecticides extensively to control them leads to resistance development. When aphicides are used heavily on pomegranates or grapevines, the remnants are mainly concentrated in the fruits. Contamination with pesticides is undesirable because these fruits are consumed fresh (Li and Han, 2004; Pertot et al., 2017). As a result, scientists are looking for new pesticides that are more efficient against pests, less hazardous to natural enemies, and less destructive to the environment (Fouad et al., 2018; Ga'al et al., 2021).

Root weevils, white grubs, root worms, cutworms, sciarid flies, and armyworms are among the pests that have been controlled by EPNs (Hazir et al., 2004). When tested in field and laboratory conditions, EPNs and/or entomopathogenic bacteria (EPB) have been shown to satisfactorily control mosquitoes, pomegranate aphids, cabbageworms, scarab beetles and cherry fruit flies (Herz et al., 2006; Alghamdi et al., 2017; Yooyangket et al., 2018; Elbrense et al., 2021). The gene encoding the protease inhibitor protein has been recognized and expressed in the symbiotic bacterium *Xenorhabdus b*ovienii strains BJFS526 and Xbpi-1. This protein's impact on the pea aphid *Acyrthosiphon pisum* was also investigated (Zeng et al., 2012; Jin et al., 2014). *Xenorhabdus szentirmaii* is a one-of-a-kind source of antimicrobial peptides that are effective against virtually all known phytopathogens (Fuchs et al., 2014; Fodor et al., 2022).

To date, in several countries, including Saudi Arabia, both EPNs and their associations have not been sufficiently examined in terms of their diversity and application. Considering all the plant protection perspectives, as well as climatic, geographic and regulation aspects, the most reasonable approach is to search for potential biological plant protection (EPN/EPB) agents native locally.

Therefore, various bacterial strains that could be used as suitable control organisms need to be assessed to develop a new biological control technique. The genetic diversity of Saudi Arabian and Egyptian EPN genotypes was examined using RAPD and ISSR markers after an EPN species, *Steinernema* sp., was isolated from the soil of pomegranate trees in Taif, Saudi Arabia (Aljuboori et al., 2022). Geographically, Taif is an elevated location in Saudi Arabia, with valleys, steep mountains, and agricultural plateaus. There is an abundance of potential insect hosts; thus, the diversity of EPNs and EPB is expected to be very rich in this region. The goals of this research was to identify EPB associated with *Steinernema* found in Taif, Saudi Arabia, and to assess their activity against the pomegranate and grapevine aphids, *A. punicae* and *A. illinoisensis*, under laboratory conditions. On the basis of these goals we hypothesized that EPB associated with *Steinernema* would be good controlling agents for the control of *A. punicae* and *A. illinoisensis*.

Materials and methods

Insects

Fresh leaves and buds of pomegranate and grapevine trees infested with *A. punicae* and *A. illinoisensis*, respectively, were harvested on the same experimental day from pomegranate and grapevine farms in Taif, Saudi Arabia.

Isolation of *Steinernema*-associated bacteria

In this study, EPN *S. feltiae* strain NYH (MTöth et al., 2005) was originated from the Laboratory of Fodor Andras, Pannonia University, Keszthely, Hungary. According to the Akhurst (1980) method modified by (Vitta et al., 2018), bacterial symbionts of *S. feltiae* were isolated from infective dauer juveniles (IJs) or from the haemolymph of deceased *Galleria mellonella* larvae that was infected with *S. feltiae* IJs. Briefly, to isolate EPB from EPN infective juveniles, IJs were collected and centrifuged three times using sterilized tap water after being obtained from Galleria white traps. Some were placed in sterile petri plates with a drop of physiological saline (M9) solution before being moved to 5% chlorox. Individuals were moved to a series of sterilized M9 drops after a 2-min incubation period, and then fractured with a sterile platinum wire. The drop of M9 was diluted and deposited onto an NBTA indicator plate [nutrient agar + triphenyl tetrazolium chloride (0.004%) and bromothymol blue (0.025%)] and incubated at 28°C for 48 h. For isolation of EPB from *G. mellonella* cadavers, the dead *G. mellonella* larvae were washed in 100% ethanol for 1 min to be surface-sterilized before being placed in a sterile Petri dish to dry. Following that, a sterile sharp needle was used to penetrate the third segment of the *G. mellonella* larvae's head to allow an influx of the haemolymph containing symbiotic bacteria. The haemolymph samples were distributed and streaked over NBTA media using a sterile loop, as previously described. Bacteria were frequently cultured every 24 h until pure isolated colonies were acquired, and then stored at −80°C with 20% glycerol (*v*/*v*) for further study. To generate the cell-free conditioned filtrates or cell suspensions, in 5 ml of Luria-Bertani (LB) broth, one colony of every isolate of relevant bacteria was seeded and cultured overnight at 28°C shaking at 220 rpm. Furthermore, 100-ml culture aliquots were shaken at room temperature overnight before being introduced to flasks with 400 ml of the identical media and agitated at 200 rpm for 5 days. To obtain a cell-free filtrate, the supernatant was filtered by a 0.22 μm Millipore filter, then the pellet was resuspended in sterile distilled water. Following that the filtrate was kept at 4°C for subsequent dilution with sterile distilled water to get concentrations of 600, 400, 200, and 100 μl/ml. The bacterial cell suspension was adjusted at OD_{600} to 1.0 using a spectrophotometer. A 10-fold serial dilution spread plate was used, with a bacterial suspension concentration of 1×10^8 (CFU/ml). Each bacterial cell solution was diluted to obtain concentrations 10^8, 10^6, 10^4, and 10^2 CFU/ml.

Identification of *Steinernema*-associated bacteria

The genomic DNA of the isolated bacteria was extracted from the bacterial pellets using the Bacterial Genomic DNA Miniprep Kit (QIAprep Spin Miniprep Kit). The bacterial genomic DNA was stored at −20°C prior to use in a PCR. To identify bacterial species, PCR-based analysis and *16S rRNA* gene sequencing (1,504 base pairs, bp) were completed using the inter-universal primers 785F (GGATTAGATACCCTGGTA) and 907R (CCGTCAA TTCMTTTRAGTTT; Tailliez et al., 2006).

Phylogenetic tree analysis

To identify the bacterial species associated with *S. feltiae*, a comparison of the partially edited nucleotide sequences (16S rRNA) was performed using the BLASTN program from the NCBI. The 16S rRNA sequences were compared to the NCBI database "16S rRNA sequence (Bacteria and Archae)." The alignments of all 16S sequences were done operating the program of MUSCLE with 50 iterations and were presented in the CLC viewer. The evolutionary history was inferred using the most probability method supported the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−1426.34) is shown. The proportion of trees within which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the most composite likelihood (MCL) approach and so selecting the topology with superior log

likelihood value. Evolutionary analyses were performed employing MEGA7 (Kumar et al., 2018) with 1,000 bootstrap values.

Bioassay

The five *Steinernema*-associated bacterial isolates (cells or filtrates) were selected for use in the toxicity bioassay: *Stenotrophomonas tumulicola* strain T5916-2-1b (Isolate 7), *Pseudochrobactrum saccharolyticum* strain CCUG (Isolate 13), *Lysinibacillus xylanilyticus* strain BN-13 (Isolate 1), *Serratia liquefaciens* strain TU-6 (Isolate 6) and *Lysinibacillus xylanilyticus* strain TU-2 (Isolate 2). Accession numbers have been given in Table 1. The toxicity of the bacterial isolates was evaluated on *A. punicae* and *A. illinoisensis via* a topical application method described by Eidy et al. (2016), with slight modification. In brief, five Petri dishes (9 cm) lined with filter paper (Whatman number 2) were prepared for each bacterial cell or filtrate concentration, and then four Taify pomegranate or grapevine leaf discs with a diameter of 1.5 cm were cut out and placed on the filter paper in each dish to feed the aphids. Then, 5 µl of each concentration of bacterial cell suspension or supernatant was dropped directly onto the bodies of the aphids. The individual adult aphids were carefully relocated using a fine camel hairbrush onto the leaf discs in the Petri dishes. In the control conditions, insects were treated with the same volume of distilled water or sterile filtered LB media for each aphid species. After that, the Petri dishes were wrapped with Parafilm and held under laboratory conditions of 25 ± 1°C, 65 ± 3% relative humidity and a 12 l:12 D light–dark cycle. The mortality rate of the aphids was recorded after exposure to the bacterial suspensions or cells for 6, 12, 24 and 48 h. If an insect's appendages did not move when pushed with a fine-point brush, it was declared dead. Each bioassay was performed with five replicates on different dates. The experiment was repeated twice. Furthermore, LC_{50} and LC_{90} values of both the EPB cells and filtrates were determined using Probit analysis (Finney, 1971).

Statistical analysis

A two-way variance analysis (ANOVA) was operated to evaluate the aphid mortality rate, followed by Duncan's multiple range tests. The results were presented as mean ± standard error ($M \pm SE$). The COSTAT program was used to conduct all analyses. (Version 6.400). Using SPSS Version 23, the values of LC_{50} and LC_{90}, the 95% confidence limits of the lower and upper values, slope and intercept and the χ^2 values of the tested EPB were *t*-tested ($p < 0.05$), where *p*-values less than 0.05 were significantly considered.

TABLE 1 Bacterial accession numbers.

Isolates	Bacterial strain	Accession number
Isolate 1	*Lysinibacillus xylanilyticus* strain BN-13	OP001649
Isolate 2	*Lysinibacillus xylanilyticus* TU-2	OP578131
Isolate 6	*Serratia liquefaciens* strain TU-6	OP578132
Isolate 7	*Stenotrophomonas tumulicola* strain T5916-2-1b	OP002058
Isolate 13	*Pseudochrobactrum saccharolyticum* strain CCUG 33852	OP002063

Results

Identification of EPBs by sequencing the 16S rRNA gene

A BLASTN search of the rRNA_type strains/16S_ribosomal_RNA database returned the following results. Isolate 1 showed 87.50% identity with *Lysinibacillus fusiformis* strain DSM 2898 (NR_042072.1) and with *Lysinibacillus fusiformis* strain NBRC 15717 (NR_112569.1). Similarly, Isolate 2 showed 88.15% identity with *Lysinibacillus fluoroglycofenilyticus* strain cmg86 (NR_148289.1) and with *Lysinibacillus sphaericus* strain DSM 28 (NR_042073.1), *Bacillus ndiopicus* strain FF3 (NR_149205.1), *Lysinibacillus macrolides* (NR_114920.1), *Solibacillus isronensis* (NR_115952.1) and *Lysinibacillus boronitolerans* (NR_041276.1). However, isolate 2 showed identity (98% similarity) with *Lysinibacillus xylanilyticus* strain TU-2. Isolate 6 showed 99.26% identity with *Serratia liquefaciens* strain ATCC 27592 (NR_122057.1), and Isolate 7 showed 99.56% identity with *Stenotrophomonas maltophilia* strain NBRC 14161 (NR_113648.1), *Stenotrophomonas tumulicola* strain T5916-2-1b (NR_148818.1) and *Stenotrophomonas pavanii* strain ICB 89 (NR_116793.1). Isolate 13 showed 88.26% identity with *Pseudochrobactrum saccharolyticum* (NR_042473.1).

The phylogenetic tree analysis results (Figure 1) validated our morphological identification as indicated that Isolate 7 belonged to a *Stenotrophomonas tumulicola* T5916-2-1b and that Isolate 6 belonged a *Serratia liquefaciens* strain TU-6. However, isolate 1 belonged to *Lysinibacillus xylanilyticus* strain BN-13. Whereas, isolate 2 were found to be closely related to *Lysinibacillus xylanilyticus* strain TU-2. Additionally, isolate 13 was found to be closely related to *Pseudochrobactrum* sp.

Insecticidal activity

Toxicity of bacterial cell suspensions to *Aphis illinoisensis* and *Aphis punicae*

The data presented in Table 2 show that the mortality rate of *A. illinoisensis* adults varied from 0 to 100% after topical application of five bacterial cell suspensions at four concentrations for four exposure times. These results confirmed that the efficiency of the bacterial isolates was directly associated with concentration (Table 2). The data show that cells from all of the bacterial isolates had a significant impact on the mortality rates of *A. illinoisensis* adults to some extent ($p < 0.05$), as they caused various levels of mortality in the grapevine aphid ($p < 0.05$). Table 2 also shows that the *A. illinoisensis*

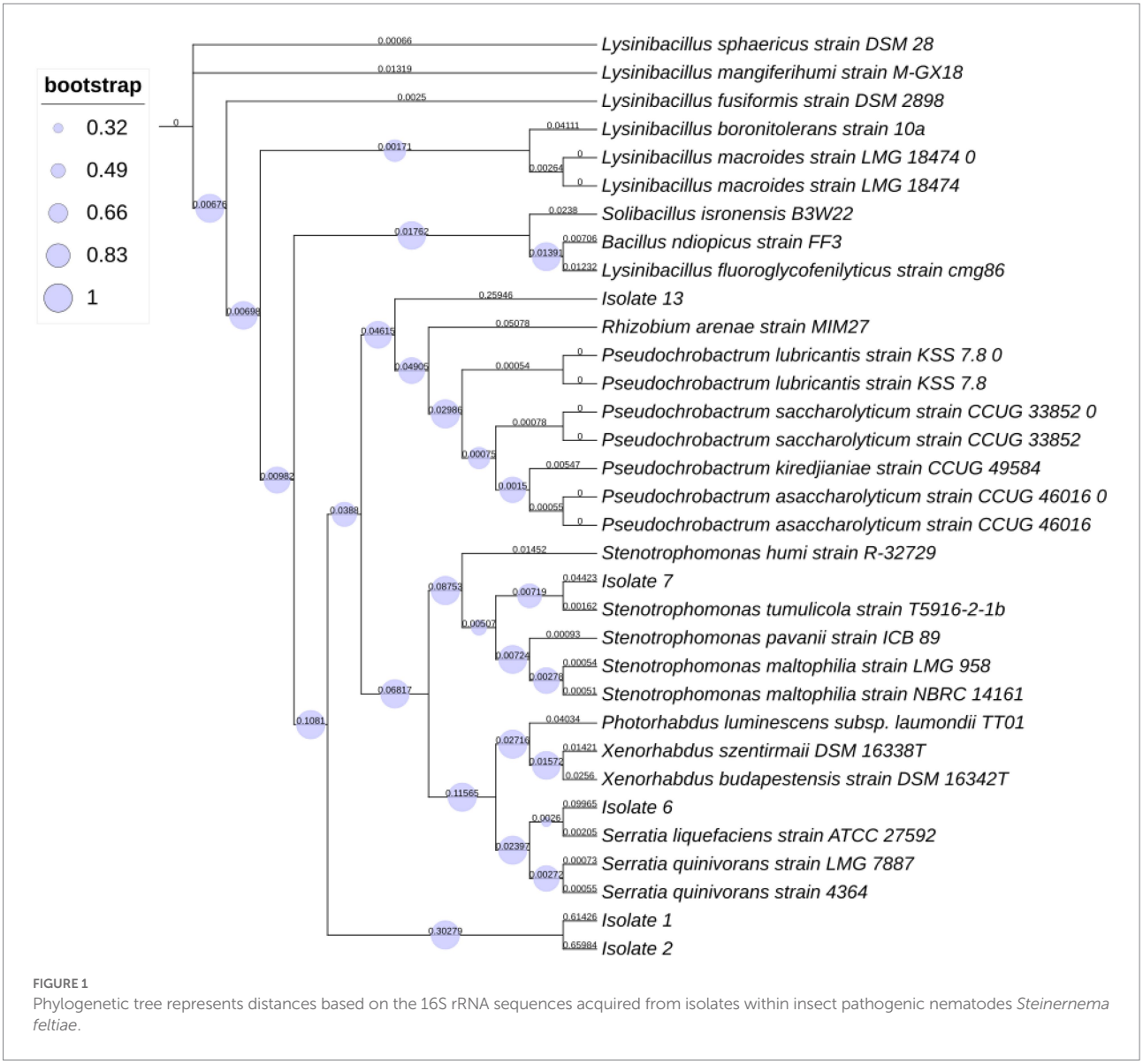

FIGURE 1
Phylogenetic tree represents distances based on the 16S rRNA sequences acquired from isolates within insect pathogenic nematodes *Steinernema feltiae*.

adults were susceptible ($p < 0.05$) to all of the bacterial isolates at all tested concentrations and exposure periods. Average mortality rates of 77 and 45.8% were recorded for *S. tumulicola strain* T5916-2-1b and *S. liquefaciens* strain TU-6, respectively. Bacterial cells of *Lysinibacillus xylanilyticus* strain BN-13 were less effective, inducing a mean adult mortality rate of 12.8% ($p < 0.05$). When individuals were exposed to 10^8 CFU/ml of *S. tumulicola* for 24 and 48 h, the greatest *A. illinoisensis* mortality rate (100%) was detected. There was also a real correlation between mortality rate and exposure time ($p < 0.05$). As a result, the mortality rate increased dramatically as the exposure period increased., and an adult mortality rate of 44.2% was recorded 48 h post-exposure. High mortality rates were associated with high concentrations of *S. tumulicola* and *S. liquefaciens* at all tested times, and considerable mortality rates were associated with *L. xylanilyticus* strain TU-2. In contrast to the bacterial cells of *S. tumulicola* causing significant aphid mortality at all tested times when at a low concentration (10^2 CFU/ml), the mortality rate associated with *L. xylanilyticus* strain BN-13. Was not significantly different from that of the control treatments (absence of bacteria-derived products), specifically with a 6-h exposure (0%).

The data in Table 3 confirmed that the bacterial cells of *S. tumulicola* and *S. liquefaciens* were the most effective against *A. illinoisensis* 48 h after treatment, with LC_{50} values of 2.75×10 and 6.76×10^3 CFU/ml and LC_{90} values of 8.13×10^3 and 3.24×10^7 CFU/ml, respectively. Table 3 also shows that the cells of *L. xylanilyticus* strain TU-2 were the third most virulent and that *Pseudochrobactrum saccharolyticum* strain CCUG and *L. xylanilyticus* strain BN-13 were the least efficient against *A. illinoisensis* after different exposure durations with LC_{50} values of 7.41×10^4, 1.26×10^7 and 4.37×10^9 CFU/ml, respectively. For the *A. illinoisensis* population, the highest degree of homogeneity was found in *S. tumulicola* and *S. liquefaciens* with slope values of 2.95 and 2.33, respectively, and the other tested bacterial cell species exhibited low slope values, indicating heterogeneity in the aphid response to these bacterial isolates (Table 3).

TABLE 2 Toxicity of five bacterial species cells against the grapevine aphid, *Aphis illinoisensis* under laboratory condition.

Bacterial species	Concentration (CFU ml^{-1})	[a]Mortality %				Bacterial species means
		6 h	12 h	24 h	48 h	
Stenotrophomonas tumulicola	10^2	[b]48 ± 4.9	60 ± 0	60 ± 0	68 ± 4.9	**77a**
(Isolate 7)	10^4	56 ± 4	64 ± 4	72 ± 4.9	88 ± 4.9	
	10^6	72 ± 4.9	80 ± 0	88 ± 4.9	96 ± 4	
	10^8	84 ± 4	96 ± 4	100 ± 0	100 ± 0	
Pseudochrobactrum	10^2	12 ± 4.9	20 ± 0	32 ± 4.9	36 ± 4	**34.5d**
saccharolyticum	10^4	16 ± 4	32 ± 4.9	36 ± 4	40 ± 0	
(Isolate 13)	10^6	20 ± 0	36 ± 4	40 ± 0	44 ± 4	
	10^8	36 ± 4	44 ± 4	52 ± 4.9	56 ± 4	
Lysinibacillus xylanilyticus strain	10^2	8 ± 4.9	16 ± 7.5	20 ± 6.3	28 ± 4.9	**40.3c**
TU-2.	10^4	24 ± 4	32 ± 4.9	36 ± 4	36 ± 4	
(Isolate 2)	10^6	40 ± 0	44 ± 4	56 ± 4	60 ± 0	
	10^8	44 ± 4	52 ± 4.9	68 ± 4.9	80 ± 0	
Serratia liquefaciens	10^2	24 ± 4	28 ± 4.9	32 ± 4.9	48 ± 4.9	**45.8b**
(Isolate 6)	10^4	24 ± 4	36 ± 4	40 ± 0	48 ± 4.9	
	10^6	28 ± 4.9	40 ± 6.3	48 ± 4.9	68 ± 4.9	
	10^8	44 ± 4	56 ± 4	76 ± 4	92 ± 4.9	
Lysinibacillus xylanilyticus	10^2	0 ± 0	4 ± 4	4 ± 4	4 ± 4	**12.8e**
strain BN-13	10^4	8 ± 4.9	8 ± 4.9	12 ± 4.9	12 ± 4.9	
(Isolate 1)	10^6	8 ± 4.9	12 ± 4.9	16 ± 4	20 ± 0	
	10^8	16 ± 4	16 ± 4	28 ± 4.9	36 ± 4	
Control		0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0f**
Exposure time means		**25.5d**	**32.3c**	**38.2b**	**44.2a**	

[a]Each treatment was represented by five replicates, each with 20 adults insect.
[b]Numbers in each column indicates to mortality ± standard error.
Means with different letters within the same column or row differ significantly ($p < 0.05$ using Duncan's multiple range test).

TABLE 3 Aphicidal activity of five bacterial species cells against *A. illinoisensis* after 48h of exposure.

Bacterial species	LC_{50} CFU ml^{-1}(95% LCL–UCL)	LC_{90} CFU ml^{-1}(95% LCL–UCL)	Slope ± SE	Intercept	X^2	*p*-Value
S. tumulicola	2.75×10 (1–1.8)*	8.13×10^3 (3.4–4.7)	2.95 ± 0.43	−0.49	2.39	0
P. saccharolyticum	1.26×10^7 (4.8–9.4)	2.75×10^{15} (12.96–17.3)	0.24 ± 0.03	−1.19	1.21	0.007
L. xylanilyticus strain TU-2	7.41×10^4 (4.3–5.4)	1.35×10^{10} (9–11.9)	0.76 ± 0.28	−0.73	1.83	0
S. liquefaciens	6.76×10^3 (1.1–4)	3.24×10^7 (6.98–9)	2.33 ± 0.42	−0.64	2.40	0.001
L. xylanilyticus strain BN-13	4.37×10^9 (8.5–11.7)	3.16×10^{17} (15.4–19.1)	0.23 ± 0.07	−2.55	1.08	0

*Figures in parenthesis are expressed as a power of 10.
LC_{50}, lethal concentration that kills 50% of insects; LC_{90}, lethal concentration that kills 90% of insects; LCL, lower confidence limit; UCL, upper confidence limit; X^2, Chi-square value; SE, standard error; and *p*-value, probability.

The toxic activity data of five *Steinernema*-associated bacterial species against *A. punicae* under laboratory conditions are presented in Table 4. These EPB cells were found to have a significant effect on adult aphid mortality ($p < 0.05$). Adult lethality was significantly greater in the *S. tumulicola* isolate (82%) than in the other isolates. Individual mortality rates increased substantially as bacterial cell concentration and exposure time increased ($p < 0.05$). On aphid infection, there was a strong interaction between EPB species, bacterial cell concentration, and exposure period ($p = 0.0113$), whereas the interaction between bacterial cell concentration and exposure time was insignificant ($p = 0.5263$). The maximum mortality rate (100%) was observed with individuals exposed to 10^8 CFU/ml of *S. tumulicola*, 6–48 h post treatment (compared to treatment with distilled water), and the lowest mortality rate (4%) was recorded with the adults exposed to 10^2 CFU/ml of *L. xylanilyticus* strain BN-13, 6 h after the treatment (Table 4).

As shown in Table 5, of all the tested bacterial species, *S. tumulicola* cells were the most effective in terms of toxicity against *A. punicae* adults 48 h after treatment, with an LC_{50} of

TABLE 4 Aphicidal activity of five bacterial species cells on the pomegranate aphid, *A. punicae* under laboratory condition.

Bacterial species	Concentration(CFU mL^{-1})	[a]Mortality %				Bacterial species means
		6 h	12 h	24 h	48 h	
S. tumulicola	10^2	[b]52 ± 4.9	64 ± 4	64 ± 4	72 ± 4.9	**82a**
	10^4	60 ± 0	68 ± 4.9	76 ± 4	92 ± 4.9	
	10^6	84 ± 4	88 ± 4.9	92 ± 4.9	100 ± 0	
	10^8	100 ± 0	100 ± 0	100 ± 0	100 ± 0	
P. saccharolyticum	10^2	16 ± 4	24 ± 4	36 ± 4	40 ± 6.3	**38.5d**
	10^4	20 ± 0	36 ± 4	40 ± 0	44 ± 4	
	10^6	24 ± 4	40 ± 0	44 ± 4	48 ± 4.9	
	10^8	40 ± 0	48 ± 4.9	56 ± 4	60 ± 0	
L. xylanilyticus strain TU-2	10^2	12 ± 4.9	20 ± 6.3	24 ± 4	32 ± 4.9	**44.8c**
	10^4	28 ± 4.9	36 ± 4	40 ± 0	40 ± 0	
	10^6	44 ± 4	48 ± 4.9	64 ± 4	64 ± 4	
	10^8	48 ± 4.9	60 ± 0	72 ± 4.9	84 ± 4	
S. liquefaciens	10^2	28 ± 4.9	32 ± 4.9	36 ± 4	52 ± 4.9	**49.8b**
	10^4	28 ± 4.9	40 ± 0	44 ± 4	52 ± 4.9	
	10^6	32 ± 4.9	44 ± 4	52 ± 4.9	72 ± 4.9	
	10^8	48 ± 4.9	60 ± 0	80 ± 0	96 ± 4	
L. xylanilyticus strain BN-13	10^2	4 ± 4	8 ± 4.9	12 ± 4.9	12 ± 4.9	**17.3e**
	10^4	12 ± 4.9	12 ± 4.9	16 ± 4	16 ± 4	
	10^6	12 ± 4.9	16 ± 4	20 ± 0	24 ± 4	
	10^8	20 ± 0	20 ± 0	32 ± 4.9	40 ± 0	
Control		0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0f**
Exposure time means		**29.7 d**	**36 c**	**41.7 b**	**47.5 a**	

[a]Five replicates of each treatment were used in this experiment, each with 20 adults insect.
[b]Numbers in each column indicated to mortality ± standard error.
Within the same column or row, means with different letters differ significantly ($p < 0.05$ using Duncan's multiple range test).

TABLE 5 Toxicity of five bacterial species cells against *A. punicae* after 48h of exposure.

Bacterial species	LC_{50} CFU ml^{-1} (95% LCL–UCL)	LC_{90} CFU ml^{-1} (95% LCL–UCL)	Slope ± SE	Intercept	X^2	*p*-Value
S. tumulicola	2.69 × 10 (1.04–1.7)*	1.55×10^3 (2.8–3.8)	3.68 ± 0.57	−0.57	3.40	0
P. saccharolyticum	1.62×10^6 (3.9–8.4)	7.59×10^9 (7.1–13.5)	0.75 ± 0.28	−0.53	1.87	0.008
L. xylanilyticus strain TU-2	2.51×10^4 (3.8–4.9)	3.89×10^9 (8.5–11.2)	2.27 ± 0.30	−1.33	2.98	0
S. liquefaciens	2.34×10^3 (1.6–4.4)	1.75×10^7 (6.4–10.4)	2.56 ± 0.69	−1.21	3.50	0
L. xylanilyticus strain BN-13	1.55×10^9 (8.5–13.3)	1.35×10^{15} (14.4–17.9)	1.51 ± 0.34	−1.76	3.61	0

*Each figure represented as a power of 10.
LC_{50}, lethal concentration that killing 50% of insects; LC_{90}, lethal concentration that kills 90% of insects; LCL, lower confidence limit; UCL, upper confidence limit; X^2, Chi-square value; SE, standard error; and *p*-value, probability.

2.69×10^1 CFU/ml and an LC_{90} of 1.55×10^3 CFU/ml. In comparison, *S. liquefaciens* cells recorded an LC_{50} of 2.34×10^3 and an LC_{90} of 1.75×10^7 CFU/ml (Table 4), and *L. xylanilyticus* strain BN-13 cells recorded higher LC_{50} and LC_{90} values of 1.55×10^9 and 1.35×10^{18} CFU/ml, respectively. It was also clear that *S. tumulicola* and *S. liquefaciens* isolates exhibited high slope values (3.68 and 2.56), which indicates homogeneity in the pomegranate aphid response to these bacteria (Table 4).

Toxicity of bacterial filtrates to *Aphis illinoisensis* and *Aphis punicae*

The same tendency was observed when the influence of bacterial filtrate on the mortality of *A. illinoisensis* adults was examined (Table 6). According to these findings, individual mortality was also found to be highly influenced by bacterial species, filtrate concentration, and exposure time ($p < 0.05$). *S. tumulicola* exceeded all of the other tested bacteria in *A. illinoisensis* mortality at all

TABLE 6 Aphicidal activity of five bacterial species filtrates on the grapevine aphid, *A. illinoisensis* under laboratory condition.

Bacterial species	Concentration(μL mL^{-1})	[a]Mortality %				Bacterial species means
		6 h	12 h	24 h	48 h	
S. tumulicola	**100**	[b]44 ± 4	56 ± 4	56 ± 4	64 ± 4	**73.5a**
	200	52 ± 4.9	60 ± 0	68 ± 4.9	84 ± 4	
	400	68 ± 4.9	76 ± 4	84 ± 4	92 ± 4.9	
	600	80 ± 0	92 ± 4.9	100 ± 0	100 ± 0	
P. saccharolyticum	**100**	8 ± 4.9	16 ± 4	28 ± 4.9	32 ± 4.9	**30.5d**
	200	12 ± 4.9	28 ± 4.9	32 ± 4.9	36 ± 4	
	400	16 ± 4	32 ± 4.9	36 ± 4	40 ± 0	
	600	32 ± 4.9	40 ± 0	48 ± 4.9	52 ± 4.9	
L. xylanilyticus strain TU-2	**100**	4 ± 4	12 ± 8	16 ± 7.5	24 ± 4	**36.8c**
	200	20 ± 0	28 ± 4.9	32 ± 4.9	32 ± 4.9	
	400	36 ± 4	40 ± 0	56 ± 4	56 ± 4	
	600	40 ± 0	52 ± 4.9	64 ± 4	76 ± 4	
S. liquefaciens	**100**	20 ± 0	24 ± 4	28 ± 4.9	44 ± 4	**41.8b**
	200	20 ± 0	32 ± 4.9	36 ± 4	44 ± 4	
	400	24 ± 4	36 ± 7.5	44 ± 4	64 ± 4	
	600	40 ± 6.3	52 ± 4.9	72 ± 4.9	88 ± 4.8	
L. xylanilyticus strain BN-13	**100**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**9e**
	200	4 ± 4	4 ± 4	8 ± 4.9	8 ± 4.9	
	400	4 ± 4	8 ± 4.9	12 ± 4.9	16 ± 4	
	600	12 ± 4.9	12 ± 4.9	24 ± 4	32 ± 4.9	
Control		0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0f**
Exposure time means		**22.3d**	**29.2c**	**35.2b**	**41a**	

[a]Each treatment was represented by five replicates, each with 20 adults insect.
[b]Mortality ± standard error is shown by the numbers in each column.
Means with different letters within the same column or row differ significantly ($p < 0.05$ using Duncan's multiple range test).

TABLE 7 Toxicity of five bacterial species filtrates against *A. illinoisensis* after 48h of exposure.

Bacterial species	LC_{50} μl ml^{-1} (95% LCL–UCL)	LC_{90} μl ml^{-1}(95% LCL–UCL)	Slope ± SE	Intercept	X^2	*p*-Value
S. tumulicola	70.3 (45.8–91.4)*	271.1 (225.3–350.1)	2.38 ± 0.40	−4.04	4.17	0
P. saccharolyticum	586.5 (440.3–1,129)	1888 (1261–4,929)	1.5 ± 0.023	−1.7	1.39	0.005
L. xylanilyticus strain TU-2	294.7 (249.5–351.9)	1,512 (1040–2763.1)	1.81 ± 0.23	−4.46	4.30	0
S. liquefaciens	212.9 (148.7–263.3)	700.6 (603.7–862.1)	2.19 ± 0.32	−3.35	4.23	0
L. xylanilyticus strain BN-13	958.8 (737.5–1,544)	3,318 (1924.3–9,550)	0.61 ± 0.22	−7.09	2.85	0

*Each figure represented as a power of 10.
LC_{50}, lethal concentration that kills 50% of insects; LC_{90}, lethal concentration that kills 90% of insects; LCL, lower confidence limit; UCL, upper confidence limit; X^2, Chi-square value; SE, standard error; and *p*-value, probability.

concentrations and exposure periods tested. *S. tumulicola* induced an adult mortality rate of 73.5%, and isolates *S. liquefaciens*, *L. xylanilyticus* strain TU-2, *P. saccharolyticum* and *L. xylanilyticus* strain BN-13 induced mortality rates of 41.8, 36.8%, 30.5 and 9%, respectively. *A. illinoisensis* adults were significantly killed by a bacterial cell-free suspension at 600 μl/ml (46.2%; $p < 0.05$). Exposure time significantly affected the mortality rate ($p < 0.05$); a 41% mortality rate was observed 48 h post-exposure. As appeared in Table 2, aphid mortality (means ± SE) ranged from 44 to 100%, 20 to 88%, 4 to 76%, 8 to 52, and 0 to 32%, respectively ($p < 0.05$) when *S. tumulicola*, *S. liquefaciens*, *L. xylanilyticus* strain TU-2, *P. saccharolyticum* and *L. xylanilyticus* strain BN-13 were applied at 100–600 μl/ml. Furthermore, the cell-free supernatant of *L. xylanilyticus* strain BN-13 did not cause any mortality at 100 μl/ml at any tested times, and initiates toxicity (4% mortality) up to 32% when tested at 200–600 μl/ml (Table 6).

Likewise, the data presented in Table 7 clarified that the filtrate of *S. tumulicola* isolate was more effective against grapevine aphid adults than the filtrates of all other tested isolates;

TABLE 8 Aphicidal activity of five bacterial species filtrates on the pomegranate aphid, *A. punicae* under laboratory condition.

Bacterial species	Concentration (µL ml^{-1})	[a]Mortality %				Bacterial species Means
		6 h	12 h	24 h	48 h	
S. tumulicola	**100**	[b]48 ± 4.9	60 ± 0	60 ± 0	68 ± 4.9	**77.3 a**
	200	56 ± 4	64 ± 4	72 ± 4.9	88 ± 4.9	
	400	72 ± 4.9	80 ± 0	88 ± 4.9	96 ± 4	
	600	88 ± 4.9	96 ± 4	100 ± 0	100 ± 0	
P. saccharolyticum	**100**	12 ± 4.9	20 ± 0	32 ± 4.9	36 ± 4	**34.5 d**
	200	16 ± 4	32 ± 4.9	36 ± 4	40 ± 0	
	400	20 ± 0	36 ± 4	40 ± 0	44 ± 4	
	600	36 ± 4	44 ± 4	52 ± 4.9	56 ± 4	
L. xylanilyticus strain	**100**	8 ± 4.9	16 ± 7.5	20 ± 6.3	28 ± 4.9	**40.8 c**
TU-2	**200**	24 ± 4	32 ± 4.9	36 ± 4	36 ± 4	
	400	40 ± 0	44 ± 4	60 ± 0	60 ± 0	
	600	44 ± 4	56 ± 4	68 ± 4.9	80 ± 0	
S. liquefaciens	**100**	24 ± 4	28 ± 4.9	32 ± 4.9	48 ± 4.9	**45.8 b**
	200	24 ± 4	36 ± 4	40 ± 0	48 ± 4.9	
	400	28 ± 4.9	40 ± 6.3	48 ± 4.9	68 ± 4.9	
	600	44 ± 4	56 ± 4	76 ± 4	92 ± 4.9	
L. xylanilyticus strain	**100**	0 ± 0	4 ± 4	8 ± 4.9	8 ± 4.9	**13.3 e**
BN-13	**200**	8 ± 4.9	8 ± 4.9	12 ± 4.9	12 ± 4.9	
	400	8 ± 4.9	12 ± 4.9	16 ± 4	20 ± 0	
	600	16 ± 4	16 ± 4	28 ± 4.9	36 ± 4	
Control		0 ± 0	0 ± 0	0 ± 0	0 ± 0	**0 f**
Exposure time means		**25.7 d**	**32.5 c**	**38.5 b**	**44.3 a**	

[a]Each treatment was represented by five replicates, each with 20 adults insect.
[b]Numbers in each column indicates to mortality ± standard error.
Means with different letters within the same column or row differ significantly ($p < 0.05$ using Duncan's multiple range test).

the LC_{50} and LC_{90} at 48 h after treatment were 70.3 and 271.1 µl/ml, respectively. It's also worth mentioning that *L. xylanilyticus* strain BN-13 and *P. saccharolyticum* isolates, having values of LC_{50} (958.8 and 586.5 µl/ml) and LC_{90} values of 3,318 and 1888 µl/ml, respectively, after 48 h were the least effective (Table 7). The highest degree of homogeneity for *A. illinoisensis* was observed for *S. tumulicola* (slope value of 2.38), and Low slope values were recorded for the other bacterial filtrate concentrations examined, indicating that the grapevine aphid response to these concentrations was heterogeneous (Table 7).

The mortality rates of *A. punicae* were statistically significant ($p < 0.05$) at all concentrations from 6 to 48 h (Table 8). The data's regression analysis revealed that mortality of *A. punicae* adults significantly increased with bacterial concentration ($R^2 = 0.938$; $p < 0.05$). When they were treated with 600 and 100 µl/ml, respectively, they had the highest (49.3%) and lowest (23.3%) mortality rates. Among all tested bacterial species, *S. tumulicola* was the most toxic: it induced a 77.3% mortality rate in *A. punicae* adults, whereas *S. liquefaciens*, *L. xylanilyticus* strain TU-2, *P. saccharolyticum* and *L. xylanilyticus* strain BN-13 isolates induced mortality rates of 45.8, 40.8, 34.5 and 13.3%, respectively. The overall mortality of the *A. punicae* adults after treatment with 100–600 µl/ml of *S. tumulicola* filtrate ranged from 48 to 100%, while it ranged from 24 to 92% for *S. liquefaciens*, and from 8 to 80% for *L. xylanilyticus* strain TU-2. In contrast, it ranged from 0 and 36% and from 12 to 56% for *L. xylanilyticus* strain BN-13 and *P. saccharolyticum*, respectively. Table 8 shows that the *A. punicae* adults were extremely vulnerable ($p < 0.05$) to both the *S. tumulicola* and *S. liquefaciens* filtrates, since they recorded 100 and 92% mortality 48 h post treatment. *S. tumulicola* induced 100% individual mortality at 24 and 48 h post-treatment, and *S. liquefaciens* induced 76 and 92% mortality at 600 µl/ml at the same exposure times, respectively. There was also an increase in adult mortality as the bacterial filtrate concentration and exposure time increased (Table 8).

The LC_{50} and LC_{90} values for each isolate at 48 h after treatment against *A. punicae* adults are shown in Table 9. The LC_{50} and LC_{90} values for *S. tumulicola* were 65.4 and 218.6 µl/ml. The LC_{50} and LC_{90} values for isolates *S. liquefaciens*, *L. xylanilyticus* strain TU-2, *P. saccharolyticum* and *L. xylanilyticus* strain BN-13 were 212.9 and 587.5 µl/ml, 312.1 and 755.3 µl/ml, 483.4 and 1,406.2 µl/ml, and 784.2 and 1,808.3 µl/ml, respectively. In addition, the *A. punicae* individuals exhibited different degrees of homogeneity in response to the tested

TABLE 9 Toxicity of five bacterial species filtrates against *A. punicae* 48h post-exposure.

Bacterial species	LC_{50} µl ml^{-1}(95% LCL–UCL)	LC_{90} µl ml^{-1}(95% LCL–UCL)	Slope ± SE	Intercept	X^2	*p*-Value
S. tumulicola	65.4 (42.2–84.8)	218.6 (183.9–274.6)	2.45 ± 0.37	−4.44	1.58	0
P. saccharolyticum	483.4 (353.9–864.3)	1406.2 (1119.6–2027)	0.59 ± 0.21	−1.59	1.41	0.006
L. xylanilyticus strain TU-2	312.1 (265.6–358.1)	755.3 (660.6–9.4.8)	1.79 ± 0.23	−4.31	5.02	0
S. liquefaciens	212.9 (184.2–229.1)	587.5 (516.7–696)	1.85 ± 0.24	−4.06	1.47	0
L. xylanilyticus strain BN-13	784.2 (657.2–1,043)	1808.3 (1206.8–4,806)	1.35 ± 0.27	−4.21	2.16	0

*Each figure represented as a power of 10.
LC_{50}, lethal concentration that kills 50% of insects; LC_{90}, lethal concentration that kills 90% of insects; LCL, lower confidence limit; UCL, upper confidence limit; X^2, Chi-square value; SE, standard error; and *p*-value, probability.

bacterial filtrates. The slope values ranged from 0.59 to 2.45 (Table 9). Additionally, *S. tumulicola* had the highest degree of homogeneity for *A. punicae*, with a slope value of 2.45.

Discussion

The first phase of this research resulted in the successful recovery and isolation of EPB from *S. feltiae*. Five bacterial isolates associated with EPN *S. feltiae* were identified. These findings are consistent with and add to those previously reported by (Alotaibi et al., 2021 and Noureldeen et al., 2022), who isolated *Xenorhabdus* sp. and *Photorhabdus* sp. from the EPNs *Steinernema* sp. and *Heterorhabditis* sp., respectively, in the same region and recorded their complex activities against *Meloidogyne incognita*, which infects pomegranate under greenhouse conditions. The five isolated species of *Steinernema*-associated bacteria found here were molecularly identified and termed isolates; based on phylogenetic tree analysis, *L. xylanilyticus* strain TU-2, *L. xylanilyticus* strain BN-13, *S. tumulicola*, *S. liquefaciens* and *P. saccharolyticum*. In the present study, we found that these five isolates were symbiotically associated with *S. feltiae*, which were discovered for the first time in Saudi Arabia. The obtained data are in agreement with those earlier stated by (Ogier et al., (2020), on four species of *Steinernema* and their associated bacteria. In that study, two species, *Pseudomonas chlororaphis* and *Pseudomonas protegens*, which are often members of the associated microbiota possibly, engaged in *Steinernema*'s parasitic lifecycle, exhibited entomopathogenic capabilities, implying a role in the virulence and pathobiome membership of *Steinernema*. Also our results are confirmed by (Moawad and Al-Barty, (2011), Dirksen et al., (2016), Hartman et al., (2017), and Lacerda Júnior et al., (2017).

Regarding the insecticidal activity, it was clear that the five bacterial isolates were more effective on the pomegranate aphid under laboratory conditions than on the grapevine aphid, which showed low susceptibility. It was also clear that *S. tumulicola* and *S. liquefaciens* could control the pomegranate and grapevine aphids. The obtained results also accomplished that *S. tumulicola* isolate was better than *S. liquefaciens* against both *A. punicae* and *A. illinoisensis* adults; however, *A. illinoisensis* was more resistant. *S. tumulicola* isolate either cells or filtrates, showed aphicidal activity against *A. punicae* and *A. illinoisensis* systematically or through direct contact, causing lethality to the adult stage of both aphid species, and the accumulative mortality approached 100% 48 h following treatment. These results were in accordance with those of (Alotaibi et al., 2021) who observed that the two *Xenorhabdus* bacterial species EMA and EMC causing significant higher *E. ceratoniae* larvae mortality than that caused by the *Photorhabdus* species TT01. In a recent study conducted by Elbrense et al., (2021), it was discovered that *H. bacteriophora* and its symbiont, *Photorhabdus* sp., were more virulent against *Pieris rapae* and *Pentodon algerinus*. Our findings are in conformity with the results of (Jabeen et al., 2018), who measured the amount of bacterial chitinases produced by *S. maltophilia* and their termiticidal potential, and (Amer et al., 2021), who claimed that *S. maltophilia* had antimicrobial activity against a variety of multidrug-resistant bacteria and fungi. Likewise, *Rhizoctonia solani*, a phytopathogen, was inhibited by *S. maltophilia*, probably due to antibiosis and the production of lytic enzymes that eliminate fungi (Berg, 1996). Following research, it was shown that the synthesis of novel bioactive compounds is mostly due to *S. maltophilia*'s metabolic diversity, including molecules that could be employed in bio-control against microbes and insects (Ribitsch et al., 2012). Overall, bacterial isolates are showing more toxicity toward the aphids than the bacterial cell free extract. This is most likely because during the extraction process, significant metabolites of bacterial crude cell extract were lost, or they were never present. Second, various EPB species have variable protein densities, which is why different EPB results have varied. It has been established that the protein encoding density of several *Xenorhabdus* species varies greatly (Kim et al., 2017) While the crude cell extract was shown to be less effective than other fractions of EPB, the data clearly show that it contains certain harmful proteins that remain there after centrifugation and are responsible for death. However, the relationship between biological processes and temperature is also real and significant.

Conclusion

In current study, we found that these five bacteria isolates were associated with *S. feltiae*, and be identified as the first recorded at Taif, Saudi Arabia. The effectiveness of these bacteria

was examined against the two important insect pests of pomegranate and grapevine, *A. punicae* and *A. illinoisensis*. Our results offer a reliable base for promising biocontrol methods and agents that could be used in managing piercing-sucking insects. Further investigations are needed, especially regarding other associated microorganisms for developing new environmentally friendly insect pests control toward suitable agricultural production. In the future, the inoculation of these bacteria can be used directly as biocontrol agents or they can be used in combination with other available methods of biocontrol for better managements of insect pests.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number OP001649, OP002058, OP002063, OP578131, and OP578132.

Author contributions

SSA and AN: conceptualization and writing—original draft. HD, SSA, and AN: data curation. HD, AN, SJ and AA-B: formal analysis. SSA, AA, and AN: investigation. HD, AN, MZ, and AA-B: methodology. SSA: project administration. SSA, SA, BA, and AN: resources. HD and AA: validation. AN and BA: visualization. SSA, LK, AB, UK, and AN: writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Development of solid agents of the diphenyl ether herbicide degrading bacterium *Bacillus* sp. Za based on a mixed organic fertilizer carrier

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The long-term and widespread use of diphenyl ether herbicides has caused serious soil residue problems and threatens the agricultural ecological environment. The development of biodegrading agents using high-efficiency degrading strains as pesticide residue remediation materials has been widely recognized. In this study, the strain *Bacillus* sp. Za was used to prepare solid agents for the remediation of diphenyl ether herbicides-contaminated soil. The ratio of organic fertilizer was 1:3 (pig manure: cow dung), the inoculum amount of Za was 10%, the application amount of solid agents was 7%, and the application mode was mixed application, all of which were the most suitable conditions for solid agents. After the solid agents were stored for 120 days, the amount of Za remained above 10^8 CFU/g. The degradation rates of the solid agents for lactofen, bifenox, fluoroglycofen, and fomesafen in soil reached 87.40, 82.40, 78.20, and 65.20%, respectively, on the 7th day. The application of solid agents alleviated the toxic effect of lactofen residues on maize seedlings. A confocal laser scanning microscope (CLSM) was used to observe the colonization of Za-*gfp* on the surface of maize roots treated in the solid agents, and Za-*gfp* mainly colonized the elongation zone and the mature area of maize root tips, and the colonization time exceeded 21 days. High-throughput sequencing analysis of soil community structural changes in CK, J (solid agents), Y (lactofen), and JY (solid agents + lactofen) groups showed that the addition of solid agents could restore the bacterial community structure in the rhizosphere soil of maize seedlings. The development of solid agents can facilitate the remediation of soil contaminated with diphenyl ether herbicide residues and improve the technical level of the microbial degradation of pesticide residues.

KEYWORDS

***Bacillus* sp. Za, diphenyl ether herbicides, solid agents, organic fertilizer, soil remediation**

Introduction

As an important category of herbicide family, diphenyl ether herbicides have been widely studied and applied in the field of agriculture (Xie et al., 2018). They inhibit the porphyrin biosynthesis pathway of broadleaf weeds, giving rise to lipid peroxidative damage and membrane permeability damage in the cell membrane, resulting in the death of weeds (Scalla et al., 1990; Park et al., 2021). The main diphenyl ether herbicides are lactofen, fomesafen, oxyfluorfen, fluoroglycofen, aclonifen, acifluorfen, and bifenox. Generally, diphenyl ether herbicides are highly efficient, stable, pertinent and have been widely used for a long time in cereal crop and grain production areas in China (Diao et al., 2009). However, they are difficult to eliminate from the natural environment, and the issue of diphenyl ether herbicide residues in soil has become increasingly significant. The residue of these herbicides not only poison subsequent crops, such as maize, sorghum, wheat, cabbage, sugar beet, and flax, but also inhibit the activity of soil enzymes, ultimately affecting soil fertility and reducing crop yields (Mukherjee et al., 2007). In addition, they cause water pollution through surface leakage, with negative impacts on aquatic plants and animals (Wang et al., 2018).

Diphenyl ether herbicides have various degradation modes after entering the environment, including photolysis, hydrolysis, and microbial degradation (Singh and Singh, 2016). However, microbial degradation is mainly achieved through microbial activity, with a high removal effect. To date, many degrading strains that can catabolize diphenyl ether herbicides have been isolated. For example, 50 mg/ml fomesafen was shown to be effectively degraded (88.7%) by *Pseudomonas zeshuii* BY-1 within 3 days (Feng et al., 2012). *Pseudomonas* sp. FB8 and *Bacillus* sp. FE-1 were used to remediate fomesafen-contaminated soil (Yang et al., 2011; Cui et al., 2018), whereas *Brevundimonas* sp. LY-2 completed the biotransformation of lactofen (Liang et al., 2010). In another study, *Edaphocola flava* HME-24 degraded 96.7% of 50 mg/ml lactofen within 72 h (Hu et al., 2020). *Bacillus* sp. YS-1 was shown to degrade 50 mg/l lactofen at an efficiency rate that reached 97.6% within 15 h (Shang et al., 2022). Additionally, *Bacillus* sp. Za could degrade 94.8% of 50 mg/l lactofen within 4 days, and the phytotoxicity of the degradation product to maize was decreased significantly compared with that of lactofen (Zhang J. et al., 2018). These isolated bacteria provide strain resources for the remediation of sites contaminated with diphenyl ether herbicides.

Degradation bacteria are often easily affected by environmental factors, so it is difficult to play their environmental remediation function well (Krueger et al., 2015). However, the preparation of high-efficiency degrading strains into agents is considered to be an effective method (Galloway et al., 2017; Yuan et al., 2020). Bacterial agents can be divided into liquid and solid ones, depending on the dosage form (Zhu et al., 2019). However, liquid agents often have a short shelf life and show unstable effects (Wen et al., 2021). Compared with liquid agents, solid ones have many advantages, such as easy storage and transport, a longer action time, the protection of degrading bacteria from environmental influences, and the provision of a suitable microenvironment for bacterial survival (Ruan et al., 2018; Zhu et al., 2021). There have been some reports on the commercialization of high-efficiency degrading strains for the remediation of soil and water (Sayed and Behle, 2017a; Mohd Yusof et al., 2019). The solid agents prepared by the cyclic aromatic hydrocarbon-degrading bacteria greatly improved the degradation of the organic pollutant pyrene in soil (Wang et al., 2020). Using *Pseudomonas stutzeri* Y2 to prepare solid agents, the bacterial degradation of S-triazine herbicides in industrial wastewater and corn fields could be promoted (Zhang B. et al., 2020). The development of microbial agents has great application prospects in the degradation of environmental pollutants, and the application effect has been satisfactory. However, there are few reports on the development of bacterial agents for the degradation of diphenyl ether herbicides.

The microbial community structure of soil is often affected by various external factors. Some studies have shown that not only the long-term use of pesticides will affect the microbial community (Jacobsen and Hjelmsø, 2014), but that also the application of microbial agents will change the biodiversity and community structure (Li et al., 2022). Even though, different types of pesticides and microbial agents have different effects on the soil bacterial community structure, and the specific effects of pesticides and microbial agents on this structure need to be further analyzed.

In this study, the solid agents were prepared with the diphenyl ether-degrading bacterial strain *Bacillus* sp. Za isolated in our laboratory. The preparation process of the solid agents was optimized, and factors influencing phytotoxicity to maize by solid agents were explored. This study also analyzed the changes in the bacterial community structure after the application of solid agents in herbicide-contaminated soil. The results facilitate the development of herbicide-degrading strains, as well as future large-scale production and practical application.

Materials and methods

Chemicals, strains, and media

Lactofen, bifenox, fluoroglycofen, and fomesafen (analytical grade, purity > 99%) were purchased from Beijing Bailingwei Technology Co., Ltd. The HPLC-grade reagents were purchased from Sigma Aldrich, United States, and all other reagents were of analytical grade. Pig manure, as organic fertilizer, was purchased from Gansu Sudi Fertilizer Co., Ltd., and cow dung was obtained from Nanjing Sanmei Co., Ltd. The above-mentioned carrier was naturally air-dried for 3 d and passed through a 40-mesh sieve, followed by sterilization at 121°C for later use.

Bacillus sp. Za (Zhang J. et al., 2018) was preserved in our laboratory in LB medium with the following composition (g/L): 5.0 yeast extract, 10.0 peptone, and 10.0 NaCl. Mineral salts medium (MSM) contained (g/L): 1.0 NaCl, 1.5 K_2HPO_4, 0.5 KH_2PO_4, 1.0 NH_4NO_3, and 0.2 $MgSO_4.7H_2O$. Za fermentation medium contained (g/L): 25.74 molasses, 14.21 soy peptone, 3.0 NH_4Cl, 3.5 K_2HPO_4, 1.0 KH_2PO_4, 2.0 NaCl, and 0.4 $MgSO_4{\cdot}7H_2O$.

Soil preparation and maize cultivation

Soil was collected from the Qiqiaoweng ecological wetland in Nanjing, which was loamy clay soil. At the site, no diphenyl ether herbicides had been applied, and the soil physical and chemical properties were as follows: pH 6.89, available P 14.36 mg/kg, available K 109.08 mg/kg, and organic matter 15.67 g/kg. The soil was naturally air-dried in a ventilated area, and the sundries were removed and passed through a 10-mesh sieve. According to the concentration of 10 mg/kg dry soil, lactofen, bifenox, fluoroglycofen, and fomesafen were added to the soil separately to obtain one portion of soil containing a single herbicide residue for use. The maize seeds (Meiyu No. 3, Jiangsu Tomorrow Seed Technology Co., Ltd.) were sterilized with 10% H_2O_2 solution and placed in the dark for germination, seeds with the same germination degree were selected for follow-up experiments (Qian et al., 2022).

Preparation of solid agents of strain Za

Pig manure and cow dung were selected as carriers of solid bacterial agents, and a two-factor and three-level orthogonal experiment was designed according to the mixing ratio of the carrier and the inoculation amount of strain Za. The mixing ratio levels of pig manure and cow dung organic fertilizer were set as 3:1, 1:1, and 1:3, and the levels of strain Za inoculum were set as 5, 10, and 15%. The carriers were prepared according to the above carrier ratios, and 100 g of each was taken, sterilized at 121°C twice, and air-dried for later use. Under sterile conditions, the fermentation broth (the amount of Za reached 10^{10} CFU/ml) was inoculated into each carrier at inoculum levels of 5, 10, and 15%, and placed in a 30°C incubator for 3 days to check the viability, the average number of Za was taken as logarithmic treatment. Subsequently, weighed 5 g of solid agents treated in different treatments and added them to a sterilized conical flask containing 45 ml of sterile water, the plate method was used to detect the number of strain Za cells. The recovery rate of strain Za was the ratio between the Za after inoculation and the initial inoculation. According to the optimal mixture ratio and the optimal inoculum amount, the solid agents were prepared with different types of organic fertilizer carriers, then they were stored in a dry and cool place, and the dynamic changes in the Za count were observed within 120 days.

Effects of the application amount and mode on the degradation of solid agents

The solid agents prepared and stored for 30 days (with the strain Za count of 10^9 CFU/g) were applied to the soil containing 10 mg/kg lactofen, bifenox, fluoroglycofen, and fomesafen at 1, 3, 5, 7, and 9%, the water content was adjusted to 20% with deionized water and maintained. After 4 days, samples were taken to detect the residual amounts of the four herbicides in soil and calculate the degradation rate. The experiment was performed in three groups, and each treatment was placed in an incubator to simulate the natural environment.

Following the same procedure as described above, the solid agents were applied according to the optimal amount for covering application, mixed application, and acupoint application, respectively. Soil containing the herbicides without inoculation with the solid bacterial agent was used as control. On days 1, 3, 5, and 7, samples were taken to detect the residues of the four herbicides in soil.

Relief effect of lactofen to maize phytotoxicity by solid agents and observation of Za-*gfp* colonization in maize rhizosphere

Bacillus sp. Za-*gfp* was constructed according to the method for labeling strains with GFP (Zhang et al., 2022), and used to prepare solid agents under optimal conditions. Soil containing the residues of lactofen was loaded into pots, and the optimal inoculum amount and application mode obtained were used to apply solid agents according to different treatments. The different treatments were set as follows: Control (without any addition), J (solid agents), Y5 (5 mg/kg lactofen), JY5 (5 mg/kg of lactofen + solid agents), Y10 (10 mg/kg of lactofen), and JY10 (10 mg/kg lactofen + solid agents). At the same time, the maize seeds with the same germination stage were picked and transplanted. The maize seedlings were sampled on the 7th, 14th, and 21st days, respectively, and physiological indicators such as stem and leaf length, root length, and fresh weight were measured. The maize seedling roots of groups Control and J (solid agents) were collected on the 7th, 14th, and 21st days for microscopic observation. At sampling, the root system was removed from the soil, gently shaken, and all soil particles were washed off from the root surface with sterile water, subsequently, the roots were cut into 0.5-cm-long parts with a clean blade. The root segments were divided into root cap, meristem, elongation area, and mature area, then they were fixed in a 48-well plate filled with 2.5% glutaraldehyde fixative, sliced on a glass slide, and observed under a confocal laser scanning microscope (CLSM, Leica TCS SP3). The excitation wavelength was 488 nm, and the collected signal range was 480–525 nm.

Analysis of the soil community structure

The rhizosphere soil of maize seedlings was used as the research object. The collected seedlings were dug up with the root zone soil, and the root system was shaken gently to remove the soil that was weakly attached to the root system, the remaining soil was the rhizosphere soil. The maize seedlings of Control, J, Y10, and JY10 groups were selected as the research objects, and the sampling time points were set at days 0, 7, 14, and 21. The total soil DNA was extracted and sent to Shanghai Meiji Biomedical Technology Co., Ltd. for subsequent analysis by 16S rRNA amplification and sequencing of bacteria. The PE reads obtained by MiSeq sequencing were subjected to optimization steps such as quality control, filtering, and splicing. The Qiime2 process was used to analyze the microbial diversity, and the Dada2/Deblur noise reduction method was applied to obtain the representative sequence and abundance information of the Amplicon Sequence Variant. Based on the sequencing results, follow-up *Alpha* diversity analysis, *Beta* diversity analysis, community composition analysis, and species difference analysis were carried out (Wang et al., 2007).

Chemical analysis

Lactofen, bifenox, fluoroglycofen, and fomesafen residues in soil were extracted with an equal volume of dichloromethane by shaking at 150 rpm for 1 h, and centrifugation at 8,000 × *g* for 10 min. All supernatants were collected and acidified to pH 2.5. The upper solution was discarded, the remaining solution was blown dry, dissolved in methanol, and analyzed by high-performance liquid chromatography (HPLC) (Dionex UltiMate 3,000). The chromatograph was equipped with a C18 reverse-phase chromatographic column (4.6 × 250 mm, 5 μm, Agilent Technologies, Palo Alto, CA, United States), the column temperature was 40°C, and the UV detector was set as 270 nm. The mobile phase was water: acetonitrile: phosphoric acid (40:60:0.5, v: v: v) and the flow rate was 1 ml/min.

Data processing and analysis

One-way ANOVA and Duncan's test to compare means were used to analyze the data. The statistically significant difference was set at p-values < 0.05. Using Microsoft Excel 2015 and IBM SPSS Statistics 20, the data were processed and analyzed for significant differences. Graphs were generated by the GraphPad Prism v. 8.0.2.263 software (San Diego, CA, USA).

Data availability

All of the sequencing raw data involved in this manuscript had been deposited in the NCBI database (BioProject ID: PRJNA895898), and could be download from the link: https://www.ncbi.nlm.nih.gov/sra/PRJNA895898.

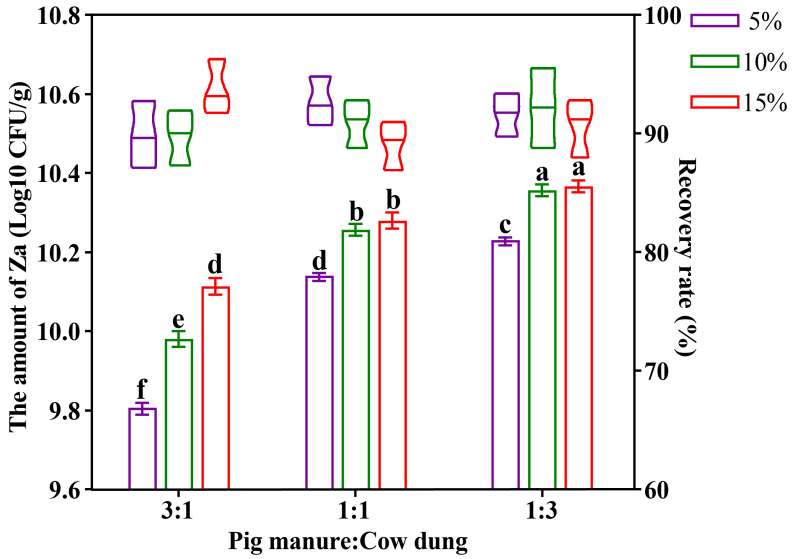

FIGURE 1
Optimization of organic fertilizer carrier ratio and Za inoculum amount in preparation of solid agents. The abscissa represented the mixing ratio of the organic fertilizer carrier (pig manure: cow dung), the icon (5%, 10%, and 15%) showed the levels of strain Za inoculum, the amount of Za was shown as a bar graph, the recovery rate of Za was shown as the violin plot.

Results

Optimization of organic fertilizer carrier ratio and Za inoculum amount in the preparation of solid agents

A two-factor and three-level orthogonal test was designed for the carrier mixture ratio and inoculum size of the solid bacterial agent, the test design and results are shown in **Supplementary Table 1A**. After one-way analysis of variance, the organic fertilizer carrier ratio and Za inoculum amount showed no significant effect on the recovery rate of strain Za in the solid agents. After two-way ANOVA, factor 1 ($F = 42.881$, $p = 0.002 < 0.05$) and factor 2 ($F = 13.185$, $p = 0.017 < 0.05$) had significant effects on the amount of Za, and factor 1 was more obvious (**Supplementary Table 1B**). Based on **Figure 1**, when the ratio of pig manure and cow dung organic fertilizer in the carrier was 1:3, the amount of strain Za was higher. Simultaneously, the amount of Za increased with the increase in the inoculum amount, but when the inoculum amount reached 15%, there was no significant difference in the amount of strain Za compared with an inoculum amount of 10%. Therefore, the optimal carrier mixture ratio in the preparation of the solid preparation of strain Za was 1:3 (pig manure: cow dung), the optimal inoculum amount of Za was 10%, and the amount of Za in the solid agents could reach 2.34×10^{10} CFU/g.

Quantity dynamics of Za of solid agents with different carriers during the storage period

Pig manure organic fertilizer carrier, cow dung organic fertilizer carrier, and mixed carrier were used to prepare solid agents. At days 30, 60, 90, and 120, samples were collected to detect the number of Za in solid agents, the results are shown in **Figure 2**. When stored for 30 days, its number in each carrier reached the highest level. Among them, the number of Za contained in the cow dung organic fertilizer carrier reached 1.28×10^{10} CFU/g, and that in the mixed carrier was 1.09×10^{10} CFU/g. Subsequently, the amount of Za contained in the organic fertilizer carrier decreased gradually. When stored for 120 days, the level of Za contained in the cow dung organic fertilizer carrier was only 2.80×10^{8} CFU/g. The carrier with the highest level of Za was the mixed carrier, with 7.50×10^{8} CFU/g.

Influence of application amount and mode on the degradation effect of solid agents

Lactofen, bifenox, fluoroglycofen, and fomesafen herbicides at a concentration of 10 mg/kg dry soil were applied to the soil, and the solid agents were applied in a mixed application amount at 1, 3, 5, 7, and 9%. The concentration of herbicide residues was detected after 4 days. As shown in **Figure 3**, when the application amount was 1%, the degradation effect of the solid agents was poor. The possible reason was that when the amount of Za in soil was low, the strain showed difficulties in growth and reproduction in a short period. Low biomass of strain Za impeded its effective colonization in the maize rhizosphere, and limited the degradation of herbicide residues. At an application level of 7%, the solid agents could degrade 87.36, 84.40, 83.16, and 63.96% of 10 mg/kg lactofen, bifenox, fluoroglycofen, and fomesafen in soil, respectively, and at 9%, the degradation rates were 88.40, 86.76, 84.47, and 66.32%, respectively. When the application amount was 9%, the degradation effect was not significantly improved compared with 7%. At an application level of 7%, Za could stably colonize the maize rhizosphere and efficiently degrade the residues of diphenyl ether herbicides. Considering the costs and benefits of the solid agent application, the optimal application amount of solid agents was 7%.

The solid agents were applied in three ways, namely covering application, mixed application, and acupoint application. The changes in herbicide residues were detected on the 1st, 3rd, 5th, and 7th days. On the 1st day, the degradation effect of solid agents on herbicide residues in soil was not obvious. The possible reason was that after the solid agents were applied to the soil, they needed to grow and reproduce in soil to form a certain number of scales before the degradation effect was significant. On the 7th day, in the mixed application treatment, the degradation rates of solid agents on lactofen, bifenox, fluoroglycofen, and fomesafen in soil reached 87.40, 82.40, 78.20, and 65.20%, respectively. The degradation effect was better than that achieved *via* acupoint application and covering application. From the perspective of the overall degradation effect, the best application mode was obtained for the mixed application (**Figure 4**).

Decrease the phytotoxicity of lactofen to maize by solid agents

The pot experiment was carried out on the phytotoxicity of lactofen to maize and the removal of phytotoxicity by solid agents at the seedling stage of maize, and the results are shown in **Figure 5**. The growth indicators of Control and J groups (solid agents) at various maize seedling stages were highly consistent, proving that the application of solid agents did not affect the growth of maize seedlings (**Figure 5A**). Stem and leaf length, root length, and fresh weight of maize in group Y5 (5 mg/kg lactofen) were 15.6 ± 1.9 cm, 17.3 ± 1.21 cm, and 1.83 ± 0.27 g, whereas in Y10 (10 mg/kg lactofen), they were 12.7 ± 1.06 cm, 8.2 ± 1.51 cm, and 1.45 ± 0.28 g on the 7th day, respectively, which were significantly lower than those of the Control. The difference became more and more significant

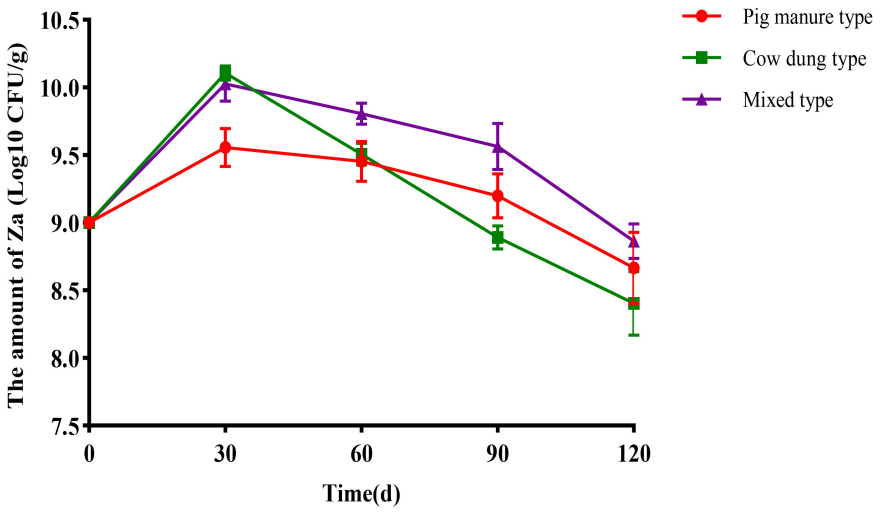

FIGURE 2
Quantity dynamics of strain Za with different carriers. The inoculation amount of Za bacterial solution was 10%, the error bars indicated standard deviations for three samples.

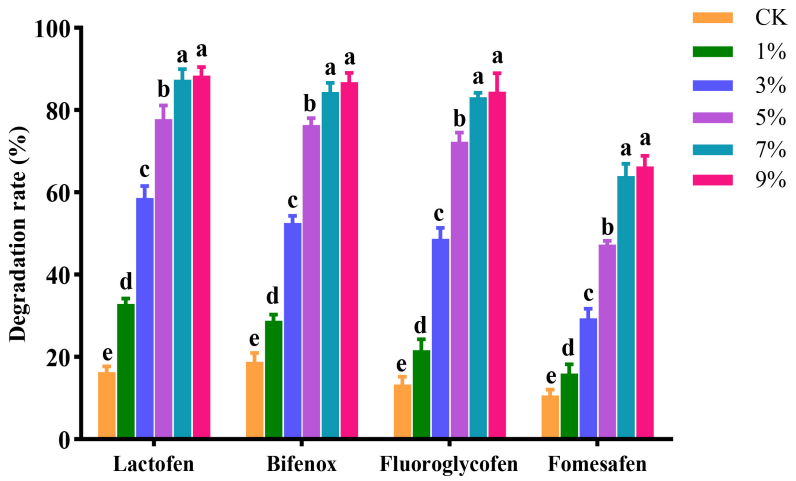

FIGURE 3
Effect of application rate on degradation of diphenyl ether herbicides by solid agents.

on the 14th and 21st days. However, the application of solid agents could decrease the phytotoxicity to maize seedlings. The growth indices of JY5 (5 mg/kg lactofen + solid agents) and JY10 (5 mg/kg lactofen + solid agents) were significantly higher than those of Y5 and Y10 after the 7th day of the maize seedling stage. On the 7th day, the growth indices of the maize seedlings in JY10 and Y10 were similar. On the 21st day, the stem and leaf length and root length of Y10 were 21.3 ± 1.69 cm and 10.8 ± 1.57 cm, and those of JY10 were 35.1 ± 2.86 cm and 25.6 ± 2.51 cm, respectively, the fresh weight of maize seedlings in JY10 (10.48 ± 1.53 g) was three times that of Y10 (2.96 ± 0.51 g) (**Figures 5B–D**). The growth indices of maize seedlings in JY5 were also significantly higher than those in Y5. Based on these results, the application of solid agents could significantly reduce the phytotoxicity to maize caused by the residues of lactofen in soil (**Figure 5**).

Colonization observation of strain Za-*gfp* in maize rhizosphere

Some pesticide-degrading bacteria can colonize the root surface of maize seedlings (Zhang H. et al., 2018; Qian et al., 2022). Based on this, this study explored whether the solid agents could effectively release strain Za-*gfp* in soil and enable its colonization on the surface of maize roots. The root samples

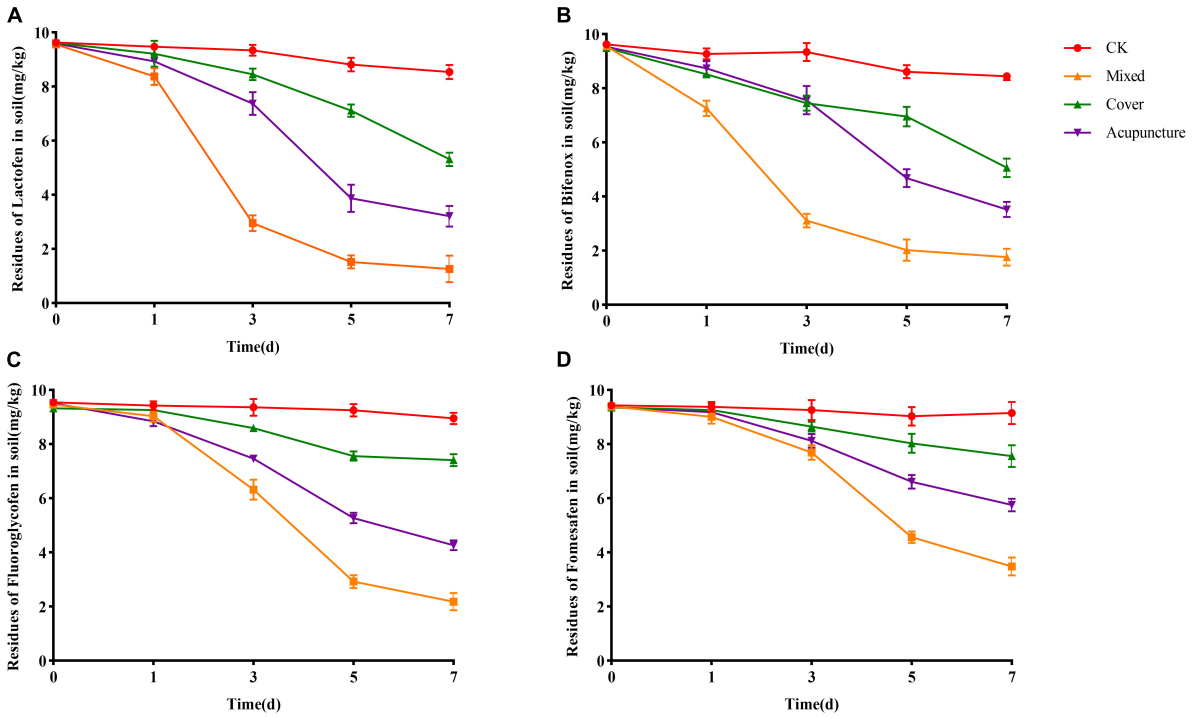

FIGURE 4
Effects of application mode of solid agents on the degradation of diphenyl ether herbicides. **(A)** Lactofen; **(B)** Bifenox; **(C)** Fluoroglycofen; **(D)** Fomesafen.

at 7, 14, and 21 d were observed under a CLSM. As seen in Figure 6, Za-*gfp* in the solid agents could be effectively released into the plant rhizosphere soil, resulting in the colonization of the root surface. Strain Za-*gfp* mainly colonized the elongation and the mature area of maize root tips, and no colonization was observed in the root cap. The colonization effect was more significant on the 7th day, with a large fluorescence range and a high intensity. A strong green fluorescence could still be observed in the meristem, elongation and mature areas of the maize root system sampled on the 21st day. Most likely, the strain Za-*gfp* in the solid agents could be rapidly released and colonized the maize root system after application to the soil, and the colonization time could exceed 21 days.

Soil bacterial community structure during the degradation of lactofen by solid agents

The Shannon index and the Chao index represent the diversity and richness of microbial community, respectively. The *Alpha* diversity of the rhizosphere soil samples from the different treatments showed that the application of 10 mg/kg lactofen to the soil caused a sharp decrease in the diversity of soil bacteria, and the difference reached a highly significant level (Figure 7A). When the solid agents were applied in the presence of 10 mg/kg lactofen, both the Chao and the Shannon index increased significantly, reaching to the level of the Control (Figures 7A,B). The application of solid agents could reverse the adverse effects of herbicide residues on the diversity of the soil bacterial community. The *Beta* diversity showed that the residues of lactofen could change the bacterial community structure, and the treatments with herbicide residues were significantly different from the Control (Figure 7C). The application of solid agents had a certain recovery effect on the soil bacterial community.

The relative abundances of bacteria at the phylum level under different treatment conditions are shown in Figure 8A. The phyla with relative abundance ratios >0.05 were Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, and Chloroflexi. Proteobacteria and Firmicutes are the most reported diphenyl ether herbicide-degrading bacteria. The relative abundance of Proteobacteria in groups Y10 and JY10 was significantly higher than that in the Control, leading to the speculation that some strains of Proteobacteria can grow with lactofen as a carbon and nitrogen source. The relative abundances of Proteobacteria in groups J and Control were similar, indicating that the addition of solid agents would not lead to changes in the abundance of Proteobacteria. The relative abundance of Firmicutes in the Control treatment gradually

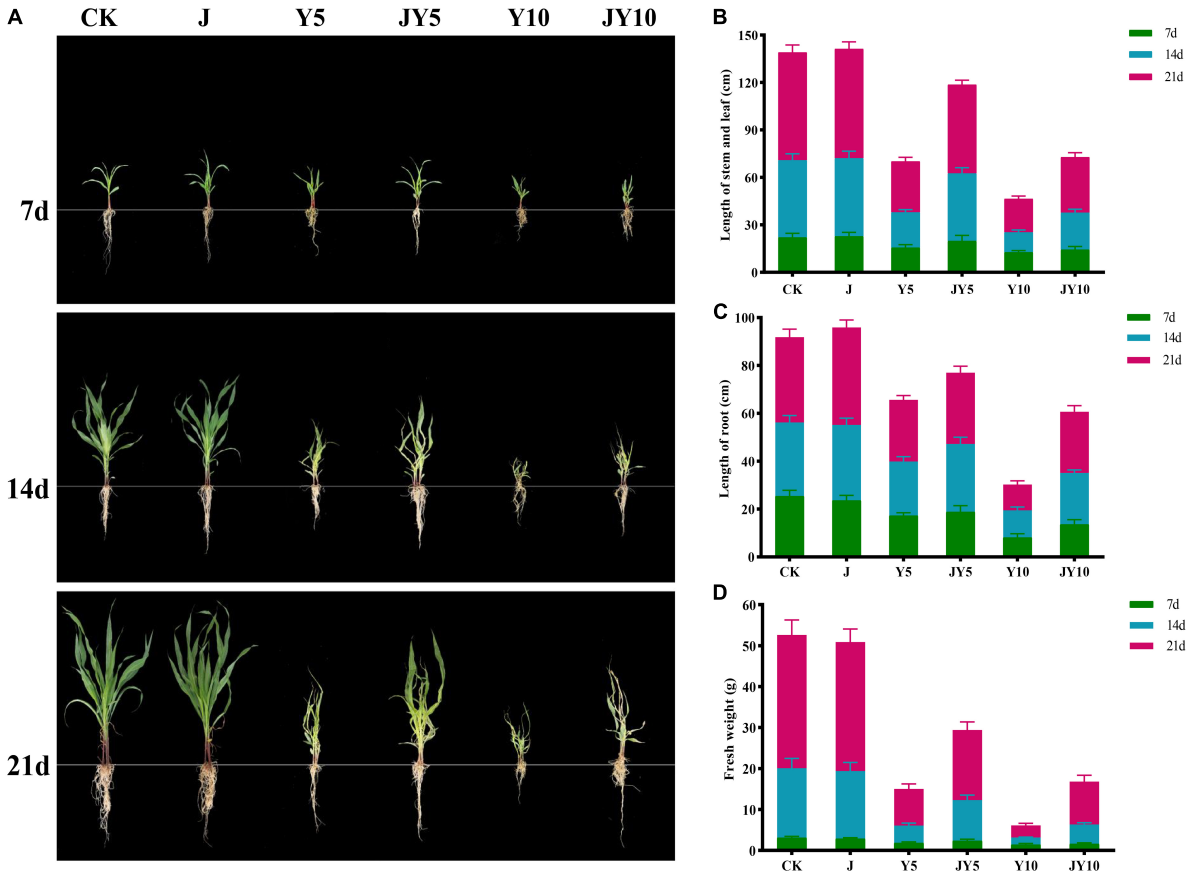

FIGURE 5
Effects of lactofen and solid agents on physiological indices of maize. **(A)** Growth status of maize under each treatment; **(B)** Length of stem and leaf (cm); **(C)** Length of root (cm); **(D)** Fresh weight (g). CK, control; J, solid agents; Y5, 5 mg/kg lactofen; JY5, 5 mg/kg lactofen + solid agents; Y10, 10 mg/kg lactofen; JY10, 10 mg/kg lactofen + solid agents.

decreased over time, and this change was more pronounced in the Y10 and JY10 groups (Figure 8A). The relative abundance of Firmicutes in the J7 group treatment was the highest among all samples, whereas in groups J and JY, it gradually decreased over time. On the 21st day, the relative abundance of Firmicutes in group J was still significantly higher than that in the other treatments.

The relative abundances of bacterial genera under different treatment conditions are shown in Figure 8B. Genera with a relative abundance ratio >0.04 were *WPS-2*, *Sphingomonas*, *JG30-KF-AS9*, *Bacillus* and *Tumebacillus*. The relative abundance of *Sphingomonas* was significantly higher than that of the Control in each treatment. Its relative abundance increased rapidly after maize planting, and *Sphingomonas* became one of the dominant genera in the rhizosphere soil of maize seedlings. The relative abundance of *Sphingomonas* gradually increased over time, with significantly higher levels in soil containing herbicide residues. It was therefore speculated that some strains of this genus can grow using lactofen in soil, and there may also be strains degrading diphenyl ether herbicides. The abundance of *Bacillus* in the Control gradually decreased over time, which may be related to the cultivation of maize. The relative abundance of *Bacillus* in group J was significantly higher than that in the other treatments, especially in group J7, which had the highest relative abundance of *Bacillus* among all samples. On the 14th day, the relative abundance of *Bacillus* rapidly decreased and remained stable, and on the 21st day, the relative abundance of *Bacillus* in the treatment with solid agents (group J) was significantly higher than that in the other treatments, most likely because strain Za-*gfp* had colonized the rhizosphere of maize (Figure 6) and had become the dominant strain in soil.

Discussion

Diphenyl ether herbicides have become important herbicides and have been widely studied and applied in agricultural field (Sheu et al., 2006). However, with the large-scale use of diphenyl ether herbicides, the problems of their

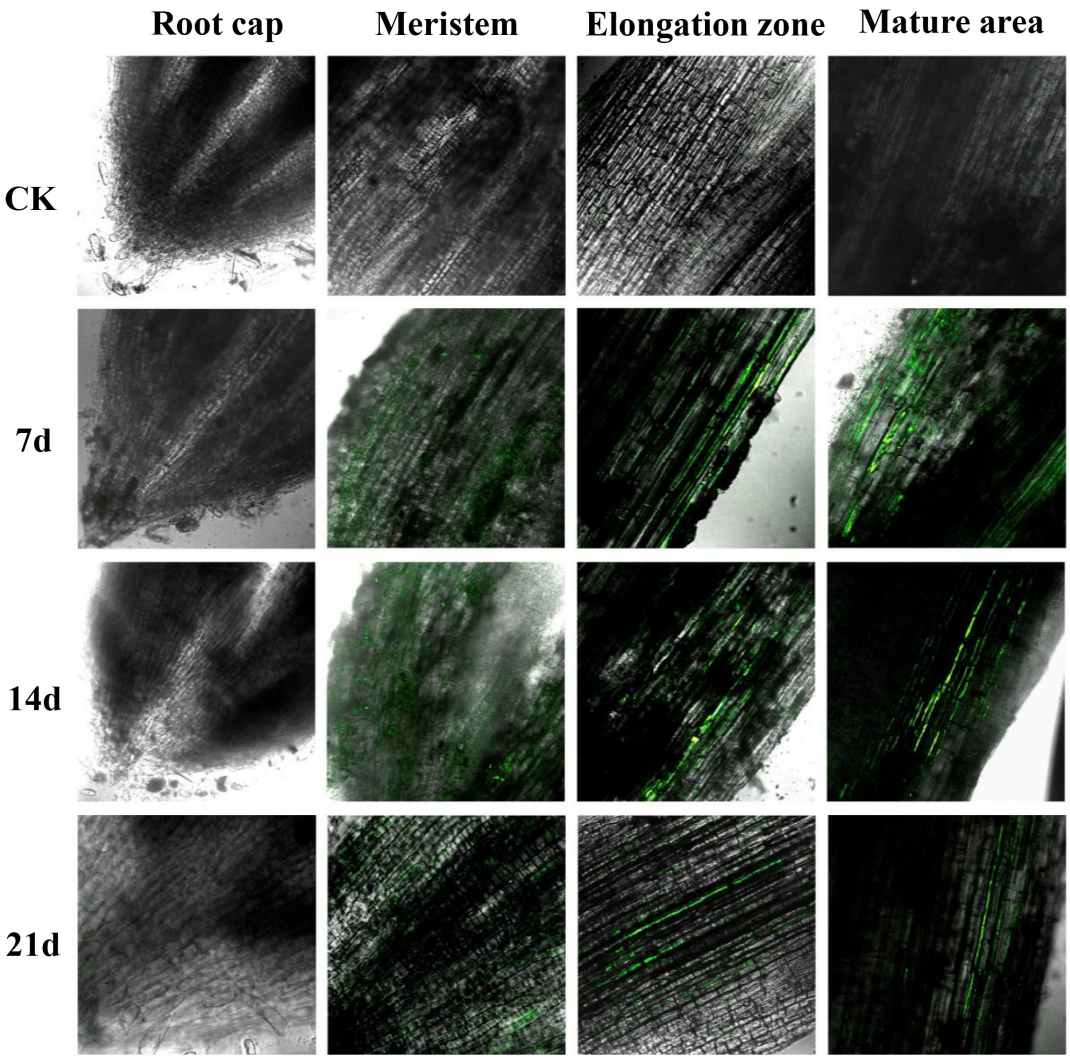

FIGURE 6
Confocal laser scanning microscope (CLSM) photos of strain Za-*gfp* in solid agents colonized on the root surface of maize seedlings. The experimental materials were obtained from the maize seedling samples of the CK and J groups (solid agents).

residues in soil and the toxic effects on organisms have become increasingly prominent (Mukherjee et al., 2007; Wang et al., 2018). Bioremediation is the most promising biotechnological method to eliminate pesticide residue pollution in soil, and some efficient diphenyl ether pesticide-degrading bacteria have been reported, including *Brevundimonas* sp. LY-2 (Liang et al., 2010), *Mycobacterium phocaicum* MBWY-1 (Chen et al., 2011), *Pseudomonas* sp. FB8 (Yang et al., 2011), *Pseudomonas zeshuii* BY-1 (Feng et al., 2012), *Lysinibacillus fluoroglycofenilyticus* cmg86 (Cheng et al., 2015), *Bacillus* sp. FE-1 (Cui et al., 2018), *Edaphocola flava* HME-24. (Hu et al., 2020) and *Bacillus* sp. YS-1 (Shang et al., 2022). The research object of this study, *Bacillus* sp. Za, was a diphenyl ether herbicide-degrading strain isolated in our laboratory (Zhang J. et al., 2018), providing valuable resources for the bioremediation of sites contaminated with diphenyl ether pesticide residues.

The development of microbial degrading agents using highly efficiency strains is widely recognized and considered to be a safe, efficient solution with great application potential (Zhang W. et al., 2020). Compared with other kinds of microbial agents, solid agents are more popular due to their easy preservation, long shelf life, and good remediation effect. The Za solid agents in this study had a viable count of 2.34×10^{10} CFU/g on the third day, which remained above 10^8 CFU/g after 120 days of storage. Within 7 days, the removal rate of diphenyl ether pesticides was about 80% with the application of solid bacterial agents. There have been some reports on the development and application of solid agents, the recovery efficiency of solid agents has been largely improved,

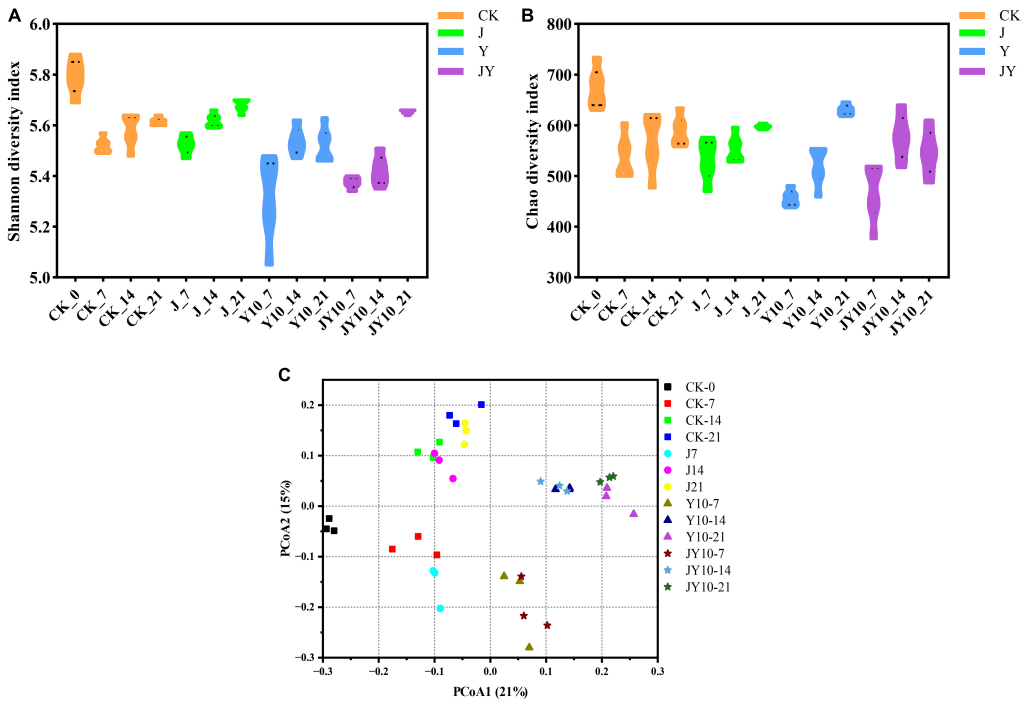

FIGURE 7
Alpha diversity and *Beta* diversity analysis of soil microbial community structure under different treatments. **(A)** Shannon diversity index for microbial communities of different treatments. **(B)** Chao diversity index for microbial communities of different treatments. **(C)** Analysis of the differences between different samples with same treatment condition. CK, no lactofen and solid agents; J, solid agents; Y, lactofen; JY, lactofen and solid agents. Among them, Y10 means 10 mg/kg lactofen, JY10 means 10 mg/kg lactofen and solid agents. The numbers 7, 14, and 21 represented the days of administration, respectively. The Shannon index comprehensively considered the richness and evenness of the community. The larger the Shannon value was, the higher the community diversity was. The Chao index reflected the richness of the community by estimating the number of species in the community, and the larger the index was, the higher the community richness was. PCoA, which could be used to study similarities or differences in sample community composition.

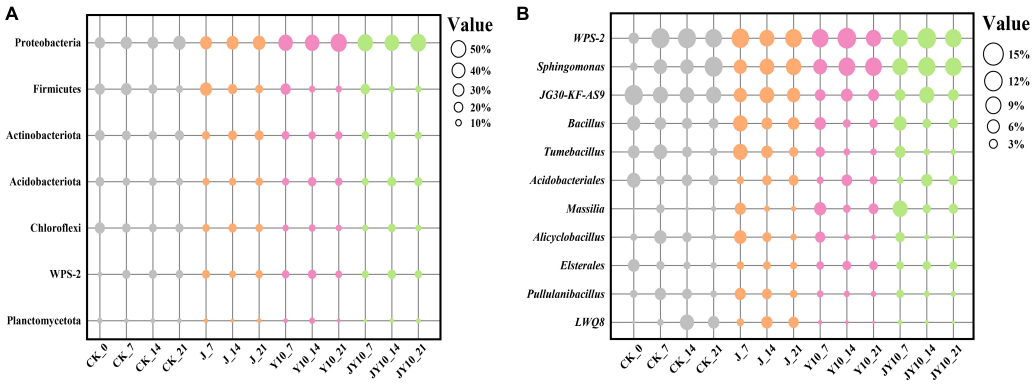

FIGURE 8
Community composition analysis of soil bacteria at phylum **(A)** and genus **(B)** levels under different treatment conditions. CK, no lactofen and solid agents; J, solid agents; Y10, 10 mg/kg lactofen; JY10 mg/kg lactofen and solid agents. The numbers 7, 14, and 21 represented the days of administration, respectively. Bubble sizes represented relative percentages of different species.

and good results have been obtained. For example, starting from the carrier aspect, the effects of kaolinite, montmorillonite, and vermiculite on the efficiency of phenol degradation by *Sphiningomonas* sp. GY2B were evaluated (Gong et al., 2016). From the perspective of the composition ratio of immobilized materials, orthogonal experiments tested the combination of four materials, namely polyvinyl alcohol, sodium alginate, activated carbon, and *Pseudomonas stutzeri* Y2, to stabilize and

extend the bacterial degradation of s-triazine herbicides through an immobilization strategy (Zhang B. et al., 2020). At the same time, the remediation effect of bacterial agents on polluted soil was also reflected through a maize growth experiment, which was consistent with the experimental purpose of this study, as shown in **Figure 5**. Compared with the treatments without solid agents (Y5 and Y10), the herbicide damage to maize seedlings was significantly alleviated with the addition of solid agents (JY5 and JY10), and over time, this phenomenon was more obvious. The CLSM photos of *Bacillus* sp. Za-*gfp* in solid agents on the root surface of maize seedlings indicated that the diphenyl ether herbicide-degrading bacteria Za-*gfp* could colonize the root surface of maize seedlings and further proved that the development of solid agents could promote the remediation of sites contaminated with diphenyl ether herbicides. By evaluating the influence of different external conditions (pH, temperature, etc.) on solid agents, the storage time of solid agents was extended from the perspective of environmental factors (More et al., 2015). Various studies have shown that the ratio of different strains and different carrier materials can affect the storage time of solid agents (Sayed and Behle, 2017b), which is in agreement with the findings of the present study. Moreover, the degradation effect of the solid agents on the four diphenyl ether herbicides also reached an ideal level, providing a suitable microbial agent for the removal of the diphenyl ether herbicides.

Both environmental pollutants and the use of microbial solid agents can alter the structure and diversity of the microbial communities. For example, long-term metal and radionuclide contamination affected the structure and function of microbial communities (Rogiers et al., 2021). The addition of synthetic and biological pesticides would directly affect the total bacterial community diversity in the rhizosphere soil (Walvekar et al., 2017). Microbial solid agents need to be prepared by selecting suitable materials as carriers, and the addition of these solid agents will also change the microbial community structure. In a previous study, an enhanced nitrogen removal from brine wastewater by adding biochar-immobilized bacteria was observed, and the biochar bacterial agent had a significant effect on the microbial community structure (Zhao et al., 2022). In another study, the highly efficient degrading bacterial strain *Daracoccus aminovorans* HPD-2 significantly promoted the degradation of Polycyclic Aromatic Hydrocarbons (PAHs) in soil, increased the abundance of embedded stratified bacteria in soil and enhanced the potential indigenous degrading bacteria at the same time (Ren et al., 2022). The carrier material selected in this study was organic fertilizer (pig manure and cow dung), and the mixing ratio of these two types of carriers was optimized to 1:3. The microbial community structure and biodiversity results showed that the residues of the pesticide lactofen reduced the soil bacteria diversity, and the application of solid agents could restore this diversity. Based on the analysis of the relative abundance of species at the bacterial phylum and genus levels, the relative abundance of Proteobacteria was significantly increased in the treatment with the addition of lactofen residues, most likely because the herbicide residues changed the soil environment and the bacterial preference. *Sphingomonas* quickly gained the dominant species status after maize planting, indicating that some strains of this genus may be involved in the process of cultivating soil bacterial communities in maize. Its relative abundance was higher in soil containing herbicide residues, leading to the assumption that there are strains of this genus that can degrade diphenyl ether herbicides. Various research results showed that environmental pollutants and microbial agents can alter microbial communities and biodiversity (Satapute et al., 2019; Giambò et al., 2022). However, the soil response to microbial preparations and the mechanism of action in soil rhizosphere are still unclear. Nevertheless, relying on modern molecular biotechnology and bioinformatics methods, it is possible to deeply explore the mechanism of action of microbial preparations.

In this study, the development and performance parameters of the solid degrading microbial agent were determined under laboratory conditions. Complex environmental factors represent a major obstacle to the production and application of solid microbial agents. Although this study applied multi-factor optimization for the development of solid agents (such as organic fertilizer mixing ratio, inoculum amount, and application mode), for practical applications, many factors still need to be considered for further optimization. At the same time, the mechanism of action of microbial agents in soil and rhizosphere environments needs to be further explored.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Author contributions

GZ designed the work, conducted the experiments, and wrote the manuscript. YT and HY participated in revising the manuscript. JL and DM conducted the experiments and analyzed the data. XH and RF guided the data analysis and revised the manuscript. All authors contributed to the study and approved the final submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1075930/full#supplementary-material

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Impact of carbendazim on cellular growth, defence system and plant growth promoting traits of *Priestia megaterium* ANCB-12 isolated from sugarcane rhizosphere

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Agrochemicals are consistently used in agricultural practices to protect plants from pathogens and ensure high crop production. However, their overconsumption and irregular use cause adverse impacts on soil flora and non-target beneficial microorganisms, ultimately causing a hazard to the ecosystem. Taking this into account, the present study was conducted to determine the high dosage of fungicide (carbendazim: CBZM) effects on the rhizobacteria survival, plant growth promoting trait and reactive oxygen species (ROS) scavenging antioxidant enzyme system. Thus, a multifarious plant growth promoting rhizobacteria (PGPR) isolate, ANCB-12, was obtained from the sugarcane rhizosphere through an enrichment technique. The taxonomic position of the isolated rhizobacteria was confirmed through 16S rRNA gene sequencing analysis as *Priestia megaterium* ANCB-12 (accession no. ON878101). Results showed that increasing concentrations of fungicide showed adverse effects on rhizobacterial cell growth and survival. In addition, cell visualization under a confocal laser scanning microscope (CLSM) revealed more oxidative stress damage in the form of ROS generation and cell membrane permeability. Furthermore, the increasing dose of CBZM gradually decreased the plant growth promoting activities of the rhizobacteria ANCB-12. For example, CBZM at a maximum 3,000 μg/ml concentration decreases the indole acetic acid (IAA) production by 91.6%, ACC deaminase by 92.3%, and siderophore production by 94.1%, respectively. Similarly, higher dose of fungicide enhanced the ROS toxicity by significantly ($p < 0.05$) modulating the stress-related antioxidant enzymatic biomarkers in *P. megaterium* ANCB-12. At a maximum 3,000 μg/ml CBZM concentration, the activity of superoxide

dismutase (SOD) declined by 82.3%, catalase (CAT) by 61.4%, glutathione peroxidase (GPX) by 76.1%, and glutathione reductase (GR) by 84.8%, respectively. The results of this study showed that higher doses of the fungicide carbendazim are toxic to the cells of plant-beneficial rhizobacteria. This suggests that a recommended dose of fungicide should be made to lessen its harmful effects.

KEYWORDS

carbendazim, PGPR, CLSM, reactive oxygen species, oxidative stress, antioxidant enzymes

Introduction

Sugarcane (*Saccharum officinarum* L.) is a commercially important crystal sugar and bioenergy-producing cash crop grown in tropical and subtropical regions around the world. China is the world's third largest sugarcane producer, which contributes to the economic prosperity of indigenous farmers (Sharma et al., 2021). However, sugarcane productivity was hampered due to diverse pathogen attacks. Among them, Pokkahboeng caused by *Fusarium verticillioides*, wilt caused by *Fusarium sacchari*, red rot caused by *Colletotrichum falcatum*, smut caused by *Sporisorium scitamineum*, and twisted leaves caused by *Phoma* sp. are re-emerging diseases of sugarcane. In China, these fungal diseases cause serious yield losses (about 5–50%) in commercial sugarcane production (Liu et al., 2017; Xu et al., 2019; Hossain et al., 2020). Most of these diseases of sugarcane have been associated with several other fungal diseases. To control the negative effects of these diseases, application of fungicides, particularly carbendazim (methyl 1H-benzimidazol-2-yl carbamate, $C_9H_9N_3O_2$), a broad-spectrum fungicide, has been widely used to protect sugarcane and other crops from fungal diseases (Shao and Zhang, 2017). Carbendazim (CBZM) has a huge global market worth over $200 million at the user level. Although some credit goes to CBZM application, the continuous application of fungicide reduces the affinity of this chemical fungicide as well as, through various mechanisms, the resistance also developed in targeted plant pathogens (Ma and Michailides, 2005; Martinez-Rossi et al., 2008). A field survey recently found approximately 15% of the CBZM-resistant *Fusarium* species complex in a chewing cane field in China (Xu et al., 2019). Due to insufficient knowledge and understanding of disease control, apart from the recommended dose (i.e., 0.1% CBZM), farmers extensive use the fungicide, resulted in serious health implications for soil fertility, non-targeted soil beneficial microorganisms, agriculture, the environment, water, air, and animal health (Pankaj et al., 2016a,b; Sharma et al., 2016; Xu et al., 2019; Bhatt et al., 2021). There is now overwhelming evidence that due to their long half-life (up to 12 months) and their relatively high stability, some of these organic compounds (OC) are classified as hazardous xenobiotics by the WHO (World Health Organization), which imposes a potential risk and could pollute every life form on the earth (Zhang et al., 2013; Bhatt et al., 2019; Mishra et al., 2021).

In an agriculture system, plants rely on beneficial soil microorganisms' processes, which include nutrient cycling and decomposition of organic matter. Any loss of soil microbial flora due to fungicide application could result in deteriorating soil health and lead to reduced field productivity. The repetitive use of fungicides or pesticides increases their persistence in the soil, which leads to affected microbial growth and performance (Gill and Garg, 2014; Al-Enazi et al., 2022). Reports are available highlighting the dose dependent effects of fungicides on plant growth promoting rhizobacteria (PGPR), i.e., with the increase of dose adverse effects of fungicide/pesticide on PGPR (Yang et al., 2011; Mundi et al., 2020). Recently, Shahid et al. (2021a) reported that treatments of various pesticides adversely affect the growth, morphology, viability, cellular respiration, EPS, and biofilm formation, as well as plant growth promoting activities, in different bacterial species. In addition, pesticides also induce oxidative stress by generating ROS (reactive oxygen species). ROS molecules, *viz.*, hydroxyl radical (^{-}OH), hydrogen peroxide (H_2O_2), superoxide radical (O_2^{-}), etc., lead to lipid peroxidation, protein denaturation, enzyme inactivation, metabolic disturbance, and alteration of the genetic materials, which finally affects the cellular integrity (Bernat et al., 2018; Shahid et al., 2021b). Thus, to neutralize the toxic effects of ROS molecules, living organisms produce antioxidant enzymatic machinery that is needed to evaluate the survival mechanisms of microbes. In addition, such a type of study is a pre-requisite to amending pesticide regulations in the near future.

Keeping in view the problem of fungicide toxicity to soil-beneficial plant growth promoting bacteria, the present study was intended to determine carbendazim toxicity on sugarcane rhizobacterial physiology, plant growth-promoting (PGP) activity, and the antioxidant enzyme defense system.

Materials and methods

Soil sampling, enrichment, and isolation of rhizobacteria

In the present study, randomly selected a total of five rhizosphere soil samples were collected from the fungicide applied sugarcane field near the Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China. From collected rhizospheric soil samples, stone, soil debris, and plant parts were removed and subjected to shade drying for 24–48 h. The composite sieved rhizospheric soil sample was enriched in mineral salt medium (MSM) amended with 500 μg/ml of CBZM (50% w/v of the active ingredient) for 96 h for the isolation of fungicide tolerant bacteria. After incubation of the fungicide enriched soil sample, the bacteria isolation was carried out by the serial dilution method on mineral salt agar plates supplemented with 500 μg/ml CBZM and incubated for 72–96 h at 28°C. After the incubation, a total of 18 dominant bacterial colonies (ANCB-1 to ANCB-18) were screened and conserved in 50% glycerol at −80°C for further use.

Intrinsic tolerance of isolated rhizobacteria against carbendazim

The intrinsic fungicide tolerance ability of the obtained 18 rhizobacterial isolates was determined through minimal salt agar (MSA) medium (g/L: KH_2PO_4 1.0 g, K_2HPO_4 1.0 g, NH_4NO_3 1.0 g, $MgSO_4.7H_2O$ 0.2 g, $FeSO_4.7H_2O$ 0.01 g, $CaCl_2.2H_2O$ 0.02 g, agar powder 18.0 g, and pH 6.5). Briefly, actively growing fresh rhizobacterial cultures (10^8 cells/ml) were spot inoculated on MSA plates containing varying concentration of fungicide CBZM, *viz.*, 500, 1,000, 1,500, 2,000, 2,500, and 3,000 μg/ml. After the inoculation, all the inoculated medium plates were incubated at 28 ± 2°C for 48–72 h. Followed by highest fungicide, tolerant rhizobacteria was selected for further study.

Morphological, biochemical, and metabolic characterization of the rhizobacteria

The selected bacteria isolate ANCB-12 showed a higher level of CBZM tolerance was morphologically and biochemically characterized by following Bergey's Manual of Determinative Bacteriology (Tindall et al., 2010). Furthermore, isolate ANCB-12 was metabolically characterized by carbon source utilization pattern using BIOLOG GNIII MicroPlate™ (Biolog, Inc., Hayward, CA, USA) following the manufacturer's instruction.

Molecular identification by phylogenetic analysis

By adopting the standard protocol of Pospiech and Neumann (1995), genomic DNA was extracted from the actively grown rhizobacterial culture. To amplify the 16S rRNA gene, the universal 16S rRNA gene primers PA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and PH (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used (Sharma et al., 2015). The 50 μl PCR reaction mixture contains 50 ng of DNA template, 10× reaction buffer, 2.5 mM dNTPs, 10 pM of each primer, and 3 U of *Taq* polymerase. PCR condition in the thermal cycler was as follows: at 94°C initial denaturations for 4 min, followed by denaturation at 94°C for 45 s of 35 cycles, annealing at 51°C for 1.5 min, and at 72°C elongations for 90 s, and 72°C final extensions for 7 min. The Amplified 16S rRNA gene PCR product was purified using the BioFlux PCR purification kit (China) by following the manufacturer's protocol. The purified PCR product was sequenced and analyzed for percent (%) similarities with the available sequences in the GenBank database at the NCBI website. The nucleotide sequence of the bacteria ANCB-12 was deposited in the GenBank sequence database (ON878101). Based on evolutionary distances and Jukes–Cantor coefficient calculation, a neighbor-joining (NJ) phylogenetic tree was constructed through MEGA-X software (Sharma et al., 2019).

Growth kinetics study of selected isolate

Among the all tested bacterial isolates, isolate ANCB-12 showed the highest tolerance of carbendazim and was selected for growth kinetic study. The selected isolate was inoculated in liquid MSM containing 0, 500, 1,000, 2,000, and 3,000 μg/ml concentration of CBZM and incubated at 150 rpm for 60 h at 28 ± 2°C. To make the growth kinetic curve, the optical density at 600 nm was taken every 12 h.

Cell viability under carbendazim stress

Cell viability in terms of CFU count was carried out by spreading the 100 μl aliquots of 48 h grown culture of each CBZM stress level. CFU was counted after 48 h of incubation at 28 ± 2°C. The CFU was calculated as:

$$\text{Colony forming unit (CFU)} = \text{Number of colonies} \times \text{dilution factor/volume plated.}$$

Bacterial cell membrane injury: A confocal microscopic analysis

The fungicide-induced bacterial membrane integrity and mortality were determined by confocal laser scanning microscopy (Xia et al., 2022). In brief, bacteria culture was inoculated in MSM liquid medium supplemented with 3,000 μg/ml CBZM fungicide and incubated in a rotatory shaker for 48 h at 28°C. After the incubation of the treated and un-treated cultures, the pellet was collected by centrifugation and 2–3 times washed with PBS (phosphate buffer saline). In 100 μl of fungicide treated and un-treated bacterial suspension, 5 μl of propidium iodide (PI) and 5 μl of acridine orange (AO) were added and incubated for 10 min at 28 ± 2°C. Then the samples were centrifuged at 5,000 rpm for 10 min to remove the unbound dyes, and the pellets were resuspended in PBS buffer. Samples were transferred to glass slides and observed under confocal laser scanning microscope (CLSM) (Leica Confocal Microscope, Germany).

Effect of fungicide on plant growth-promoting attributes of the rhizobacteria

All the PGP activities of CBZM fungicide treated and un-treated rhizobacteria were assayed by using a standard method as given in the below section.

Indole acetic acid production

The indole acetic acid (IAA) production of treated and untreated bacterial cultures was determined by following the method of Bric et al. (1991). In 2 ml of cell-free rhizobacterial culture, perchloric acid and Salkowski reagent were added and incubated for 30 min at 28°C under dark conditions. IAA was quantified by taking absorbance at 530 nm using a UV-VIS spectrophotometer (Shimadzu, Model UV-1601) spectrophotometer and the amount of IAA production was calculated by using the standard curve of IAA (Bano and Musarrat, 2003).

Phosphate solubilization

Phosphate (P) solubilization activity was tested on National Botanical Research Institute Phosphate (NBRIP) medium (Mehta and Nautiyal, 2001). Fungicide treated and un-treated rhizobacterial cultures were spot inoculated on NBRIP agar plates and incubated for 24–96 h at 28 ± 2°C. The appearance of a clear halo zone around the bacterial growth indicated the positive results of P-solubilization. Quantitative estimation of P solubilization was performed by the method described by Fiske and Subbarow (1925) using NBRIP broth (Mehta and Nautiyal, 2001).

Activity of ACC deaminase

Activity of ACC deaminase (ACCD) activity was analyzed by following the method of Penrose and Glick (2003). Rhizobacterial cultures were grown in a DF salt minimal medium supplemented with 3 mM ACC or 0.1 M $(NH_4)_2SO_4$ as the sole nitrogen source. After incubation at 28 ± 2°C for 96 h, the bacterial growth on the DF salt minimal agar plate amended with 3 mM ACC showed positive results for ACC deaminase activity. For quantitative estimation of ACCD activity, rhizobacterial cultures grown in liquid minimal salt medium with or without 3 mM ACC were centrifuged, and collected cells were washed 2–3 times with Tris–HCl (0.1 M, pH 7.5), followed by in cells, 1 ml of Tris–HCl (0.1 M, pH 8.5) was added. A further 5% toluene solution was added for labilization.

Siderophore production

Siderophore production efficiency was assessed by spot inoculation of freshly grown rhizobacterial culture on a CAS (Chrome azurol S) agar medium plate. After the incubation at 28 ± 2°C for 24–72 h, the appearance of a yellow-to-orange color zone around the rhizobacteria colony is an indication of positive results of the siderophore production (Schwyn and Neilands, 1987) method. Through the CAS-shuttle assay (Payne, 1994), quantitative estimation of siderophore production was determined in MM9 medium (amended glucose 1% w/v). The loss of blue color of the reaction mixture due to the removal of the iron from the dye complex indicates the siderophore present in the supernatant. The quantity of siderophore production was determined by taking OD at 630 nm and quantifying it using the formula:

$$\%\ \text{Unit of siderophore} = \text{Ar} - \text{As}/\text{Ar} \times 100$$

where Ar denotes the reference (CAS reagent) OD at 630 nm and As denotes the test sample OD at 630 nm.

Ammonia production

The ammonia production of fungicide treated and un-treated cultures was compared by following the standard protocol of Cappuccino and Sherman (1992). A total of 20 μl of actively grown rhizobacterial culture was inoculated in 10 ml of freshly prepared peptone water and shaken for 24–48 h at 28 ± 2°C followed by the addition of 0.5 ml of Nessler's reagent. The appearance of a dark yellow to brown color indicates positive ammonia production.

Protein estimation

The total protein content of fungicide treated and un-treated rhizobacterial cells was estimated by following the standard method of Bradford (1976). The amount of protein in the rhizobacterial cells was determined spectrophotometrically at 595 nm, and protein content was calculated by using the standard curve of bovine serum albumin (BSA).

Determination of lipid peroxidation

The lipid peroxidation (LPO) was analyzed by measuring the end product malondialdehyde (MDA), according to the method of Heath and Packer (1968). Finally, the optical density of the obtained supernatant was measured at 532 nm (all lipids) and 600 nm (all lipids except for MDA), and the amount of MDA was expressed in nmol/ml.

Antioxidant enzymes assay

Freshly grown fungicide-treated and un-treated rhizobacterial cultures were centrifuged at 10,000 rpm for 10 min, and cell pellets were harvested. In the collected cell pellet, Tris–HCl was added to obtain a homogenous bacterial suspension. Furthermore, rhizobacterial cell suspensions were sonicated, and then sonicated cell suspensions were centrifuged (at 12,000 rpm for 10 min) to eliminate the cell debris. The supernatant containing cellular extract was used for the assay of antioxidant enzyme activity (Srivastava et al., 2017). Antioxidant enzymes like superoxide dismutase (SOD) (ADS-W-KY011), catalase (CAT) (ADS-W-KY002), glutathione peroxidase (GPX) (ADS-W-G003), ascorbate peroxidase (APX) (ADS-W-VC005), and glutathione reductase (GR) (ADS-W-FM029), activities were measured using an enzyme-linked immune sorbent assay (ELISA) kit (Jiangsu, China) by following the manufacturer's protocol.

Statistical analysis

Data obtained from this study are the mean of three individual replications ± standard error. SPSS (ver. 16.0) statistical software (IBM, Armonk, NY, USA) was used to analyze the data through ANOVA (analysis of variance) and DMRT (Duncan's multiple range test) at $p \leq 0.05$.

Results and discussion

Isolation and characterization of the rhizobacterial isolate

In the present study, a total of 18 dominant rhizobacterial colonies, i.e., from ANCB-1 to ANCB-18, were obtained from the collected rhizosphere soil sample of sugarcane on MSA medium plate supplemented with 500 μg/ml carbendazim. On the basis of the initial fungicide stress and plant growth promoting traits assay, a multiple PGPR bacterial isolate ANCB-12, which showed its growth at a higher carbendazim concentration, was selected for further study. The selected isolate ANCB-12 was subjected to phenotypic, biochemical, and metabolic characterization. The obtained rhizobacterial strain ANCB-12 was a Gram-positive, motile, rod-shaped, endospore-forming rhizobacteria based on phenotypic characteristics. The biochemical characteristics demonstrated that isolate ANCB-12 showed positive results for citrate utilization, catalase, methyl red, oxidase, indole, lysine utilization, nitrate reduction, and ornithine utilization but was found negative for the Ortho-Nitrophenyl-β-galactoside (ONGC), Voges–Proskauer test, arginine, phenylalanine deaminase, and H_2S production (**Table 1**). The great metabolic flexibility of the bacterial isolate allows them to inhabit variable environments such as those reported in the present study. Similarly, by referring to Bergey's Manual of Systematic Bacteriology Al-Enazi et al. (2022) characterized pesticide-tolerant *Pseudomonas* sp. from the rhizosphere soil of *Vigna radiata* (L.).

Carbon utilization pattern

Soil microorganisms require some basic nutrients (such as carbon and nitrogen) for their growth and survival and to maintain their metabolic functions. Thus, microorganisms' ferment or metabolize a wide range of simple sugars, complex carbohydrates, and amino acids for their growth and energy. The utilization patterns of carbon and amino acid sources may reveal information about the microbial biochemical pathways for various biological activities as well as for comparing the various microorganisms. With this account, in the present study, the metabolic profile through the C-utilization pattern of the isolate ANCB-12 was analyzed through "BIOLOG phenotype

TABLE 1 Morphological and biochemical characterization of the rhizobacteria ANCB-12.

Tests	ANCB-12
Gram reaction	+
Shape	R
pH	6.0–8.5
NaCl tolerance	Up to 8%
Motility	+
Endospore	+
Voges–Proskauer's	–
Citrate	+
Methyl red	+
ONPG	–
Nitrate reduction	+
Catalase	+
Oxidase	+
Arginine	–
Lysine utilization	+
Ornithine utilization	+
Phenylalanine deamination	–
H2S production	–

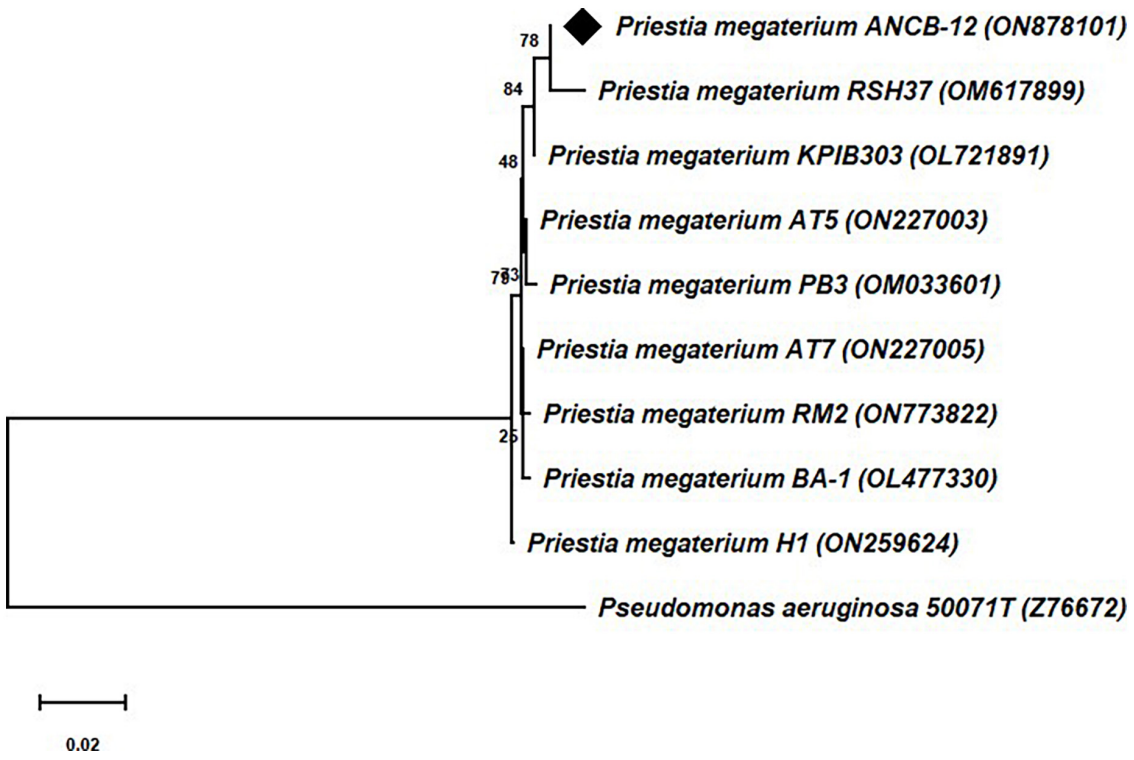

FIGURE 1
Phylogenetic tree of 16S rRNA gene sequence of the isolate ANCB-12 with similar bacterial sequences of the NCBI database. The tree was constructed by using the neighbor joining method with 1,000 bootstrap re-samplings. *Pseudomonas aeruginosa* was taken as an out group.

micro-array^{TM} GNIII-carbon plate." The results of the BIOLOG assay revealed that isolate ANCB-12 was positive for 19 sugars, 2 reducing sugars, chemically sensitive for 13 substrates, 6 amino acids, 7 hexose-PO_4, 7 hexose acids, 10 carboxylic acids, esters, and fatty acids (**Supplementary Table 1**). The results of carbon utilization through BIOLOG assay showed that the tested rhizobacterial isolate was metabolically active. The results are in line with the study of Li et al. (2017) and Singh et al. (2020) who used BIOLOG based C-utilization assay to the exploration of metabolically active rhizobacteria of sugarcane.

Molecular identification by phylogenetic analysis

Molecular identification of the rhizobacteria was carried out through structural 16S rRNA gene sequencing analysis. The comparison of the obtained nearly ~1.5 kb 16S rRNA gene sequences through BLAST search with the available bacterial sequences in the NCBI Genbank public databases indicates that the isolate ANCB-12 is closely related to *Priestia megaterium* with 100% sequence similarity. The obtained 16S rRNA gene nucleotide sequence of the rhizobacterial isolate ANCB-12 was submitted to GenBank with accession number ON878101.

Figure 1 shows a phylogenetic tree made from the isolate ANCB-12 and similar bacterial 16S RNA gene sequences of NCBI database. The tree was made using the neighbor-joining method and 1,000 bootstrap samples.

16S rRNA gene sequencing analysis is one of the most widely used methods for bacterial identification and phylogeny/taxonomy analysis (Jamali et al., 2020). In accordance with our results, Sharma et al. (2019, 2021) have also isolated rhizospheric bacteria from rhizospheres of chickpea and sugarcane from different agro-climatic regions, and molecularly identified them through 16S RNA partial gene sequencing analysis.

Rhizobacterial growth kinetics and viability analysis

To control the plants' diseases and to enhance agriculture production, an overdose of pesticides and fungicides imposes a negative effect on non-targeted beneficial microorganisms. Thus, in the present study, the effects of carbendazim fungicide on the growth of 18 isolated rhizobacterial (ANCB-1 to ANCB-18) were evaluated. At the initial screening level, all 18 isolates were spot inoculated on the MSA medium plates stressed

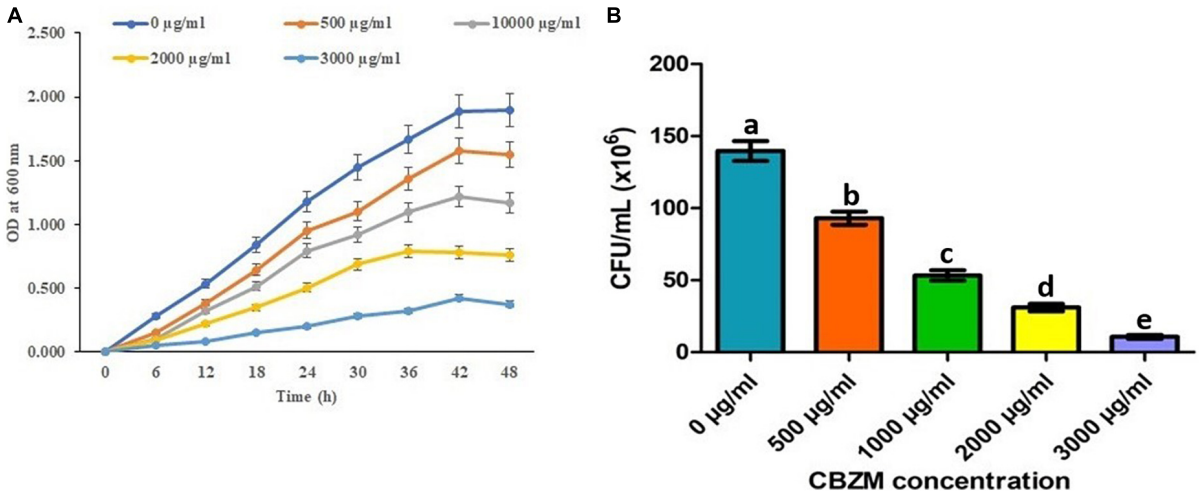

FIGURE 2
Effect of different concentrations of CBZM on rhizobacteria ANCB-12 growth **(A)** and variability in terms of CFU count **(B)** up to 48 h time duration. The data represent the mean ± SEM (n = 3) of three replicates. The error bars display the standard error mean (SEM). a–e indicate the statistically significant value. Letters on each bar denote significant variances ($p \leq 0.05$) according to the DMRT test.

by adding different concentrations of fungicide ranging from 0, 0.05% (500 µg/ml), 0.1% (1,000 µg/ml, recommended dose), 0.2% (2,000 µg/ml), and 0.3% (3,000 µg/ml). After the incubation time, results showed that all the tested rhizobacteria did not show growth. Only one isolate, ANCB-12, was found to be significantly tolerant to higher fungicide stress, so isolate ANCB-12 was chosen for a growth kinetic study in a liquid medium with varying concentrations of carbendazim (**Figure 2A**). Generally, pesticides can change the growth of tolerant or degrading bacteria (Peters et al., 2014). In the present study, the growth of the isolate ANCB-12 revealed a distinct pattern of growth when exposed to increasing concentrations of fungicide. Results in the form of a growth curve showed that in an un-treated medium, the isolate revealed luxurious growth (**Figure 2A**), while as the concentration of fungicide increased, the growth was sluggish as an adaptation period and a long lag phase was observed before reaching exponential growth. Isolate tolerates up to 0.25% fungicide concentration, whereas compared to the lower fungicide concentration, the higher concentration had an adverse toxic effect on bacterial life. Results showed the maximum concentration (0.3%) of fungicide has a killing effect on bacterial cell CFU. Lower growth and CFU with increasing concentration of fungicide may be due to the intake of fungicide, which influences the damage of the cell membrane and metabolic process of the bacteria (**Figure 2B**; Reshma et al., 2018). Tolerance to fungicides is assumed to be a distinctive characteristic among soil-living beneficial microorganisms, which are led by unique physiological, metabolic, and genetic features (Khan et al., 2020). In 2012, from the mustard rhizosphere, Ahemad and Khan isolated *Pseudomonas putida* PS9 which exhibited a different level of tolerance to various fungicides ranging from 1,400 to 3,200 µg ml^{-1} concentrations. Similarly, a *Bacillus subtilis* BC8 strain isolated from the cabbage rhizosphere soil showed a different growth pattern under different concentrations of pesticides and fungicides ranging from 0 to 3,200 µg ml^{-1} (Shahid et al., 2019). The results of the current study are also in accordance with the observation of Nagaraju et al. (2017), who reported the lethal effect of fungicides, herbicides, and zinc solubilizing bacteria.

Impact of fungicide on rhizobacterial cell membrane

In the present study, an advanced confocal laser scanning microscopy (CLSM) technique was used to analyze the toxic effects of the fungicide CBZM on rhizobacterial membrane permeability. Results of CLSM analysis showed that as the fungicide treatment level increased, a huge number of red fluorescence releasing rhizobacterial cells were observed (**Figure 3**). This indicated the highly toxic effect on the viability of rhizobacterial cells, which led to cell damage over controlled un-treated cells (**Figure 3**). However, no red-colored cells were detected in CLSM images of control CBZM un-treated cells (**Figure 3A**). This is because toxic fungicides increase cell permeability, allowing dyes to enter the cell and bind with intracellular components and nucleic acids. The results of the present study are in line with the study of Kudoyarova et al. (2017), who differentiated between dead and live cells of *B. subtilis* strain BC8 in CLSM analysis. Similar to our results, similar observations were obtained in the studies of Shahid et al. (2019, 2021a,b), who reported an

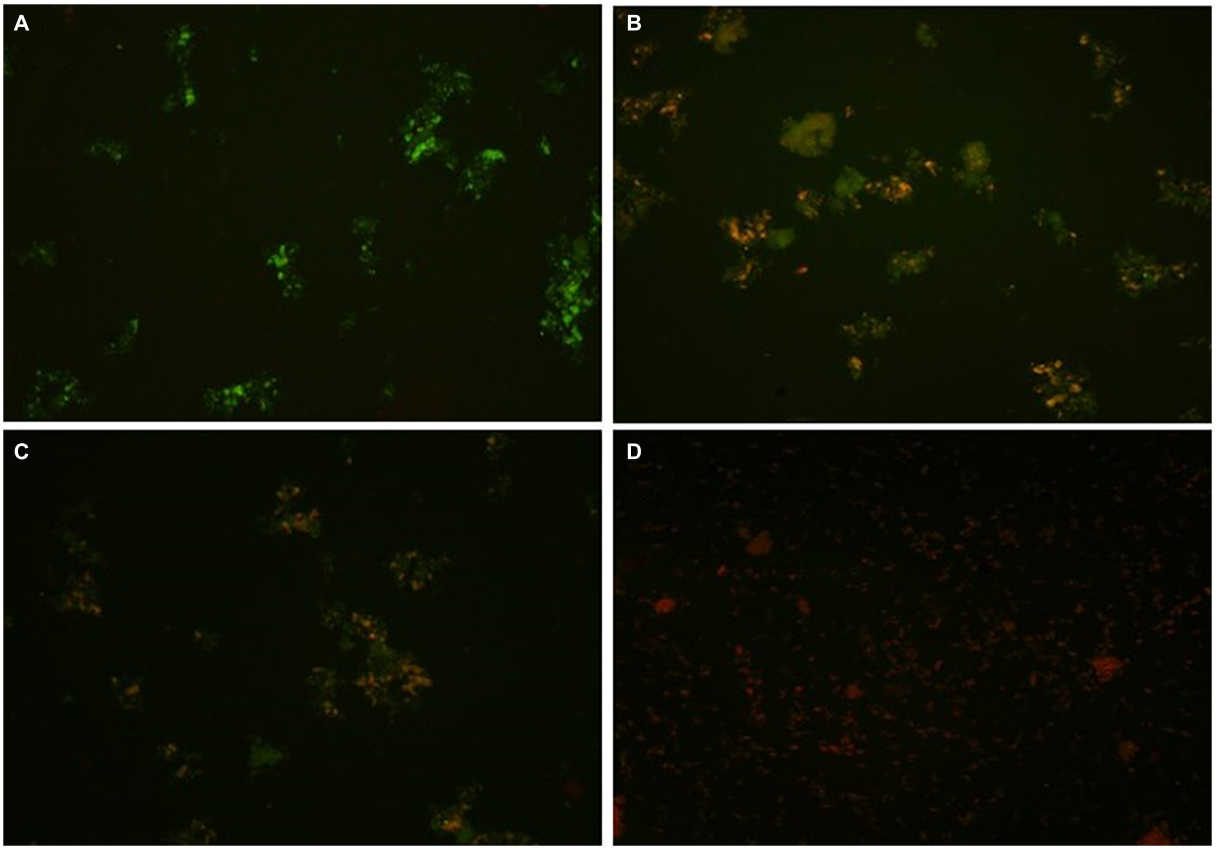

FIGURE 3
Confocal laser scanning microscopy (CLSM) based membrane permeability analysis of isolate ANCB-12 under different CBZM concentrations. **(A)** Untreated cell, **(B)** 1,000 μg/ml CBZM treated cell, **(C)** 2,000 μg/ml CBZM treated cell, and **(D)** 3,000 μg/ml CBZM treated cell.

increasing number of dead cells of *Bradyrhizobium japonicum, Pseudomonas* sp., and *Enterobacter cloacae* after treatment with increasing concentrations of fungicides.

Impact of carbendazim stress on plant growth promoting attributes of ANCB-12 isolate

Indole-3-acetic acids

Plant growth promoting rhizobacteria producing IAA plays an imperative role in cell division and differentiation, especially in root development and overall plant growth (Grover et al., 2021). We analyzed the IAA producing ability of ANCB-12 under CBZM treatment conditions. Under controlled condition (in the absence of fungicide), isolate ANCB-12 secreted a considerable amount of IAA, 88.8 μg/ml (T-1). However, it was noticed that the IAA production was significantly ($p \leq 0.05$) reduced with increasing concentrations of fungicide. Results showed that the increasing concentrations of fungicide reduced the synthesis of IAA by 17.5, 58.8, and maximally 91.6% at 1,000 μg/ml (T-2), 2,000 μg/ml (T-3), and 3,000 μg/ml (T-4) fungicide levels, respectively, over the un-treated control (**Figure 4A**). Higher dosage of pesticides may bind with cellular molecules and affect the metabolism, which may be the cause of reduced IAA. A similar result was observed in *Paenibacillus* sp. treated with organochlorine pesticides (Mundi et al., 2020).

Phosphate solubilization

Phosphate solubilization is another important feature of soil bacteria, by which soil microbes provide the soluble form of phosphate to the plant. The release of low molecular weight organic acids by phosphate solubilizing bacteria (PSB) converts insoluble phosphate to soluble phosphate (Alori et al., 2017). In the present study, quantitative estimation of phosphate-solubilization of ANCB-12 isolate under graded concentrations of carbendazim was evaluated by using the phosphate-solubilizing specific standardized NBRIP liquid medium (**Figure 4B**). Results of quantitative estimation revealed that a considerable amount of TCP (340.9 μg/ml) was solubilized in fungicide deficient liquid medium (controlled condition: T-1) by the rhizobacterial isolate ANCB-12. Results

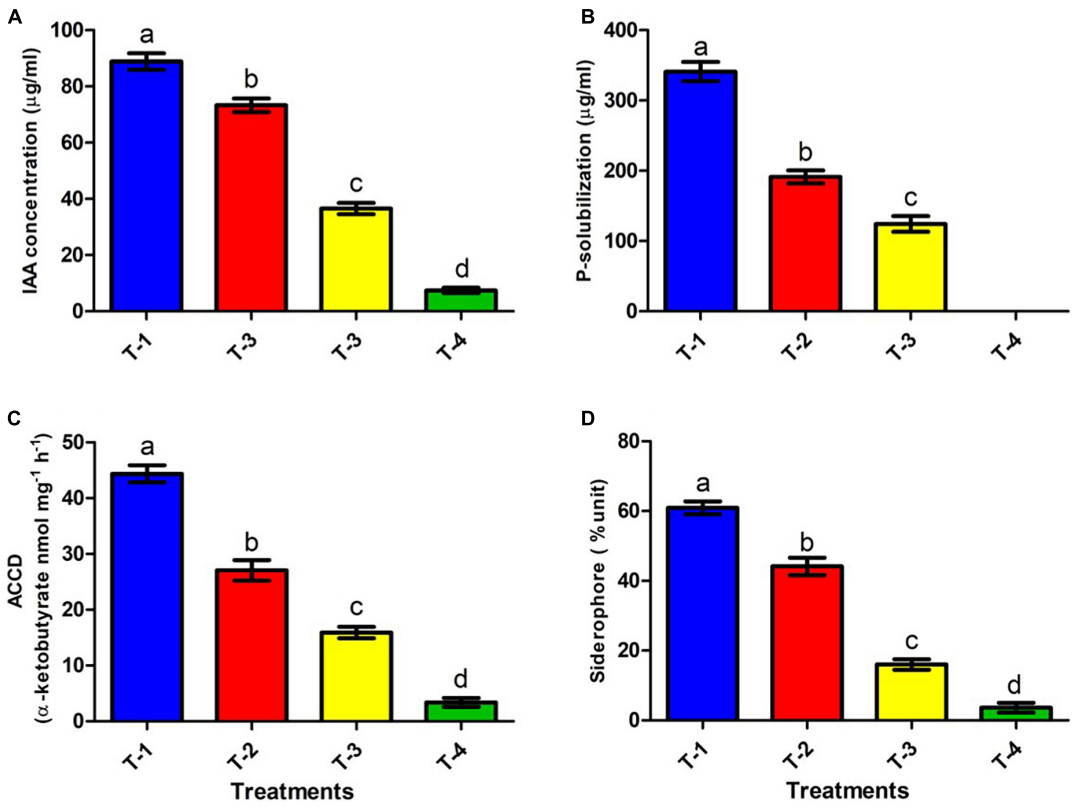

FIGURE 4
Effect of CBZM stress on plant growth promoting attributes of rhizobacteria isolates ANCB-12. (T-1) No CBZM concentration, (T-2) 1,000 µg/ml CBZM, (T-3) 2,000 µg/ml CBZM, and (T-4) 3,000 µg/ml CBZM. **(A)** IAA production, **(B)** phosphate solubilization, **(C)** ACC deaminase activity and **(D)** siderophore production. The data represent the mean ± SEM (n = 3) of three replicates. The error bars display the standard error mean (SEM). Letters on each bar denote significant variances ($p \leq 0.05$) according to the DMRT test.

showed that with increasing concentration of CBZM, i.e., at 1,000 µg/ml (T-2) and 2,000 µg/ml (T-3) a decline of P-solubilizing activity by 43.8 and 63.5%, respectively, was observed over control (T-1) (**Figure 4B**). However, at a higher 3,000 µg/ml (T-4) CBZM concentration, there was no P-solubilizing activity observed. The results are in contrast with other findings of earlier research where *Pseudomonas fluorescens* showed a declining trend of TCP solubilization under pesticide treatment (Al-Enazi et al., 2022).

ACC deaminase activity

The rhizobacterial ACC deaminase enzyme is a pyridoxal phosphate dependent enzyme, which by reducing ethylene content, protect and promotes plant growth under various biotic and abiotic conditions including under fungicide stress. (Singh et al., 2022). The isolate *P. megaterium* ANCB-12, grown under control conditions, produced a significant amount of ACC deaminase by converting 44.3 α-ketobutyrate nmol mg^{-1} protein h^{-1}, similar to IAA production. However, the amount of ACCD was reduced with an increasing concentration of fungicide (**Figure 4C**). Results showed that the amount of ACCD was reduced by 38.9, 64.1, and a maximum of 92.3 at 1,000 µg/ml (T-2), 2,000 µg/ml (T-3), and 3,000 µg/ml (T-4) fungicide levels, respectively, over unstressed control (T-1). Several studies (Syed et al., 2021; Al-Enazi et al., 2022) have found that fungicides and heavy metals reduce the ACC deaminase activity of soil bacteria in a similar way.

Siderophore production

Iron is an essential molecule for a plant's chlorophyll generation, photosynthesis, and development of resistance against pathogens. While in bacteria, iron is crucial for physiology, metabolism, DNA replication, regulatory proteins, transcription, energy production, and plant microbial interaction. A deficiency of iron results in the disturbance of all these processes. Under such a situation, PGPR produces low-molecular-weight substances called siderophores that efficiently chelate the ferric ion (Fe^{3+}) and act as carriers for the entry of Fe (III) into the cell (Ferreira et al., 2019). In the present study, the siderophore producing ability of isolate ANCB-12 was determined on CAS broth amended with variable rates

of fungicide (Figure 4D). Results showed that, like IAA and P-solubilizing activities, the capacity of the isolate ANCB-12 for siderophore production at normal controlled conditions (T-1) was 60.9%. Furthermore, results showed that the amount of siderophore production was gradually decreased by 28.2, 73.9, and 94.1% at 1,000 μg/ml (T-2), 2,000 μg/ml (T-3), and 3,000 μg/ml (T-4) fungicide levels, respectively, over the un-treated control (Figure 4D). The results of the present study are in accordance with the observation of Kumar et al. (2019), who also observed a declining trend of siderophore under pesticide treatment.

Ammonia production

Ammonia production by PGPR is beneficial for supplying nitrogen to their host plants and promoting growth (Marques et al., 2010; dos Santos et al., 2020). In our study, *P. megaterium*

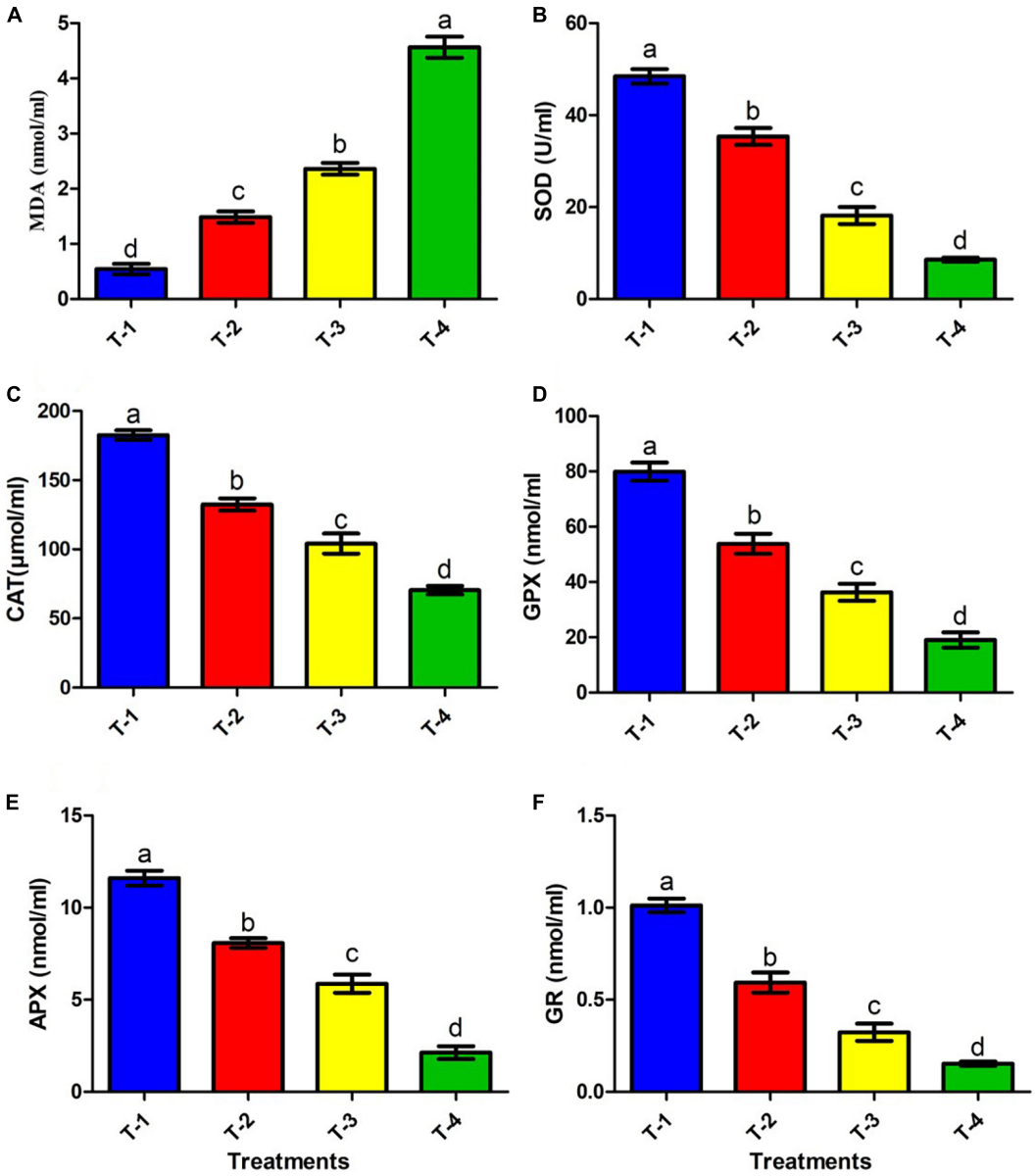

FIGURE 5
Effect of CBZM stress on antioxidant enzyme activities of rhizobacteria isolates ANCB-12. (T-1) No CBZM concentration, (T-2) 1,000 μg/ml CBZM, (T-3) 2,000 μg/ml CBZM, and (T-4) 3,000 μg/ml CBZM. **(A)** MDA content, **(B)** SOD activity, **(C)** CAT activity, **(D)** GPX activity, **(E)** APX activity and **(F)** GR activity. The data represent the mean ± SEM (n = 3) of three replicates. The error bars display the standard error mean (SEM). Letters on each bar denote significant variances ($p \leq 0.05$) according to the DMRT test.

ANCB-12's ability to make ammonia was not much affected by the higher levels of fungicide.

Lipid peroxidation (LPO)

Bacterial membrane integrity in terms of LPO due to stress conditions is a major stress marker (Heath and Packer, 1968). Therefore, considering this fact, in the present study, the membrane integrity of the isolate ANCB-12 under fungicide CBZM stress was further analyzed by MDA content. Results showed that MDA levels of the isolate ANCB-12 was 0.54 nmol/ml under control conditions (T-1) without any fungicide stress (**Figure 5A**). However, with increasing fungicide concentration, the cellular toxicity increased, which was evident by increasing MDA levels (**Figure 5A**). CBZM stress showed severe cellular toxicity and MDA content was increased to 1.48, 2.36, and 4.56 nmol/ml at 1,000 (T-2), 2,000 (T-3), and 3,000 μg/ml (T-4) CBZM concentration treatments, respectively. Results demonstrated that the levels nearly doubled with increasing fungicide treatments ($p < 0.05$; **Figure 5A**). The generation of MDA is evidence of oxidative stress within the cells. The findings are consistent with those of Shahid and Khan (2018), who found an increase in MDA levels in *B. subtilis* under CBZM and kitazin stress. In a recent study, Rovida et al. (2021) also found that herbicide-treated *Pseudomonas* sp. had a higher level of MDA.

Effect of carbendazim on antioxidant enzymes activities

Antioxidant enzymes such as SOD, CAT, GPX, and GR have been found in almost all cellular compartments and play

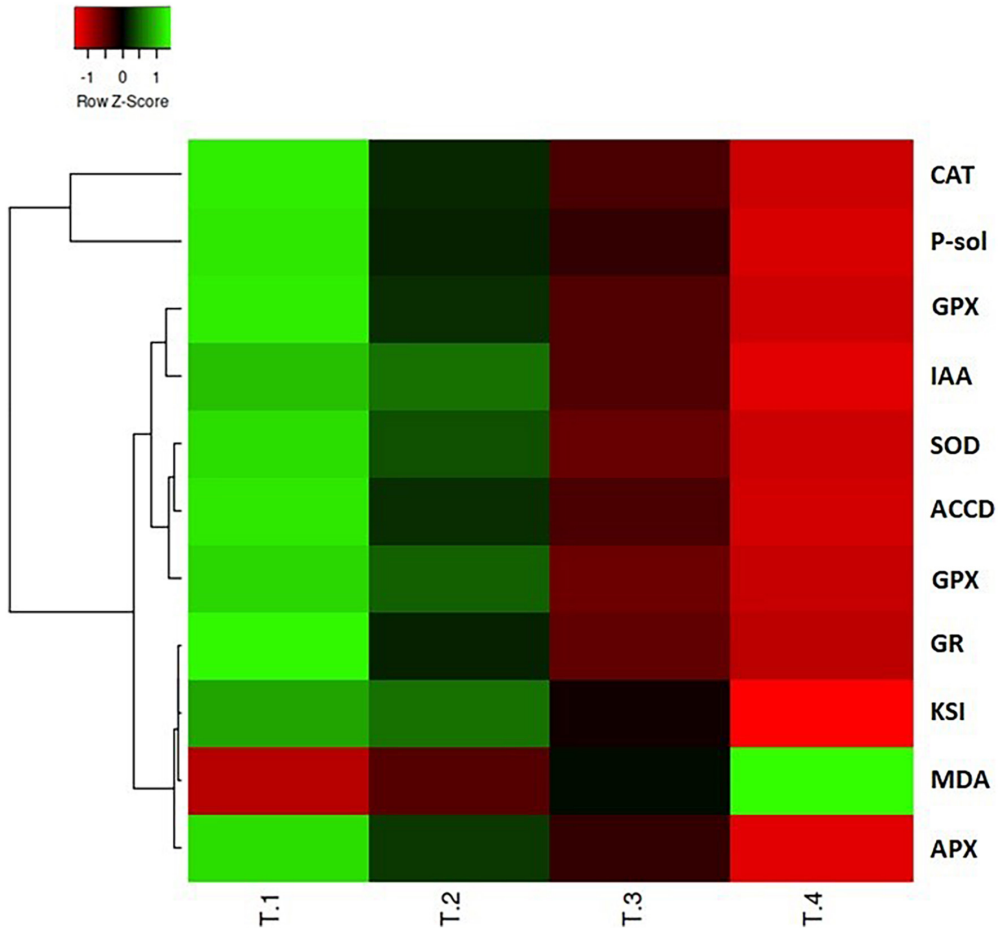

FIGURE 6
Heatmap multivariate data analysis representing the responses of various variables of PGP attributes and antioxidant enzyme defense system against the different treatments of CBZM applied. T-1: control un-treated cell; T-2: recommended 1,000 μg/ml CBZM; T-3: double dose, i.e., 2,000 μg/ml CBZM; and T-4: triple dose, i.e., 3,000 μg/ml CBZM. IAA, indole acetic acid; P-sol, phosphate solubilization; ACCD, ACC deaminase; Sid, siderophore; KSI, phosphate solubilization index; CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; APX, ascorbate peroxidase, polyphenol oxidase; GR, glutathione reductase; MDA, malondialdehyde.

a significant role in the neutralization or detoxification of toxic ROS molecules. SOD is an enzyme that is activated by ROS generation and the breakdown of singlet oxygen radical ($^1O_2^-$) or superoxide radical ($^*O_2$) into H_2O_2 and O_2 (Hasanuzzaman et al., 2020). However, this SOD induced end product H_2O_2 retains its potential to induce cell damage by conversion of H_2O_2 into hydroxyl radical (*OH). Thus subsequently, CAT, APX, and GPX detoxifies the H_2O_2 into H_2O and O_2, as well as inhibited LPO and protect cell damage (Sharma et al., 2012; Ighodaro and Akinloye, 2018). On the other hand, GR kept the intracellular glutathione pool (GSH) in a reduced state, which acts as a direct and indirect antioxidant for scavenging ROS. In the present study, we assessed the effects of different doses of fungicide CBZM on the activities of antioxidant enzymes, which are directly related to the detoxification of ROS molecules (**Figure 5**). Results showed that the addition of CBZM causes oxidative stress in the ANCB-12 rhizobacterial strain. SOD activity under non-stress conditions (0 μg/ml: T-1) was maximal (48.4 U/ml). CBZM stress gradually reduces SOD activity by 26.9, 62.5, and 82.3% at the recommended doses of T-2 (1,000 μg/ml), T-3 (double dose: 2,000 μg/ml), and T-4 (triple dose: 3,000 μg/ml) levels of CBZM (**Figure 5B**). Similarly, CAT activity was highest at normal conditions, i.e., 182.6 μmol/ml, which was significantly decreased by 27.4, 42.9, and 61.4% when exposing them to T-2, T-3, and T-4 CBZM stress concentrations, respectively, over non-CBZM stressed control (**Figure 5C**). Similar to SOD and CAT activities, GPX activity also decreased with an increasing dose of fungicide. Results showed that GPX activity maximally declined by 76.1% when exclusively exposed to the highest stress of CBZM (T-4: 3,000 μg/ml), followed by 54.5 and 32.6% at T-3 and T-4 CBZM stress levels, respectively, over control (T-1; 0 μg/ml CBZM) (**Figure 5D**). Furthermore, similar trends were also observed in APX activity, where activity maximally decreased by 81.6% at T-4 treatment of CBZM concentration, followed by 49.3 and 30.3% decreases under T-3 and T-2 CBZM stress conditions, respectively, over the non-stress control (T-1) condition (**Figure 5E**). In addition, results showed that the GR activity of bacterial cells decreased by 84.8, 68.0, and 41.3% at T-4, T-3, and T-2 CBZM stress conditions, respectively, as compared to the T-1 non-stress level of CBZM (**Figure 5F**). Overall, the results of the present study showed that with increasing fungicide stress, all the ROS scavenging antioxidants SOD, CAT, GPX, APX, and GR enzyme activity were gradually downregulated. The decrease in the activities of these enzymes is related to the increase in the free movement of H_2O_2, leading to increased cell permeability, LPO (MDA), and disruption of nuclear materials, which causes direct damage to the cell. These results are directly related to our growth kinetics, CLMS analysis, and increased level of LPO (MDA) under a gradient dose of fungicide. Similar damaging effects on antioxidant enzymes were observed with an increasing dose of pesticide stress by Shahid and Khan (2018). Our observations are also supported by similar results in *Pseudomonas* spp., *Enterobacter asburiae*, and *Mesorhizobium ciceri* under different pesticide stress conditions (Martins et al., 2011; Rovida et al., 2021; Shahid et al., 2021a).

Heatmap of Pearson's correlation analysis

Heatmap analysis revealed that the plant growth promoting attributes, LPO, and antioxidant enzyme variables displayed differential responses in the integrated heatmap against the various dosage of carbendazim fungicide treatments (**Figure 6**). The results of heatmap multivariance analysis clearly demonstrate that CBZM stress imposed adverse effects on all the tested functional and metabolic attributes of the beneficial rhizobacteria.

Conclusion

In conclusion, the results of the present study stated that an overdose of CBZM significantly reduced the growth of a PGPR strain *P. megaterium* ANCB-12, cell membrane disintegration, and beneficial plant growth promoting metabolic substances. Furthermore, increasing the concentration of the fungicide CBZM altered the defense system of ROS scavenging antioxidant enzymes, which is essential for the survival of bacterial cells under stress conditions. These findings pointed out the negative effect of high dosage of CBZM on plant beneficial bacteria present into the soil. Along with this, an eco-friendly alternative such as biofungicide needs to be developed and use in the integrative pest management (IPM) model for sustainable agriculture production.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary material**.

Author contributions

AS and Y-RL conceptualized the idea and designed the experiments. AS executed all the laboratory experiments, analyzed the data, and wrote the manuscript. X-PS contributed to resource management. AV, RS, PS, and D-JG contributed to software and necessary material. AV, SG, KV, and Y-RL critically revised the manuscript. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1005942/full#supplementary-material

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Expression, characterization, and immobilization of a novel SGNH esterase Est882 and its potential for pyrethroid degradation

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The widely-used pyrethroid pesticides have attracted public attention because of their potentials to cause environmental pollution and toxic effects on non-target organisms. Esterase is a kind of hydrolytic enzyme that can catalyze the cleavage or formation of ester bonds. it plays a pivotal role in the decomposition of pyrethroids and esters containing industrial pollutants through the hydrolysis of ester bonds. Here, a new esterase gene est882 was successfully screened, which encodes Est882, a SGNH family esterase composed of 294 amino acids. It was heterogeneously expressed, identified and immobilized. Multiple sequence alignment showed that Est882 had a typical GDS(X) conserved motif and a catalytic triad composed of Ser79, Asp269 and His275. Phylogenetic analysis showed that Est882 shall belong to a new esterase family. Biochemical characterization demonstrated that the optimum condition was 40°C and pH 9.0. Est882 immobilization was studied with mesoporous silica SBA-15 as the carrier and found to significantly improve the tolerance and stability of Est882. Its optimum pH increased to 10.0 and stabilized within pH 8.0–11.0. Free Est882 can effectively degrade various pyrethroids within 30min, with a degradation rate above 80%. The immobilized Est882 yet degraded more than 70% of pyrethroids within 30min. The present study indicated that Est882 has outstanding potential in bioremediation of a pyrethroid-polluted environment. These characteristics endow Est882 with potential values in various industrial applications and hydrolysis of pyrethroid residues.

KEYWORDS

pyrethroids, esterase, immobilization, alkali resistance, reusability, bioremediation

Introduction

Pyrethroid pesticides are a type of biomimetic synthetic insecticides modified from natural pyrethroids (Fan et al., 2012b). Because of high toxicity to insects and low toxicity to mammals, pyrethroid pesticides are considered to be safer substitutes than the highly toxic pesticides (e.g., organophosphorus) and thus are more widely used (Cycoń and

Piotrowska-Seget, 2016). Liu et al. (2020) found that 100 μg/cm^2 of metofluthrin and transfluthrin could cause significant mortality of mosquitoes within 1 h. According to the research by Kalyan et al. (2010), the percentage of cells with aberration varied from 1 to 32 when lymphocyte cells were treated with varying concentrations of cypermethrin from 3.6 to 7.6 μM (Chakravarthi et al., 2007). Despite agricultural benefits, the extensive and chronic use of pyrethroids has brought many problems, such as drug resistance of pests, toxic effects on non-target organisms (e.g., pest predators, fishes, aquatic invertebrates), abundant residues in agricultural products, water and soils, and human exposure (Yi et al., 2012; Wei et al., 2020). These findings reveal the potential harm of pyrethroids to human health and the ecosystem. Therefore, developing some effective strategies to eliminate or minimize pyrethroid residues in foods and the environment is urgent. Enzyme biotechnology provides safe, efficient and no-secondary-pollution solutions for degradation of residual pyrethroids and other pesticides (Fan et al., 2017). It is critical in bioremediation of contaminated foods, soils, and sewage treatment systems.

The key metabolic routes of pyrethroids are the oxidation by cytochrome P450 and the ester bond hydrolysis by esterases to produce intoxic acids and alcohols (Godin et al., 2007). Esterase is the general name of a class of enzymes that catalyze the hydrolysis or generation of ester bonds, playing a pivotal role in decomposition of pyrethroid compounds and esters containing industrial pollutants through the hydrolysis of ester bonds. Many pyrethroid hydrolases from different sources have been isolated and studied, such as EstPS1, PytY, EstA, Est3385, Est684, and EstP (Wu et al., 2006; Ruan et al., 2013; Cai et al., 2017; Fan et al., 2017; Luo et al., 2018; Hu et al., 2019). However, there are few studies on the alkali resistance and high hydrolysis activity of pyrethroid hydrolases. Most industrial lipolysis and esterification reactions are carried out in the alkaline environment (Kumar et al., 2019; Borges et al., 2020). The ability of hydrolases to survive and catalyze under alkaline conditions is of great importance for industrial applications, especially in bioremediation of pyrethroids-polluted alkaline environments (Wei et al., 2013).

With the development of molecular biology technology, the construction of heterologous expression system has become an important means in the field of industrial enzyme preparation development. Among them, recombinant *Escherichia coli* expression system has the advantages of easy cultivation, fast growth rate and high expression level. It is currently the most commonly used genetic engineering expression system and is widely used in basic research and modern biotechnology. Wang et al. (2009) cloned an esterase gene *pytH* encoding pyrethroid hydrolase from *Sphingobium* sp. JZ-1 and expressed it heterogeneously in *E. coli*. The recombinant PytH has extensive substrate specificity and high hydrolytic activity for various pyrethroids. Zhenqiang et al. (2015) introduced the pesticide-degrading carboxylesterase genes *mpd* and *pytH* into *E. coli* DH5α to construct recombinant plasmids, and integrated them into *Pseudomonas putida* KT2440 for expression. The engineering strain KT2440 could completely degrade six mixed pesticides (0.2 mM each) within 48 h. The construction of engineering microorganisms with broad-spectrum pesticide degradation activity is a promising strategy for bioremediation of pesticide pollution.

Enzyme immobilization technology is an effective strategy to make biotransformation economically feasible (Sheldon et al., 2021). The methods for immobilization are including physical adsorption, covalent, encapsulation or embedding, and crosslinking (Patel et al., 2016, 2017, 2019; Otari et al., 2020). Depending on the immobilization methods, significant variations in the immobilization yields, lower relative activity or loading of enzyme on the supports have been demonstrated. Physical adsorption has simple operation, low cost and maintenance of high enzyme activity, but can very easily desorb enzymes because of the dependence on the weak force between enzymes and the carrier (Cai et al., 2016). Covalent binding usually requires a modifiable and highly stable carrier with more chemical groups, but the operation process is complex (Yang et al., 2021). Although embedding is to fix the enzyme molecules in the grid structure of a porous carrier, and proceeds under mild reaction conditions, the substrate can hardly react with the enzyme due to the influence of the embedding structure. Cross-linking can effectively improve enzyme leakage, but the immobilized enzyme prepared by simple crosslinking has poor mechanical properties, and therefore usually integrated with other methods to strengthen and improve the immobilization effect (Cai et al., 2016; Ke et al., 2018; Yang et al., 2021). Hence, for the success of enzyme immobilization, the selection of appropriate methods is required (He et al., 2015; Cai et al., 2016; Yang et al., 2021). Appropriate immobilization methods are capable of immobilizing free enzymes on a recoverable carrier, so as to reduce production costs and broaden the applications of enzymes (Mogharabi et al., 2012). The application of new carrier such as mesoporous silica nanomaterials endow the immobilized enzyme technology with greater development potential and plasticity, and of them, SBA-15 has large specific surface area, and high thermal and mechanical stability, and thus becomes an ideal candidate for immobilized enzymes (Verma et al., 2020). The quality of immobilized enzymes is improved from all aspects of the immobilization process by using excellent carriers, efficient immobilization methods and process optimization.

In the present study, Est882, a novel alkali-resistant and pyrethroid hydrolytic esterase was discovered from the soils of Junggar Basin in Xinjiang, and its heterologous expression, purification and biochemical characteristics was studied in detail. Then the esterase was immobilized by adsorption crosslinking on SBA-15 as the carrier to improve its stability and reusability. In addition, the biodegradation of fenpropathrin, cypermethrin, permethrin, cypermethrin and fenvalerate by the immobilized enzyme was investigated. The whole research show that immobilized Est882 on SBA-15 may offer a promising technique to increase the degradation of enzyme for pyrethroid pesticides in the future.

Materials and methods

Chemicals and reagents

Fenpropathrin, cypermethrin, permethrin, cypermethrin and fenvalerate (purity 98%) were purchased from J&K Scientific Ltd. SBA-15 (pore size 6–11 nm) was bought from XFNANO. T4 DNA ligase, restriction endonuclease and DNA polymerase were provided by Takara. A Bradford protein concentration assay kit was offered by Sigma. All other chemicals were analytically pure commercial products. *E. coli* DH5α and *E. coli* BL21 (DE3) were offered by TSINGKE Biological Technology.

Prokaryotic expression and purification of recombinant esterase Est882

A soil sample was collected from the wild Ferula asafoetida distribution area in Shihezi on the southern edge of Junggar Basin in Xinjiang. The sample was taken from 5 to 10 cm under the surface, sealed in a sterile bag, and preserved at −20°C until DNA extraction. Total genomic DNA from the soil sample was extracted according to a reported method (Zhao et al., 2021). In the metagenomic library of soil from Junggar Basin, the gene that express the protein with esterase activity was successfully extracted using a genomic DNA extraction kit (Omega, USA). After sequencing, the target sequences were uploaded to National Biotechnology Information Center (NCBI) for registration (GenBank, accession: No.MZ429070) and named as *est882*. The target gene *est882* was amplified by polymerase chain reaction (PCR) with the forward primer TTCCATATGCGAGATCA CCGACGTCAGCT and the reverse primer CCC AAGCTT TCCGGCAGGATCTGGAGGTA (the underlined parts are the *Nde*I and *Hind*III restriction sites respectively). The vector pET28a(+) and obtained fragments digested with *Nde*I and *Hind*III were ligated by T4 DNA ligase and the recombinant gene was transformed into *E.coli* BL21 (DE3). The subsequent induction of enzyme protein expression and electrophoresis were conducted referring to previous report (Hårdeman and Sjölin, 2007; Wang et al., 2020). Prior to the phylogenetic tree of esterase was constructed on Mega 7.0 using the adjacency method, the amino acid sequences among Est882 and other esterases were analyzed on Clustal W and ESPript 3.0.

Enzyme assay

Esterase activity assay was performed with ρ-nitrophenyl (ρNP) esters as substrate. The production of ρNP esters was measured at the absorbance of 405 nm. One unit of esterase activity was defined as 1 μM of ρ-nitrophenol liberated per min under the standard assay conditions (pH 9.0, 40°C, 10 min). The assay was carried out in reaction mixture containing Tris–HCl buffer (50 mM, pH 9.0), substrate solution (1.0 mM) and purified Est882 mixed in a ratio of 18: 1: 1. To eliminate the interference of substrate spontaneous hydrolysis, all experiments were conducted in triplicate (Ksenia et al., 2012; Wang et al., 2020). An enzyme-free mixture was used as the control, and the effect of each substrate on non-enzymatic hydrolysis was subtracted from the measured value of the enzymatic sample (Fan et al., 2012a). In the same group, the highest enzyme activity was set to be 100%, and the relative enzyme activity was calculated.

Characterization of Est882

The ρNP esters with different acyl chain lengths (ρNP acetate-ρNP myristate, C2-C14) was used as the substrates for specificity test so that the optimum substrate for Est882 can be determined and used for the subsequent experiments. The optimal temperature of Est882 was tested in the range of 10°C–70°C in the solution of pH 9.0. To study the thermal stability, the purified enzyme was cultured in Tris–HCl buffer at 10°C–60°C for certain time (2–10 h), and the residual enzyme activity was detected. The optimum pH of Est882 was determined in the solution of pH 3.0–11.0 at 40°C. The buffer solution (50 mM of final concentration) used for determination of optimal pH contained citrate buffer (pH 3.0–5.0), sodium acetate buffer (pH 5.0–7.0), Tris–HCl buffer (pH 7.0–9.0) and K_2HPO_4-KOH buffer (pH 9.0–11.0). The pH stability of the purified enzyme was detected after incubating in different buffers (pH 6.0–11.0) for a certain time (5-25 h). The activities of free Est882 with the addition of optimum substrate to different final concentrations (0.5–5.0 mM) were measured at 40°C in Tris HCl buffer (pH 9.0). The *Km* and k_{cat} values were calculated by fitting the data to Michaelis–Menten equation (Wang et al., 2020).

Immobilization of Est882

Esterase Est882 was immobilized in three different materials including sodium alginate beads (Jampala et al., 2017), chitosan beads with glutaraldehyde as a crosslinking agent (Chunqing et al., 2019) and mesoporous silica SBA-15 (Fan et al., 2017). The process of Est882 fixed on the carrier of mesoporous silica SBA-15 was as follows: SBA-15 (100 mg) was dispersed in 2 ml of citric acid-disodium hydrogen phosphate (CA-DSP) buffer of certain pH, and was added with an appropriate amount of an Est882 solution (1.09 mg/ml of protein concentration, 128 U/ml of enzyme activity). Oscillated at 200 rpm and cultured at room temperature for 1 h, the mixture was added with 0.1 ml of a chitosan solution (dissolved in 5 mg/ml, 0.5% dilute hydrochloric acid-solution) and 0.1 ml of a 1.0% glutaraldehyde solution and was centrifuged to collect the precipitates after further incubation for certain time. To ensure removal of free enzyme, the precipitates were repeatedly washed with CA-DSP buffer 3 times. After vacuum drying at 37°C for 12 h, the chitosan-glutaraldehyde crosslinked SBA-15-immobilized Est882 was obtained (Est882@

SBA-15). The enzyme activity recovery of three-carrier-immobilized Est882, namely the activity ratio of immobilized enzyme to free enzyme, was detected for the same time. The immobilization conditions of Est882@SBA-15 were investigated. The optimal pH for immobilization was tested with the CA-DSP buffer at different pH (4.0–9.0). To analyze the effect of immobilization time and enzyme loading, 100 mg of SBA-15 was dispersed in 2 ml of CA-DSP buffer (pH 5.0) with 100–200 μl enzyme solution for certain time (0.5–3 h) at room temperature. 0, 0.1, 0.2 and 0.3 ml of the chitosan-glutaraldehyde solution were each added with 100 mg of SBA-15 for the investigation of the effect on the crosslinking amount. The investigation for substrate specificity of immobilized enzyme was performed in accordance with the free enzyme section above. The optimum substrate was hydrolyzed by reusing the immobilized Est882, and its reuse performance was investigated. Each reaction proceeded for 10 min in the Tris–HCl buffer (40°C, pH 9.0). After centrifugation, washing and drying, the immobilized enzyme was collected, and the fresh reaction mixture was re-added to the next cycle of reaction under the same conditions. Subsequently, the kinetic parameters of immobilized Est882 toward optimum substrate was detected under the same assay conditions as that of free enzyme described above. The residual activity of the immobilized Est882 was tested after 20 consecutive cycles. Referring to previous report (Kumar et al., 2019), the immobilization yield (IY) and efficiency (IE) were calculated as follows:

$$\text{Immobilization yield}\,(\%) = \frac{\text{The total amount of immobilized enzyme}}{\text{The total amount of enzyme initially added}} \times 100 \quad (1)$$

$$\text{Immobilization efficiency}\,(\%) = \frac{\text{Total activity of the immobilized enzyme}}{\text{Total activity of free enzyme}} \times 100 \quad (2)$$

Material characterization of immobilized Est882

Morphology of the mesoporous silica before and after SBA-15 immobilization was analyzed by a Supra55 scanning electron microscope (SEM, German Zeiss) and a JEM-2100 transmission electron microscope (TEM, JEOL, Japan). SBA-15, free Est882 and Est882@SBA-15 were observed by a NicoletiS10 Fourier transform infrared spectroscope (FTIR, American Thermofisher). An appropriate amount of sample powder was stuck to the conductive adhesive, gold-sprayed for 30 s, and observed under SEM. A proper amount of sample powder was ultrasonically dispersed with anhydrous ethanol and dripped on a copper net. After drying at room temperature, it was observed and photographed under TEM. The sample powder and KBr were mixed evenly at a certain proportion, and made into transparent sheets by pressing the tablet, which were tested on FTIR.

Gas chromatograph of pyrethroids degraded by Est882

The degradation ability of Est882 over fenpropathrin, cypermethrin, permethrin, cypermethrin and fenvalerate was tested by gas chromatography. The pyrethroids were prepared into a standard pesticide mixture at a final concentration of 5 mg/ml with a pH 9.0 Tris–HCl buffer. The reaction mixture containing 1 ml of standard pesticide mixture and 1 ml of the purified enzyme (1.09 mg/ml) reacted with 3 ml of the Tris–HCl buffer at 37°C for 30 min. The reaction mixture was saturated with sodium chloride and extracted with an equal volume of N-hexane. One microliter of the reaction liquid was used for quantitative analysis by gas chromatograph (GC; Agilent7890B, USA) on HP-5MS column (Chen et al., 2022). The conditions of GC analysis were as follows: the split ratio of 5:1; the injection volume of 1.0 μl; the injection temperature of 260°C; the column flow of 1.0 ml/min. Besides, the carrier gas was high purity nitrogen with a total flow rate of 9 ml/min (Chen et al., 2022). The concentration of pyrethrin was calculated according to the peak area. A0 was used to represent the concentration of pyrethrin of the control group, while those of the experimental group were marked as A1. The calculation formula of the degradation rate (DR) of pyrethrin is shown below:

$$\text{DR} = \frac{(\text{A0} - \text{A1})}{\text{A0}} \times 100\%$$

Results and discussion

Sequence and phylogenetic analysis

A new esterase gene was isolated from the soil metagenomic library in Junggar Basin, Xinjiang (Zhao et al., 2021). The esterase gene is in full length of 882 bp and encodes a protein of 294 amino acids (named Est882). The sequence was submitted to GenBank (accession No. MZ429070). The theoretical molecular mass is 31.44 KDa and the isoelectric point is 5.33. Protein blast analysis of NCBI shows that Est882 has some homology with other hydrolases. The encoded protein has the highest homology (88.24%) with the lysophospholipase L1-like esterase from Nocardiopsis metallicus (MBB5494627.1). Multiple sequence alignment with other esterases/lipases shows that Est882 belongs to the SGNH/GDSL hydrolase family, which has a typical conserved motif GDS (X) and a catalytic triad composed of active sites Ser79, Asp269 and His275 (Figure 1). To further validate the evolutionary link between

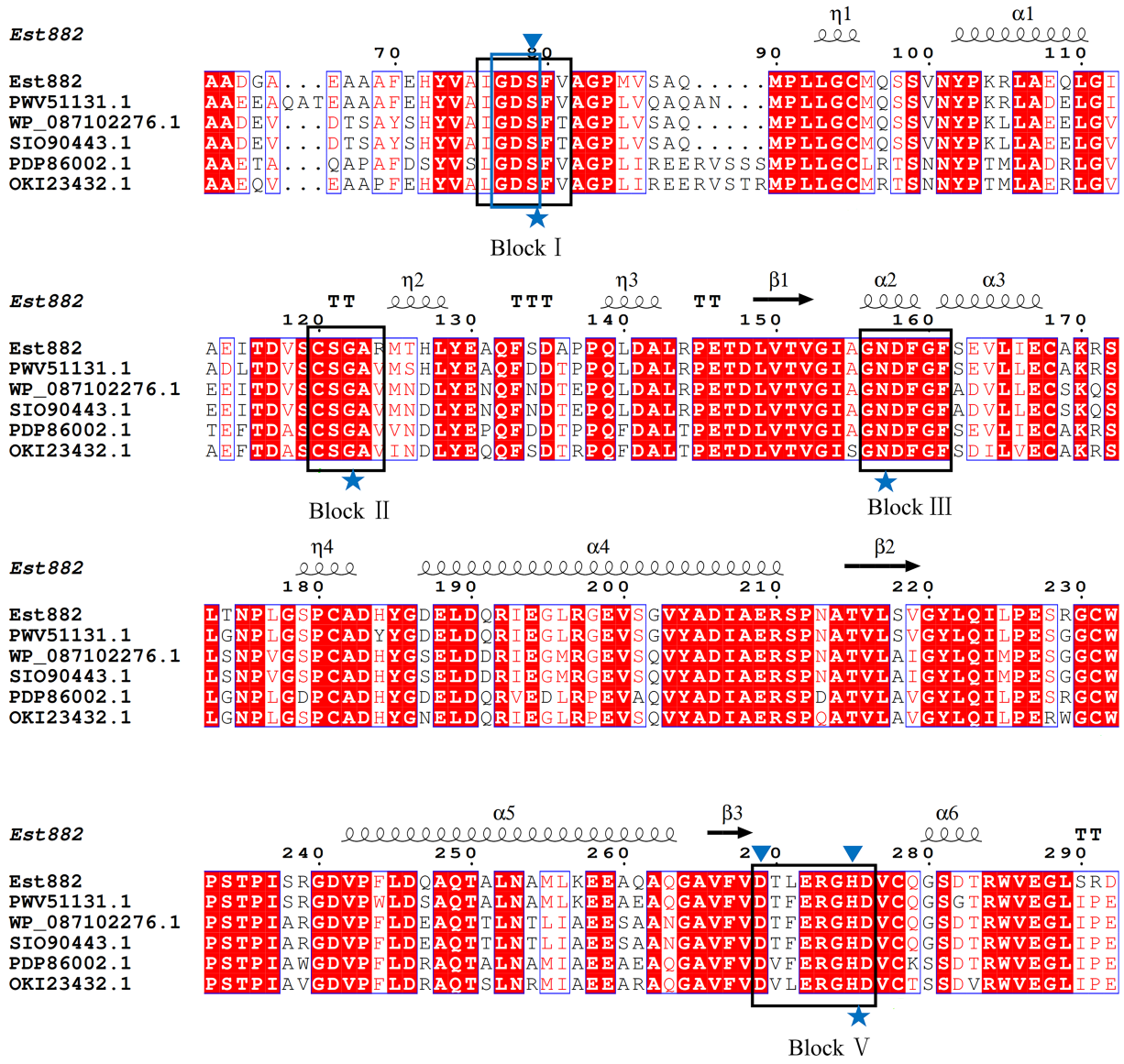

FIGURE 1
Alignment of multiple amino acid sequences of *Est882* and its homologues. Black rectangles: the four conserved domains (blocks I, II, III and V) of Est882. blue asterisks: the four constant important catalytic residues Ser-Gly-Asn-His in the conserved blocks. Blue rectangle: the conserved GDS(X) motif. Blue triangle: the amino acids (Ser79, Asp269 and His275) that make up the catalytic triad.

Est882 and other esterases, 32 esterases from 18 families and 2 homologous esterases were chosen to construct an adjacent phylogenetic tree (Figure 2). Phylogenetic analysis did not classify Est882 into the phylogenetic cluster of any known esterase family. This result indicates that Est882 and its homologues may form a new esterase family, so we speculate that Est882 is a new esterase. SGNH family hydrolases have flexible catalytic sites and unique structural properties. Some SGNH family hydrolases have been identified (Song et al., 2013; Petrovskaya et al., 2016; Jo et al., 2021). Most of the hydrolases with GDSL motifs come from plants (Dong et al., 2016; Su et al., 2020). So far, there is no systematic report on SGNH family pyrethroid hydrolases.

Expression and purification of Est882

The target gene *est882* was amplified by PCR (Supplementary Figure S1) and constructed into the vector pET-28a(+). Restriction endonuclease digestion and sequencing confirmed that the target gene was connected to the expression vector (Supplementary Figure S2). The recombinant plasmid pET-28a(+)-*est882* was transformed into *E. coli* BL21 (DE3) for expression. The protein after purification exhibited a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure 3). The protein content detected by the Bradford method was 1.09 mg/ml. SDS-PAGE showed the purified enzyme had a relative molecular mass of ~32 kDa, which is

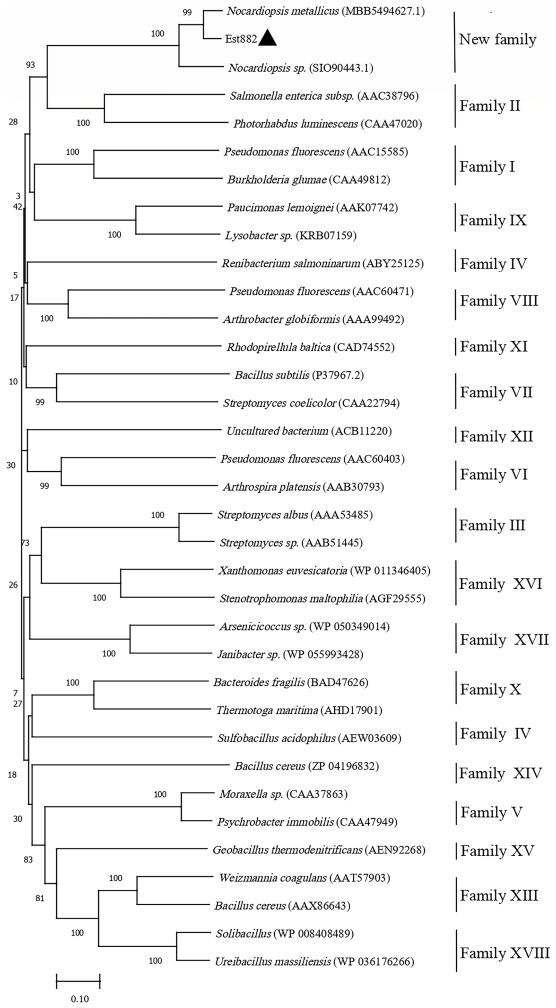

FIGURE 2
Phylogenetic analysis of *Est882* (▲) and its homologues with other esterases. The amino acid sequences (accession numbers) of all enzymes were retrieved from GenBank.

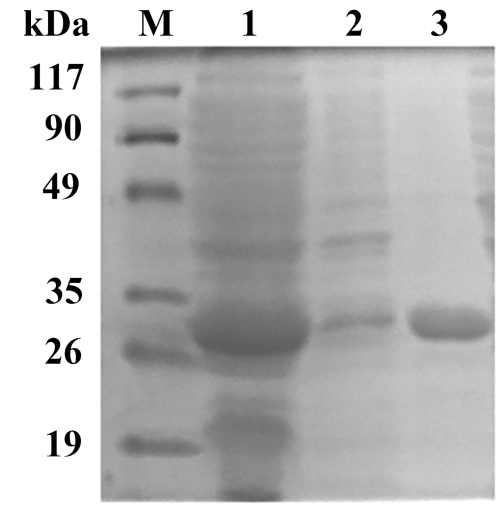

FIGURE 3
SDS-PAGE of Est882. Lanes M, 1, 2, 3: protein molecular mass marker. Cell lysate. Cell lysate supernatant. and purified *Est882, respectively.*

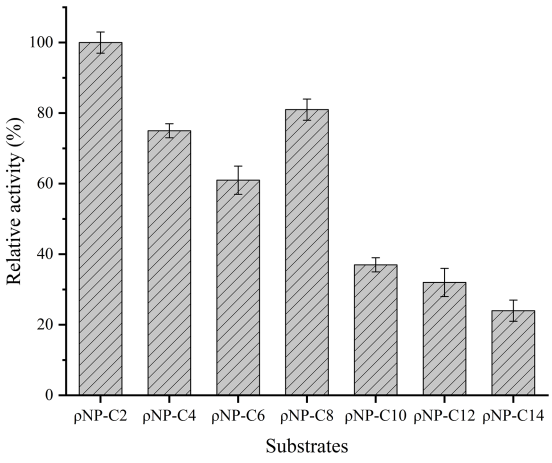

FIGURE 4
Substrate specificity of Est882. The data point is the average of the three measurements, and the error bar represents the standard deviation.

consistent with the theoretical molecular mass of the recombinant Est882 (31.44 kDa).

Substrate specificity

The substrate specificity of Est882 was determined by using ρ-nitrophenyl esters with different acyl chain lengths as substrates (Figure 4). Est882 displayed the highest activity toward ρNP-C2 among all tested ρ-nitrophenyl esters.

Effects of temperature and pH on activity and stability of Est882

With ρNP-C2 as the substrate, the effect of temperature on Es882 activity was investigated under standard conditions (Figure 5A). The optimum temperature of Est882 is 40°C. Over 50% of the maximum activity was maintained at 20°C–50°C, indicating Est882 has a wide range of temperature adaptability. Est882 was very stable below 40°C, and the remaining enzyme activity was above 70% after 10 h of incubation (Figure 5B). Similar to Est3385 from *Rhodopseudomonas palustris*. Its optimum temperature is 35°C. When the reaction temperature is 15°C, the relative activity of the enzyme is still about 60% of that at the optimum temperature (Luo et al., 2018).

The effect of pH (3.0–11.0) on the activity of Est882 was tested under standard conditions (Figure 5C). Est882 showed

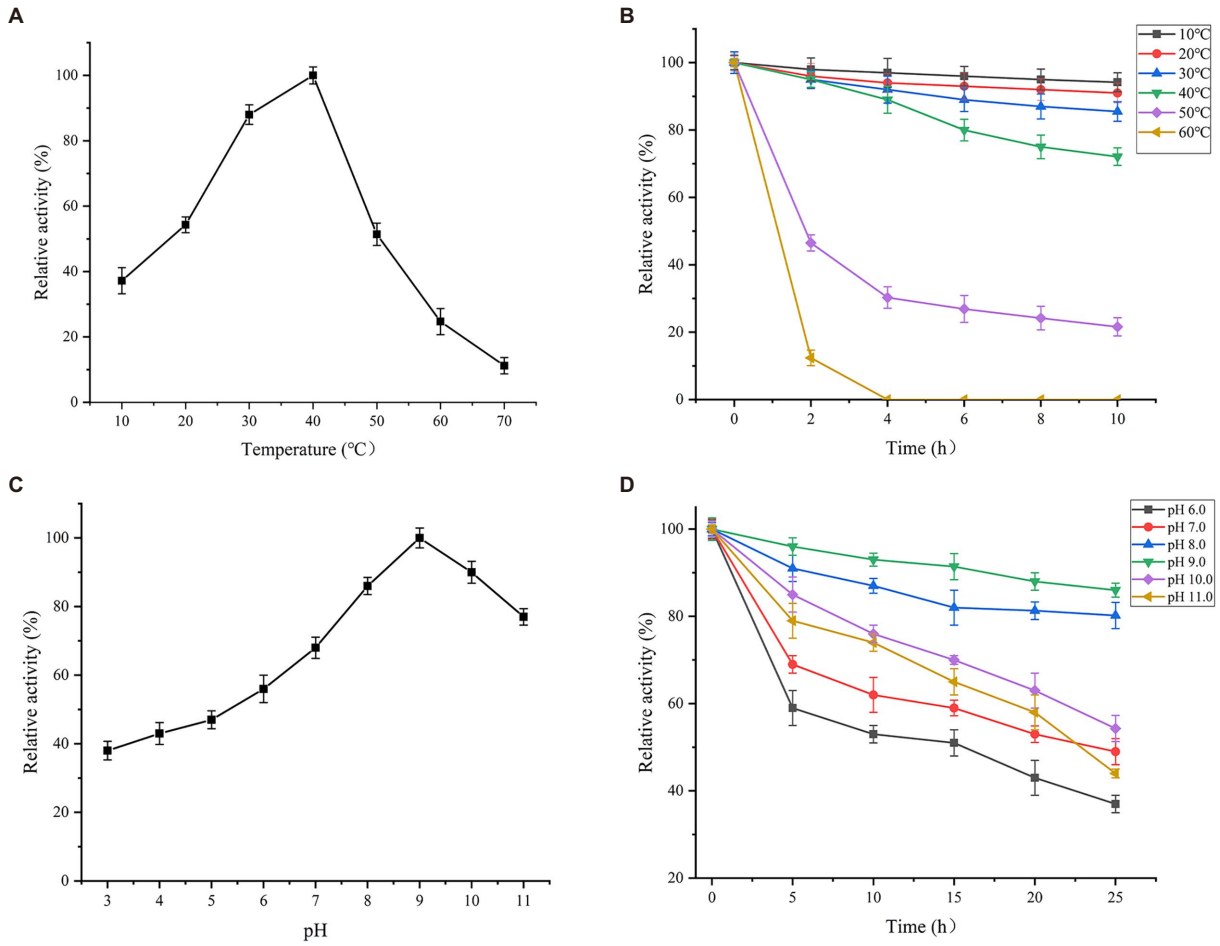

FIGURE 5
Effects of **(A)** temperature on activity, **(B)** temperature on thermal stability, **(C)** pH on activity, and **(D)** pH on stability of Est882. Symbols: square: 10°C. circle: 20°C. upward triangle: 30°C. downward triangle: 40°C. diamond: 50°C. left triangle: 60°C. Symbols: square: pH 6.0. circle: pH 7.0. upward triangle: pH 8.0. downward triangle: pH 9.0. diamond: pH 10.0. left triangle: pH 11.0.

high activity (more than 80%) in pH 8.0–10.0, and the optimum pH was 9.0, indicating Est882 is an alkaline esterase. After incubation in pH 7.0–10.0 for 25 h, the residual activity of Est882 remained more than 50% (Figure 5D), and especially, was more than 80% after 25 h of incubation at pH 8.0 and 9.0. The optimum pH of the enzyme is higher than most reported pyrethroid enzymes, except for the pyrethroid hydrolyzing carboxylesterase EstSt7 from thermophilic *Sulfolobus tokodaii* (pH 9.0; Yi et al., 2012) and the pyrethroid hydrolytic esterases Est684 from Mao-tofu genome and Pye3 from vegetable soil meta-genomic library (pH 7.0; Huang et al., 2016; Fan et al., 2017). The optimum pH of pyrethroid hydrolase Sys410 isolated from Turpan basin metagenomic library is 6.5 (Li et al., 2008; Fan et al., 2012b). Wide temperature stability and strong alkali resistance are important characteristics of hydrolases that can be used to bioremediate changeable environments. Some potential applications of alkaline-resistant enzymes in various industrial productions are reported (Dai et al., 2021). For example, the optimum pH of intracellular azoreductase enzyme from alkaliphilic *Bacillus subtilis* is 7.0 and is still active under alkaline conditions (pH 8.5). It was applied to decompose mixed azo dyes in soils polluted by textile wastewater (Su et al., 2020; Kkp et al., 2021) identified a new β-galactosidase from alkaliphilic *Paracoccus marcusii*, and found its activity remained above 90% after incubation at pH 5.0–9.0 for 3 h, indicating this enzyme is feasible for industrial preparation of prebiotic oligosaccharides. The endoglucanase from *Bacillus subtilis* Y106 has an optimum pH 6.5 and is stable within 4.0–9.0, and thus was applied to pulp modification to improve paper quality (Wang et al., 2017).

Investigation of immobilization conditions

Est882 was immobilized on chitosan, sodium alginate and SBA-15. After the same time, the recovery rates of esterase activity were 57.4%, 42.9%, and 74.2%, respectively. Therefore,

SBA-15 was selected as the carrier for further optimization (Table 1). As for the effect of buffer pH, the activity of immobilized enzyme was the highest at pH 5.0, but too high pH was unfavorable for the immobilization (Figure 6A). The immobilization effect of SBA-15 on enzyme was mainly influenced by solution pH, which can be associated with the difference of isoelectric point between enzyme and carrier. Different with the predicted isoelectric point 5.33 of Est882, that of SBA-15 is between 3.0 and 4.0 according to the previous studies that have been shown (Mehta et al., 2016; Zhong et al., 2019). In the solution of pH 5.0, the immobilization of enzyme and carrier presented better combination through the electrostatic interaction of physical adsorption when the carrier and the enzyme have opposite charges. In terms of immobilization time (Figure 6B), Est882 was rapidly adsorbed to the carrier within 1–2 h, the enzyme activity in the supernatant remained low after 1 h, and all the enzyme was immobilized on the carrier after 2 h. The loading amount of 100 mg SBA-15 immobilized enzyme was investigated (Figure 6C). When the enzyme dosage was 160 μl (protein concentration 1.09 mg/ml), the immobilized enzyme activity was the highest, and the optimal loading amount of carrier immobilized enzyme was 1.74 mg/g. Moreover, the optimal crosslinking amount of chitosan glutaraldehyde was 1 ml/g (Figure 6D).

TABLE 1 The activity recovery of the immobilizing *Est882* on different carriers.

Carriers	The activity recovery (%)
Chitosan beads	57.4 ± 1.2
Sodium alginate	42.9 ± 0.7
Mesoporous silica SBA-15	74.2 ± 1.5

Material characterization of immobilized Est882

Morphology of the SBA-15-immobilized enzyme was characterized by SEM and TEM. The SEM patterns show

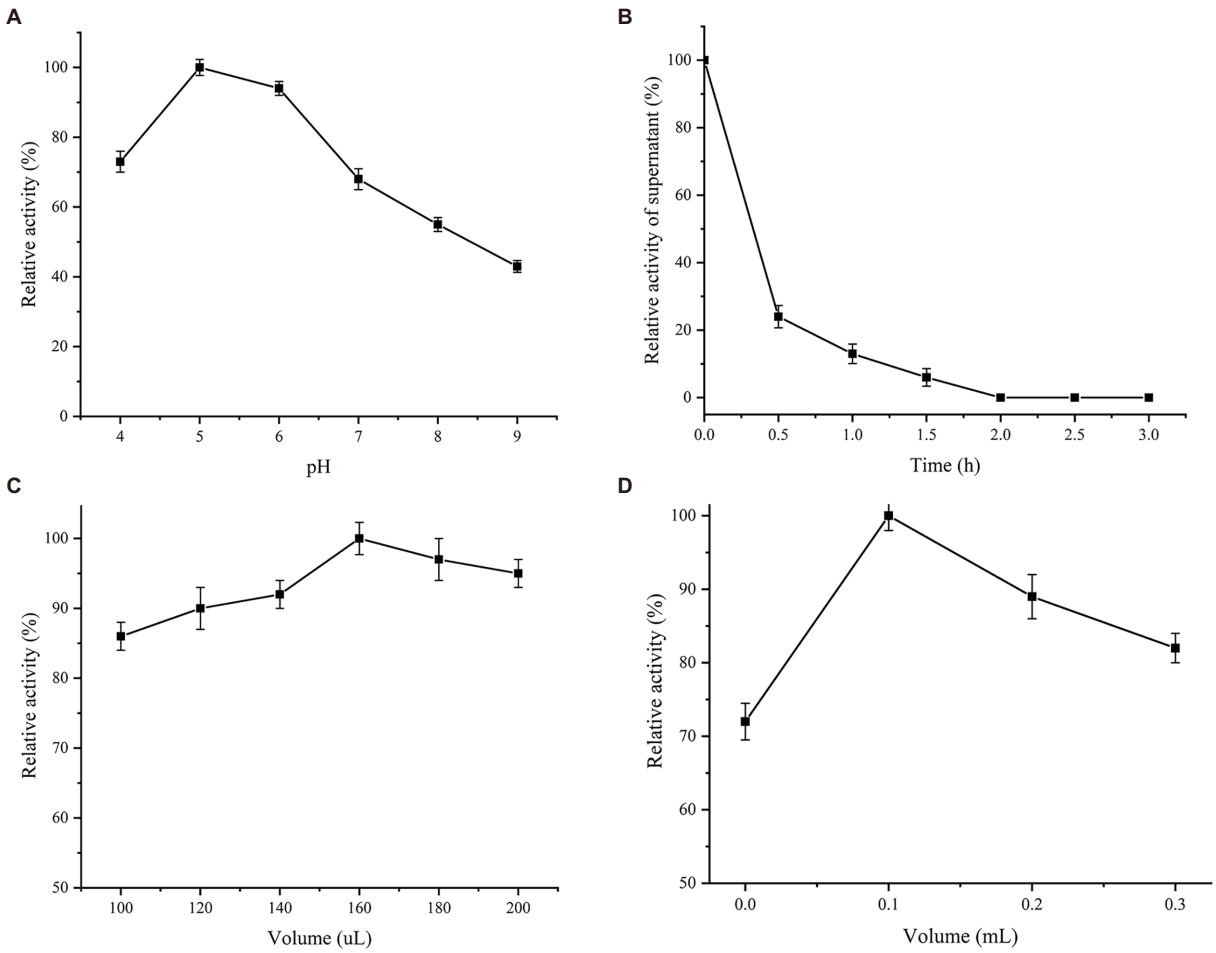

FIGURE 6
Immobilization conditions of Est882. **(A)** pH. **(B)** time. **(C)** enzyme loading. **(D)** Chitosan addition.

worm-like arrays with a certain length (Figures 7A,B). Morphology of the carrier did not change after enzyme immobilization, indicating SBA-15 was still stable after the immobilization. The TEM diagram shows that SBA-15 has uniform and ordered long-channel mesoporous structures (Figures 7C,D), and some opaque shadows appear on the surface after the immobilization, suggesting the enzyme molecules stay in the channel of the mesoporous material, which is also related to chitosan crosslinking.

FTIR demonstrates the characteristic absorption peaks at 463, 802 and 1,085 cm^{-1} of SiO_2 in SBA-15 (Figure 7E), which belong to bending, symmetric stretching and anti-stretching vibrations of Si-O-Si (Zhong et al., 2019). The typical peaks of free Est882 at 1655 and 1,545 cm^{-1} are ascribed to amide I band (C=O stretching vibration) and amide II band (N-H stretching and C-N bending vibrations). The spectrum of SBA-15 shows obvious characteristic bands of amide I band and amide II band, and bending and stretching vibrations of Si-O-Si, indicating the successful loading of Est882 on SBA-15 (Carlsson et al., 2014).

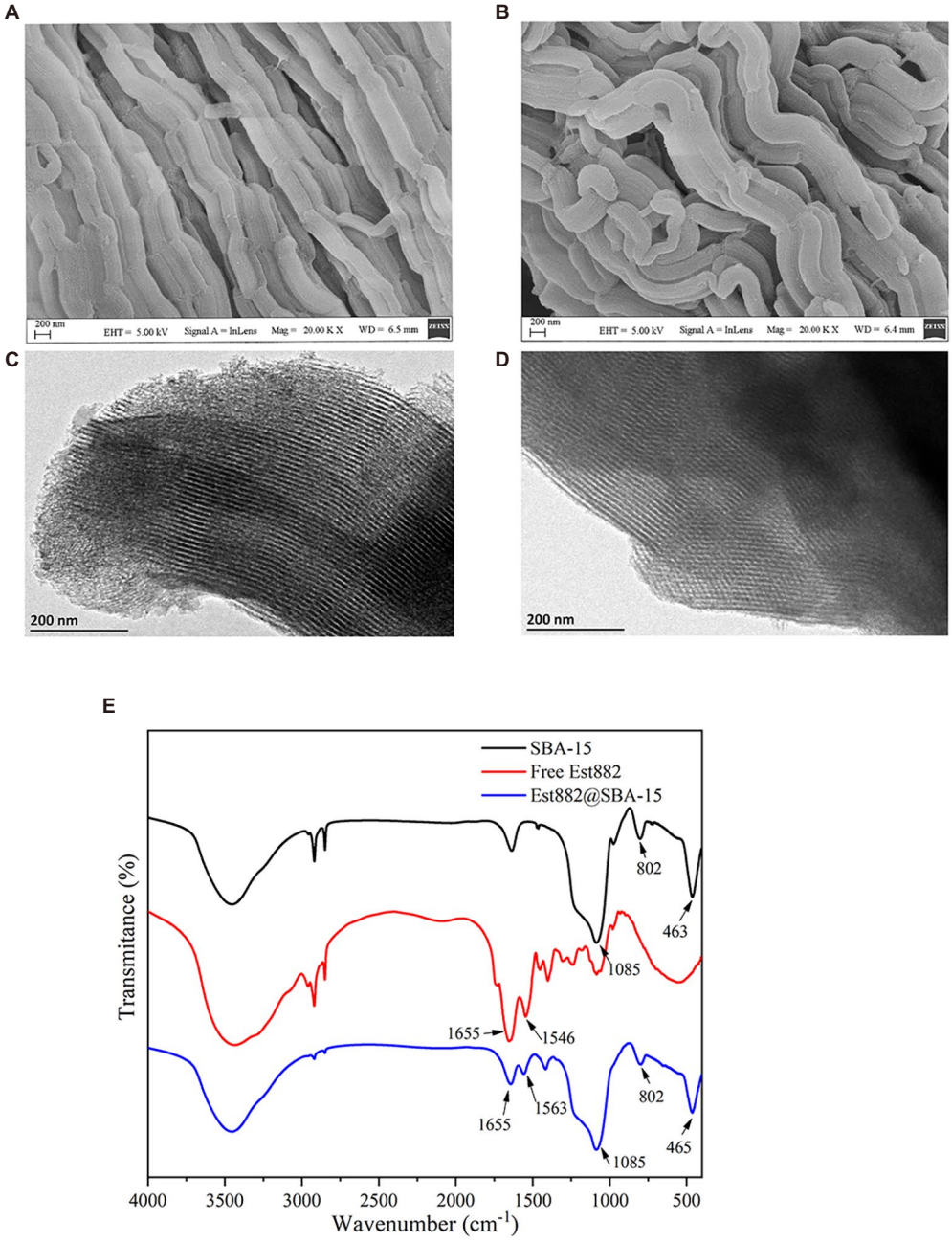

FIGURE 7
SEM **(A,B)**, TEM **(C,D)**, and FTIR **(E)** of SBA-15 immobilized Est882.

Kinetic parameters

The kinetic parameters of the free and the immobilized Est882 were tested using ρNP-C2. The *Km* and k_{cat}/Km are 0.76 mM and 468.17 s^{-1}·mM^{-1} in the free enzyme, and 0.85 mM and 272.70 s^{-1}·mM^{-1} in the immobilized enzyme, respectively, implying the *Km* of the immobilized Est882 is slightly larger. Compared with pyrethroid-degrading esterase PytY from *Ochrobactrum anthropi* whose *Km* was 2.34 mM (Zhiyong et al., 2013) and pyrethroid-hydrolyzing carboxylesterase EstSt7 from *Sulfolobus tokodaii* whose *kcat/Km* was 246.3 s^{-1}·mM^{-1} (Wei et al., 2013), Est882 exhibited a greater affinity for ρNP-C2.

Comparison of catalytic properties

The activity of the immobilized Est882 was determined in 10–60°C (Figure 8A). The optimum temperature of the enzyme with or without immobilization was 40°C, but the relative enzyme activity at 10–60°C was more than 50% in the immobilized Est882, but that at 60°C was only 24.7% in free Est882. When at above 40°C, the thermal stability of the immobilized enzyme was significantly higher than that of the free enzyme (Figure 8B). After incubation at 50°C for 10 h, the residual activities of the immobilized and free enzymes were 47.2 and 21.6%, respectively.

The optimal pH of immobilized Est882 increased to pH 10.0 from that of free Est882 (pH 9.0; Figure 8C). The stability of pH was investigated after incubation in different buffers (Figure 8D). The residual enzyme activity of immobilized Est882 remained above 60% after incubation at pH 7.0–11.0 for 25 h, and especially was 82.1% in the pH 10.0 buffer compared with the free enzyme (54.3%).

Enzyme immobilization was applied to pyrethroid hydrolase to develop biocatalysts with enhanced stability and reusable efficiency (Cai et al., 2016). In this study, esterase Est882 was immobilized on SBA-15 by adsorption-crosslinking, and the recovery rate of enzyme activity increased to 86.5% under the optimum immobilization conditions. The optimum

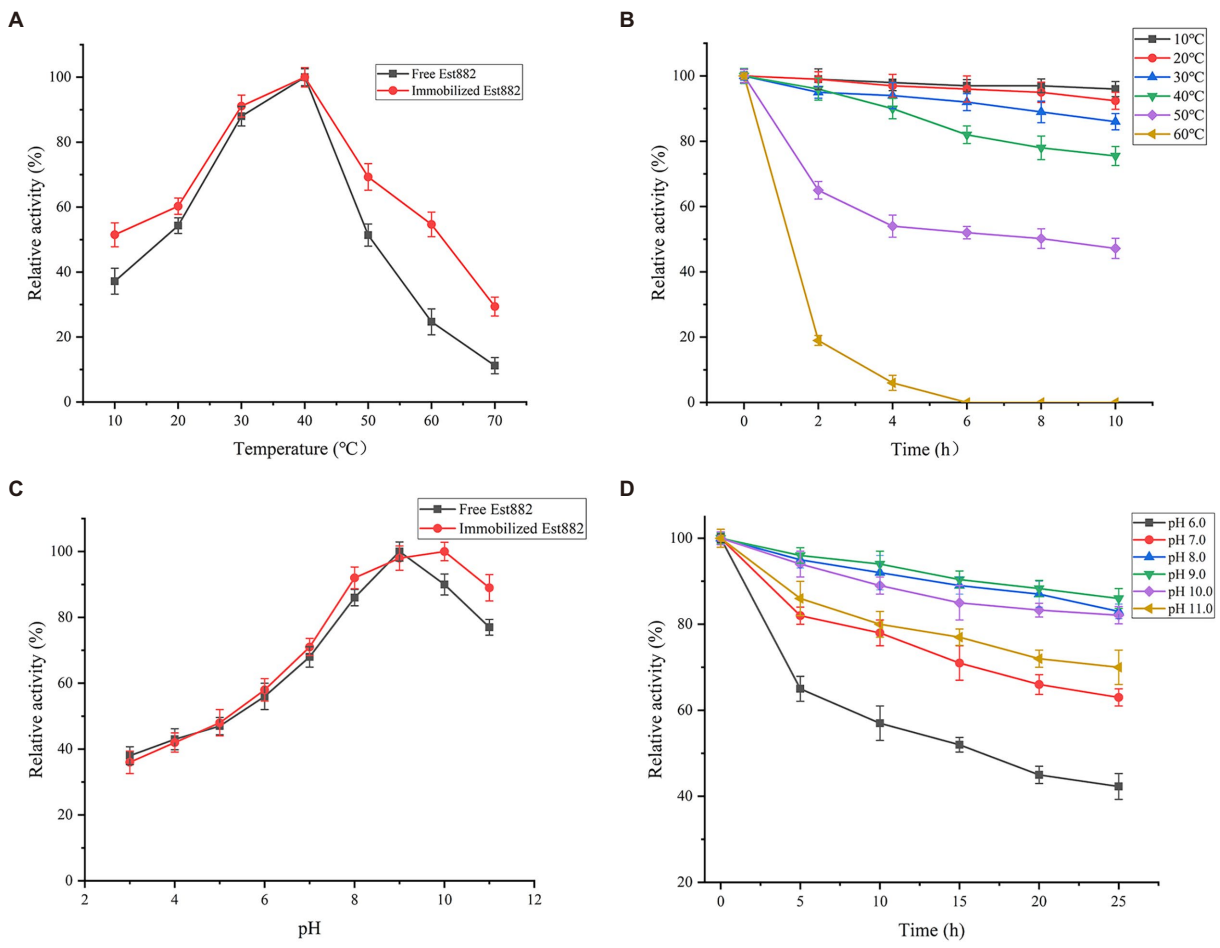

FIGURE 8
Effects of **(A)** temperature and **(C)** pH on the activity of free Est882 (square) and immobilized Est882 (circle). **(B)** Thermal stability and **(D)** pH stability of immobilized Est882. Symbols: square: 10°C. circle: 20°C. upward triangle: 30°C. downward triangle: 40°C. diamond: 50°C. left triangle: 60°C. Symbols: square: pH 6.0. circle: pH 7.0. upward triangle: pH 8.0. downward triangle: pH 9.0. diamond: pH 10.0. left triangle: pH 11.0.

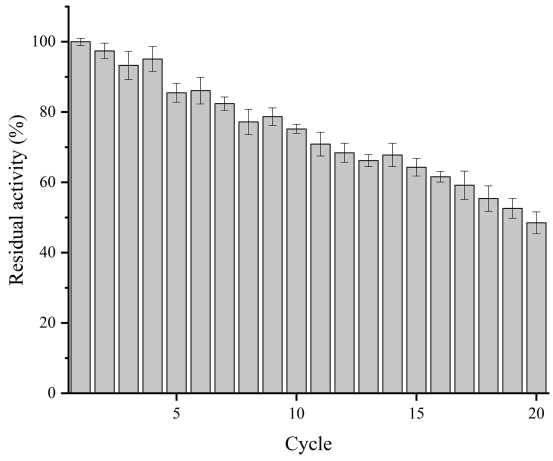

FIGURE 9
Reusability of immobilized Est882.

pH of immobilized Est882 increased from 9.0 to 10.0, and it was more tolerant to alkaline pH. Similarly, Zhang et al. (2012) used SBA-15 to immobilize laccase, and improved the optimal pH from 4.0 to 5.0. Eldin et al. (2000) immobilized penicillin G acylase on methylmethacrylate, and raised its optimum pH from 6.0 to 9.0.

Reusability of immobilized Est882

The reuse performance of immobilized Est882 was studied for 20 cycles (Figure 9). It was highly stable in the first 10 cycles and retained more than 75% of the initial activity. After 15 and 20 cycles, 64.3 and 48.5% of the initial activity were maintained, respectively. Its reusability is higher compared with the feruloyl esterase immobilized on mesoporous silica by physical adsorption after 10 cycles (44%; Eldin et al., 2000) and the alkaline hydrolase PA27 immobilized in ammonium sulfate by glutaraldehyde cross-linking after 10 cycles (70.2%; Jang et al., 2014). However, it is not as stable as acetylesterase LaAcE, which was crosslinked with glutaraldehyde and immobilized by ammonium sulfate and retained about 85% of its original activity after 10 cycles (Wang et al., 2018).

Gas chromatograph of pyrethroids degraded by Est882

Five common pyrethroids were used as substrates to investigate the degradation ability of Est882. Gas chromatograph showed that Est882 can effectively hydrolyze various pyrethroids. After 30 min of reaction at pH 9.0 and 37°C, the control without enzyme was incubated under the same conditions (Figure 10A). The hydrolysis rates of free Est882 over fenpropathrin, cyhalothrin, cypermethrin, cypermethrin and fenvalerate were 86.2%, 90.3%, 84.9%, 87.6%, and 88.3%, respectively (Figure 10B; Table 2). The hydrolysis rates of the five pyrethroids by immobilized Est882 were 78.4%, 83.0%, 76.2%, 81.6%, and 79.1%, respectively (Figure 10C; Table 3). Compared with free enzyme, the degradation rates of pyrethroid pesticides by the immobilized enzyme decreased slightly, but still maintained about 89% of the degradation efficiency of the free enzyme.

Enzyme bioremediation is considered as a potential method for rapid degradation of pesticides, and a growing number of pesticides degrading enzymes are reported (Le et al., 2019). Hu et al. (2019) investigated the degradation ability of carboxylesterase EstA from *Bacillus cereus* BCC01 to various pyrethroids (20 mg/l), and found the degradation rates after 2 h of reaction were more than 70.4%, especially for cypermethrin (100%). Zhang et al. (2021) treated cypermethrin-contaminated vegetables (500 mg/L) with esterase from *Bacillus licheniformis* B-1, and found the degradation rate of cypermethrin residues exceeded 50% at 25°C after 20 min. The pyrethroid hydrolase Sys410 isolated and identified by Fan et al. (2012b) with a metagenomics method had high degradation ability over cypermethrin, cypermethrin, fenpropathrin and deltamethrin (5 mg/ml), and the degradation rates exceeded 95% at 37°C for 15 min. Our study showed the hydrolysis rates of Est882 over cypermethrin, cyhalothrin, cypermethrin, cypermethrin and fenvalerate were more than 80% after 30 min at 37°C, indicating Est882 is a broad-spectrum pyrethroid hydrolase with high hydrolytic activity. The hydrolysis rate of pyrethroids by immobilized Est882 is more than 70%, which is slightly lower than that of the free enzyme. The difference may be due to the fact that enzyme molecules immobilized in mesoporous materials reduce the enzyme and substrate contact, thus slightly decreasing the hydrolysis efficiency of pyrethroid pesticides. Nevertheless, the higher reusability and stability still endow the immobilized Est882 with broad application prospects in the degradation of pesticide residues in vegetables and fruits, treatment of pesticide-containing wastewater, remediation of contaminated soil and environmental monitoring, and become another direction for the development and utilization of pesticide-degrading enzymes in the future.

Conclusion

Esterase has been widely used in a series of industrial fields, especially in the bioremediation of pesticide pollution. In this study, the gene est882 was found and studied from the metagenomic library constructed earlier by our research team, The gene encodes an esterase of the SGNH family consisting of 294 amino acids. Est882 has a typical GDS (X) conserved motif and a catalytic triad composed of Ser79, Asp269 and His275. Phylogenetic analysis showed that Est882 shall belong to a new esterase family. Est882 had the highest enzyme activity at 40°C and pH 9.0. The pH stability and operational stability of the immobilized enzyme were significantly

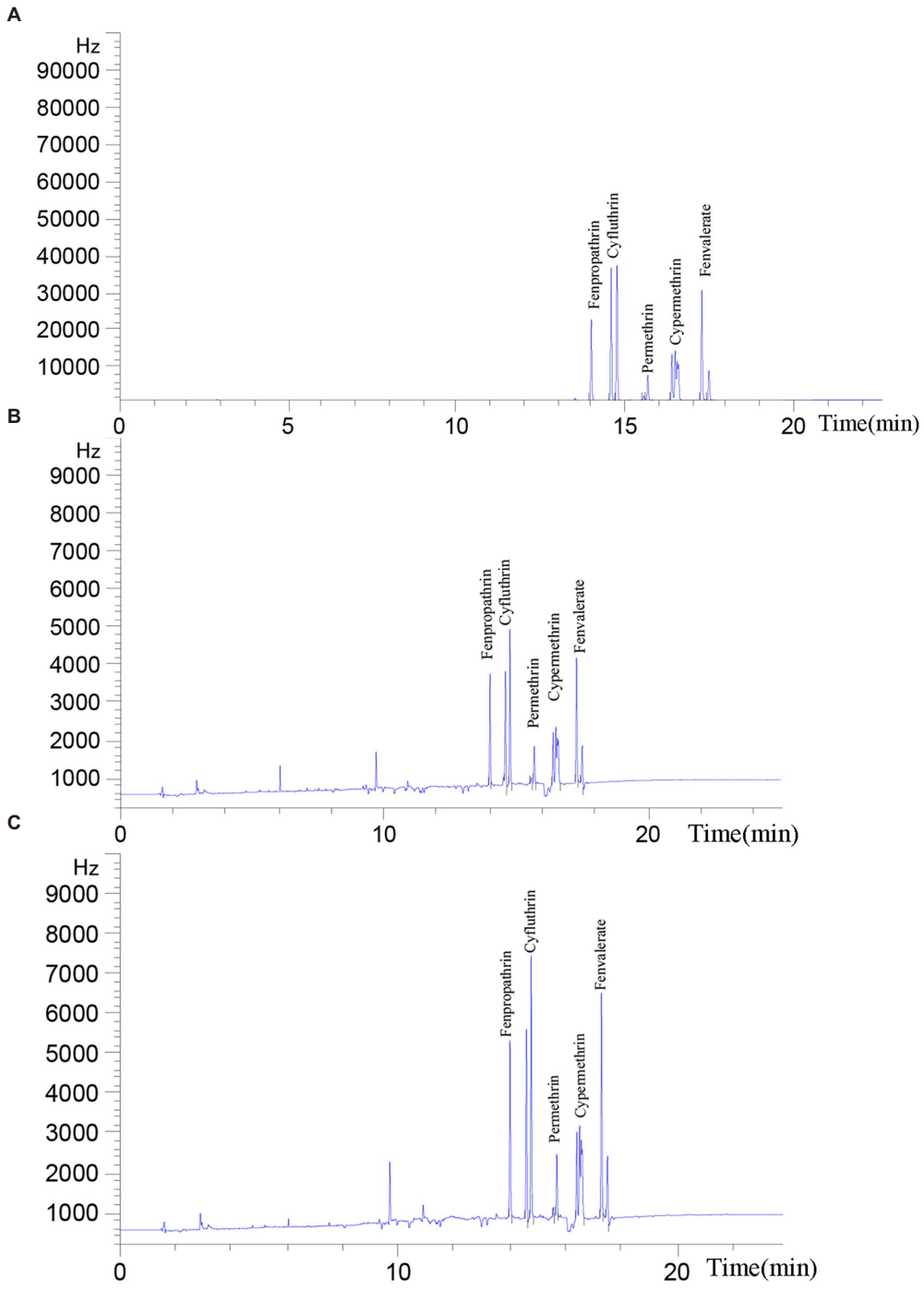

FIGURE 10
Degradation of pyrethroids by purified Est882. The reaction proceeded at 37°C for 30min, and a pyrethroid solution containing inactivated Est882 was used as a control **(A)** Ultimate pyrethroid concentrations analyzed by GC after incubation with **(B)** free Est882 and **(C)** immobilized Est882 for 30min at 37°C.

improved by immobilizing Est882 on mesoporous silica SBA-15 using the adsorption-crosslinking method, and more than 75% of the initial activity was maintained after 10 cycles. The enzyme can effectively degrade several pyrethroids in a very short time, with a degradation rate of more than 80%, showing broad substrate specificity and catalytic activity. The study of Est882, a novel esterase with broad-spectrum and efficient degradation ability, has enriched the SGNH family esterase gene resources, and the combination of favorable properties of Est882 and immobilization process provides a promising pathway for the biodegradation of pyrethroids in contaminated environments.

TABLE 2 Degradation rate of five pyrethroids by Est882.

Pyrethroids	Initial concentration (mg/L)	The concentration after degradation (mg/L)						Degradation rate (%)
		1	2	3	4	5	Mean	
Fenpropathrin	0.991	0.132	0.129	0.145	0.134	0.140	0.136	86.2%
Cyhalothrin	0.984	0.089	0.092	0.105	0.091	0.096	0.095	90.3%
Cypermethrin	0.989	0.154	0.158	0.142	0.151	0.142	0.149	84.9%
Cypermethrin	0.987	0.125	0.130	0.119	0.124	0.112	0.122	87.6%
Fenvalerate	0.983	0.119	0.117	0.102	0.113	0.124	0.115	88.3%

TABLE 3 Degradation rate of five pyrethroids by Est882@SBA-15.

Pyrethroids	Initial concentration (mg/L)	The concentration after degradation (mg/L)						Degradation rate (%)
		1	2	3	4	5	Mean	
Fenpropathrin	0.991	0.221	0.192	0.204	0.231	0.223	0.214	78.4%
Cyhalothrin	0.984	0.162	0.159	0.180	0.165	0.173	0.168	83.0%
Cypermethrin	0.989	0.227	0.235	0.238	0.251	0.225	0.235	76.2%
Cypermethrin	0.987	0.192	0.175	0.184	0.172	0.188	0.182	81.6%
Fenvalerate	0.983	0.215	0.192	0.209	0.210	0.201	0.205	79.1%

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

WZ: conceptualization, original draft preparation, and editing. WS and QX: manuscript reviewing. QG and XD: supervision. HL and YR: project administration and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author YR was employed by "Guangzhou Hua shuo Biotechnology Co. Ltd."

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1069754/full#supplementary-material

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Immobilization of bacterial mixture of *Klebsiella variicola* FH-1 and *Arthrobacter* sp. NJ-1 enhances the bioremediation of atrazine-polluted soil environments

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In this study, the effects of the immobilized bacterial mixture (IM-FN) of *Arthrobacter* sp. NJ-1 and *Klebsiella variicola* strain FH-1 using sodium alginate-$CaCl_2$ on the degradation of atrazine were investigated. The results showed that the optimal ratio of three types of carrier materials (i.e., rice straw powder, rice husk, and wheat bran) was 1:1:1 with the highest adsorption capacity for atrazine (i.e., 3774.47 mg/kg) obtained at 30°C. On day 9, the degradation efficiency of atrazine (50 mg/L) reached 98.23% with cell concentration of 1.6×10^8 cfu/ml at pH 9 and 30°C. The Box–Behnken method was used to further optimize the culture conditions for the degradation of atrazine by the immobilized bacterial mixture. The IM-FN could be reused for 2–3 times with the degradation efficiency of atrazine maintained at 73.0% after being stored for 80 days at 25°C. The population dynamics of IM-FN was explored with the total soil DNA samples specifically analyzed by real-time PCR. In 7 days, the copy numbers of both *PydC* and *estD* genes in the IM-FN were significantly higher than those of bacterial suspensions in the soil. Compared with bacterial suspensions, the IM-FN significantly accelerated the degradation of atrazine (20 mg/kg) in soil with the half-life shortened from 19.80 to 7.96 days. The plant heights of two atrazine-sensitive crops (wheat and soybean) were increased by 14.99 and 64.74%, respectively, in the soil restored by immobilized bacterial mixture, indicating that the IM-FN significantly reduced the phytotoxicity of atrazine on the plants. Our study evidently demonstrated that the IM-FN could significantly increase the degradation of atrazine, providing a potentially effective bioremediation technique for the treatment of atrazine-polluted soil environment and providing experimental support for the wide application of immobilized microorganism technology in agriculture.

KEYWORDS

***Klebsiella variicola* FH-1, *Arthrobacter* sp. NJ-1, atrazine, bioremediation, immobilization**

1. Introduction

As a type of triazine herbicide with selective and translocatable activities, atrazine is primarily used to control grasses and broadleaf weeds in the fields of maize, sorghum, and other crops (Lin et al., 2019; Ma et al., 2019). Due to its high effectiveness, low toxicity, and low cost, atrazine has become one of the most widely applied herbicides in the world (Hansen et al., 2019), severely contaminating both soil and groundwater and damaging sensitive crops due to its high solubility and extended residual characteristics (Zaya et al., 2011; Zhang et al., 2014). The stability of the

ecological environment is significantly threatened by the residues of atrazine and its metabolites, interfering the endocrine systems of humans and other animals (Luo et al., 2021). The residue of atrazine stays in soil for a long time, and the degradation half-life of atrazine in soil is 13–261 days (Gao et al., 2018). Many studies have investigated the degradation of atrazine in soil. For example, Wang et al. (2011) studied the degradation of atrazine and residual atrazine in soil by GC–MS, revealing that the half-life of atrazine was 14.1 days. However, the half-life of atrazine in soils of long-term fertilization ranges from 20.6 to 33.2 days. Atrazine is also known with deleterious effects on human and other animals. For example, atrazine could trigger the neurophysiology in common carp (Wang et al., 2018) and historical damage in the liver and tests of *Astyanax altiparanae* (Destro et al., 2021). Furthermore, atrazine has shown an adverse effect on soil microbial community, which severely threatens the sustainability of agricultural soil (Singh et al., 2018). Moreover, studies have shown that exposure to atrazine could reduce the production of testosterone, decrease the sperm motility, and increase the generation of abnormal sperms (Zhu S. H. et al., 2021). Additionally, at the maximum pollutant level (MCL) of atrazine in water (3 μg/L), the atrazine in the soil environments was also toxic to almost all members of the food chain (Rostami et al., 2021). The prolonged residual pollution following the application of atrazine has attracted increasing attention worldwide, promoting the advancements of chemical treatment, adsorption, incineration, and microbiological degradation techniques to remove atrazine from the environments (Getenga et al., 2009), with the microbial remediation rapidly recognized as one of the primary strategies for removing atrazine from the ecological environment due to its advantages of low cost, high effectiveness, and environmental friendliness (Wang et al., 2016).

Microbial degradation is the process characterized by the conversion of complex organic materials into basic inorganic matters by microbes (Miller et al., 2019). To date, the microorganisms that have been isolated to efficiently degrade atrazine include fungi, e.g., white rot fungi, *Trichoderma viride*, and *Rhizopus* (Wolf et al., 2019), bacteria, e.g., *Nocardiaceae* sp. (Plaza et al., 2021), and algae (Desitti et al., 2017; Fernandes et al., 2018). Due to their high adaptability to the environment and convenient cultivation, a variety of bacteria have been widely investigated in the applications of atrazine degradation (Kolekar et al., 2019), including *Arthrobacter* sp. (Zhao et al., 2017a), *Pseudomonas* sp. (Zhao et al., 2017b), *Penicillium* sp. (Yu et al., 2018), and *Bacillus* sp. (Huang et al., 2016). For example, Bhardwaj et al. (2015) isolated the *Pseudomonas* sp. strain EGD-AKN5 to degrade atrazine with the initial concentration of 100 mg/L by 93.30% in 3.6 days, while Kolekar et al. (2014) used *Rhodococcus* sp. BCH2 to degrade atrazine at an initial concentration of 100 mg/L at 75.0% in 7 days. It has been shown that mixed bacterial interactions are more effective in the degradation of atrazine than the single strains (Xu et al., 2019). For example, Jiang et al. (2019) obtained the degradation percentage of atrazine by a single bacterial strain *Arthrobacter* sp. DNS10 at 40.57% in 48 h, which was significantly increased to 99.18% under the co-cultivation with *Enterobacter* sp. P1. Similarly, the mixture of a bacterial strain *Ralstonia pickettii* L2 and a fungal strain *Trichoderma viride* LW-1 enhanced the degradation of chlorobenzene at an initial concentration of 220 mg/L (Cheng et al., 2017). In addition, the study found that Klebsiella has the ability to remediate polluted environment and can be used as bioremediation (Duran-Bedolla et al., 2021). Development of natural polymer-based composite carriers, in combination with nano-Fe_3O_4 to form stable agar/carrageenan-Fe_3O_4-*Klebsiella pneumoniae* composite beads, which show excellent phenol biodegradation performance (Fang et al., 2021).

The novel soil bacterial strain *K. pneumoniae* GS7-1 was applied for the degradation of zearalenone (Imade et al., 2022). *Klebsiella oxytoca* GS-4-08 has a great potential for treating real nitriles-containing wastewater, and for organic acid production (Liu et al., 2017). Immobilization is a technique to immobilize free cells in a special structural area of the carrier materials and keep them active and reusable. For instance, both *Bacillus subtilis* B99–2 (Ma et al., 2015) and *Bifidobacterium* sp. BB-12 (Fritzen-Freire et al., 2012) were immobilized to improve their stability and biocontrol effects. In general, the immobilized bacteria could not only enhance the remediation of the polluted environments (Table 1), but also gain the advantages of relatively strong stability and reusability (Zhou et al., 2016).

In our previous studies, two bacterial strains with high efficiency in atrazine degradation, i.e., *Klebsiella variicola* FH-1 and *Arthrobacter* sp. NJ-1 (Gao et al., 2020), were isolated in the laboratory settings. Furthermore, compared with the separate applications of strains FH-1 and NJ-1, the biodegradability of atrazine by the mixture of these two bacterial strains was significantly improved. The purposes of this study were to generate immobilized bacterial mixture of strains FH-1 and NJ-1 and to characterize the enhanced degradation of atrazine by the immobilized bacterial mixture in soils. The immobilized systems of bacterial suspension were developed with the immobilization matrix

TABLE 1 Atrazine-degrading microorganisms with high degradation efficiency.

Microorganism	Degradation strategy	Atrazine removal efficiency	References
Rhodobacter sphaeroides W16	Free bacteria	96.86% in 15 days	Du et al. (2011)
Arthrobacter sp. DAT1	Free bacteria	>95% in 3 days	Wang et al. (2013)
Pseudomonas sp. ADP	Free bacteria	>79% in 8 days	Lima et al. (2009)
Klebsiella variicola FH-1	Free bacteria	81.5% in 11 days	Zhang et al. (2019)
Klebsiella variicola FH-1 *and Arthrobacter* sp. NJ-1	Free bacteria	85.6% in 9 days	Gao et al. (2020)
Arthrobacter sp. ZXY-2	Immobilized bacteria using corn straw biochar	50 mg/kg in 1 h	Yu et al. (2020)
Acinetobacter lwoffii DNS32	Immobilized bacteria using synthesized La^{3+} and polydopamine	100 mg/kg in 48 h	Han et al. (2022)
Agrobacterium radiobacter J14a	Immobilized bacteria using phosphorylated-polyvinyl alcohol	>40% in 120 h	Siripattanakul et al. (2008)
Pseudomonas stutzeri Y2	Polyvinyl alcohol, sodium immobilized bacteria with alginate, activated carbon, SiO_2, and nitrogen-doped TiO_2	100% in 4 days	Zhang et al. (2020)
Penicillium sp. yz11-22 N2	Magnetic bionanomaterial including Fe_3O_4	91.2% in 120 h	Yu et al. (2018)

composed of rice straw powder, rice husk, wheat bran, and sodium alginate. The optimal culture conditions of atrazine degradation by immobilized bacterial mixture were designed by the Box–Behnken method with the effects of three factors (i.e., temperature, pH level, and initial concentration of atrazine) on atrazine degradation optimized. Furthermore, the reusability and storage stability of the immobilized bacterial mixture were also evaluated. Finally, the colonization dynamics and atrazine removal efficiency by immobilized bacterial mixture in soil were verified, i.e., the phytotoxicity of atrazine on sensitive crops (soybean and wheat) grown in atrazine-polluted soils was attenuated by the treatment of the immobilized bacterial mixture. This study provides a potential and effective bioremediation technology for the treatment of atrazine-contaminated soil environment, and an effective method for maintaining the capacity of immobilized bacterial mixture based on agricultural solid waste, trying to solve the low efficiency and poor environmental adaptability of degrading bacteria in practical application, and providing a potential and promising bioremediation method for improving the genetic stability, high degradation efficiency, and strong adaptability of herbicide-contaminated soil.

2. Materials and methods

2.1. Chemicals, bacteria, media, and plants

Atrazine (purity: 97.5%) was purchased from TCI Development Co., Ltd. (Shanghai, China) and all other chemicals used in our study were of analytical grade. Both *Klebsiella variicola* strain FH-1 (GenBank accession MH250202) and *Arthrobacter* sp. strain NJ-1 (GenBank accession MH250203) were kept frozen until use at the Pesticide Science Laboratory of Jilin Agricultural University. The rice straw powder, corn straw powder, wheat bran, rice husk, vermiculite, and waste fungal substrate were provided by the Research Center of Mycology, Jilin Agricultural University. The Luria–Bertani (LB) medium contained tryptone 10.0 g/L, yeast powder 5.0 g/L, and NaCl 10.0 g/L, with pH level adjusted to 7.0–7.5. The minimal salt medium contained sucrose 35.3 g/L, NH_4Cl 10.3 g/L, $MgSO_4$ 7 H_2O 0.4 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 1.5 g/L, and NaCl 1.0 g/L, with pH level adjusted to 7.0. The meadow black soil samples were collected from the experimental maize field (43°48′49.22″N and 125°25′18.20″E) of Jilin Agricultural University, with pH 6.4, organic matter content of 2.65%, and no application of atrazine. Fresh soil samples were collected from the underground 0–20 cm in depth, dried naturally, and sieved through 40 mesh. The seeds of both wheat (*Triticum aestivum* L. variety "Jimai No. 3") and soybean (*Glycine max* L. variety "Ji Da No.1") were obtained from the College of Plant Protection of Jilin Agricultural University and the College of Plant Science of Jilin University, respectively.

2.2. Preparation of the bacterial suspensions

Single colonies were picked from FH-1 and NJ-1 solid media and transferred to LB liquid media, incubated at 30°C and 150 r/min for 12 and 18 h, respectively. Then, the samples were centrifuged for 5 min at 7,000 rpm, with the supernatant removed; the bacterial samples of strains FH-1 and NJ-1 were then suspended with sterile water and mixed in a volume ratio of 3:2 (V:V), which was the bacterial suspensions (FN; 4.0×10^9 cfu/ml; Gao et al., 2020).

2.3. Screening of carrier materials

A total of six types of carrier materials, i.e., rice straw powder, corn straw powder, wheat bran, rice husk, vermiculite, and waste fungal substrate (sieved through 100 mesh), were weighed 5 g each and loaded into six 250 ml triangular flasks, and sterilized at 121°C for 20 min before cooling and inoculating with 10 ml of FN. The samples were shaken to mix and then air-dried at 30°C to prepare the carrier materials containing bacterial suspensions. The obtained samples were stored at 4°C and 25 ± 5°C (room temperature), respectively. The viable bacterial cell counts in different carrier materials were measured at 2 h, 10 days, 20 days, 40 days, 60 days, and 90 days. Each experiment was repeated three times to determine the optimal carrier materials and storage temperature.

2.4. Degradation of atrazine by the bacterial suspensions with varied ratios of carrier materials

The optimal carrier materials screened, i.e., rice straw powder, rice husk, and wheat bran, were mixed with the ratios of 1:1:1, 1:2:1, 1:1:2, and 2:1:1, respectively. A total of 5 g of each mixture of carrier materials were collected and autoclaved at 121°C for 20 min, added into the minimal salt medium containing 50 mg/L atrazine with or without 10 ml FN, respectively, as two treatment groups, shaken and mixed, ventilated, and dried at 30°C. The control experiment contained only atrazine. The concentration of atrazine was determined at 0, 1, 3, 5, 7, and 9 days using high performance liquid chromatogram (HPLC), respectively.

2.5. HPLC analysis

The concentration of atrazine was measured using HPLC analysis (Agilent 1260). The wavelength was set to 222 nm on the UV detector using a reverse-phase column C_{18} (4.6 × 250 mm, 5 μm) with a flow rate of 1.0 ml/min (methanol/water = 60/40, v/v), column temperature of 30°C, and an injection volume of 10 μl. The limit of detection for atrazine was 3 ng, and the limits of quantification for atrazine in water and soil samples were 0.03 and 0.02 mg/kg, respectively.

2.6. Adsorption of atrazine by the carrier materials

2.6.1. Immobilization of carrier materials

Based on the optimal ratio of carrier materials (M) obtained above, the carrier materials were added with 3% sodium alginate (w/v) to generate the immobilized carrier materials (IM). The mixture was extruded in droplets into 2% sterile calcium chloride solution (w/v) *via* a syringe and cross-linked at 4°C for 24 h to obtain the IM.

2.6.2. Adsorption kinetics

To increase the ionic strength of the solution, a total of 1.1098 g of $CaCl_2$ was weighed and dissolved in 1,000 ml of water to prepare the sterile 0.01 mol/L $CaCl_2$ solution. A total of 50 ml of 0.01 mol/L $CaCl_2$ solution were added to a 250 ml glass vial containing 50 mg/L atrazine to generate three treatments each of 0.2 g of M, 0.2 g of IM, and blank control, respectively. The samples collected in 1, 2, 4, 6, 8, 10, 12, 24, and

48 h were placed in a vibration shaker at 30°C and 150 rpm, filtered with a 0.22 μm filter membrane (Wang et al., 2020), and analyzed by HPLC. All experiments were performed in three replicates with data presented as mean ± standard deviation (SD).

2.6.3. Adsorption isotherms

Based on the results of adsorption kinetics experiment, the adsorption isotherm experiments were performed using the same procedures with initial concentration of atrazine set to 0.5, 5, 10, 20, and 50 mg/L, respectively. The samples were placed in a vibration shaker at 150 rpm for 48 h at three different temperatures of 20, 25, and 30°C to achieve the adsorption equilibrium (Magid et al., 2021). Samples were collected at 0 and 24 h to measure the adsorption. All experiments were performed with three biological replicates.

2.7. Preparation of immobilized bacterial mixture

The FN, M, and 3% sodium alginate were mixed and collected in droplets into 2% sterile $CaCl_2$ solution (w/v) *via* a syringe and cross-linked at 4°C for 24 h to prepare the immobilized bacterial mixture (IM-FN). The IM-FN obtained was loaded with bacteria of about 1.6×10^9 cells (cfu/g). The IM without bacteria was prepared and stored separately in a refrigerator at 4°C. The morphological features of carrier materials (M), immobilized carrier materials (IM), carrier materials containing bacteria suspensions, and immobilized bacterial mixture (IM-FN) were observed using the scanning electron microscopy (SEM; Zeiss EVO18, Jena, Germany).

2.8. Atrazine degradation assays

To determine the optimal culture conditions for the biodegradation of atrazine by IM-FN, the atrazine was incubated in 100 ml of minimal salt medium with varied bacterial cell concentration (i.e., 4.0×10^7, 8.0×10^7, 1.2×10^8, 1.6×10^8, and 2.0×10^8 cfu/ml), atrazine concentration (i.e., 10, 20, 50, 100, and 200 mg/L), pH levels (i.e., 6, 7, 8, 9, and 10), and temperature (i.e., 20, 25, 30, 35, and 40°C), shaken at 150 rpm, with the residual concentration of atrazine and OD_{600} measured in 9 days. The controls contained only atrazine without bacterial inoculation. The degradation efficiency of atrazine by immobilized bacterial mixture and bacterial suspensions was calculated using the degradation efficiency of atrazine of the control as the baseline (Bhatt et al., 2022). Then, the Box–Behnken module of the design-Expert.V8.0.6 was used to design the experiments with the three factors (i.e., temperature, pH level, and initial concentration of atrazine) affecting the degradation of atrazine by IM-FN optimized for response surface analysis (Supplementary Table 2).

Degradation efficiency of atrazine = $(C_0 - C_1)/C_0$, where C_0 represented the content of atrazine in minimal salt medium without inoculum and C_1 represented the content of atrazine in minimal salt medium with bacterial suspensions and immobilized bacterial mixture (Zhao et al., 2022).

2.9. Stability and reusability of immobilized bacterial mixture

A total of 10 g IM-FN were added to 100 ml minimal salt medium containing 50 mg/L atrazine with the degradation percentage of atrazine measured every 20 days for a total of 120 days. The IM-FN was incubated in 100 ml of minimal salt medium containing 50 mg/L atrazine for 9 days. Then, the IM-FN was removed, rinsed 3–5 times with sterile water, and then added to a new minimal salt medium (100 ml) containing the same concentration of atrazine; this process was repeated five times with the morphology of the IM-FN observed and the degradation percentage of atrazine measured each time.

2.10. Cloning of bacterial genes in soils colonized with immobilized bacterial mixture

Both FN and IM-FN were added to plastic pots (9 × 9 cm) filled with soil samples, respectively. The soil samples were collected after 3, 5, 7, 14, and 21 days and stored in the refrigerator at 4°C. Soil DNA extraction kit (SPINeasy DNA Kit for Soil, MP Biomedicals, LLC, Solon, OH, United States) was used to extract total soil DNA based on 1 g of soil sample from different treatments. The Zn^{2+}-dependent hydrolase gene *PydC* (Zhang J. P. et al., 2021) and the esterase gene *estD* (Dong, 2019) were cloned from strains FH-1 and NJ-1, respectively.

The standard curve was plotted using the quantitative real-time PCR (qRT-PCR) method for absolute quantification. The total DNA of the soil samples treated with FN and IM-FN was used to perform the qRT-PCR (10 μl reaction) as described in Supplementary material. The total copy numbers of *PydC* and *estD* genes were calculated by the standard curves, with the dynamics of colonization of strains FH-1 and NJ-1 in soil as the functional coordinate and IM-FN expressed in time as horizontal coordinate. The primers used in the PCR are provided in Supplementary Table 1.

2.11. Degradation of atrazine in soils by immobilized bacterial mixture

Soil samples were randomly collected underground 0–15 cm, air-dried, and sieved to 2 mm, from the experimental field of Jilin Agricultural University (43°48′49.22″N and 125°25′18.20″E) without atrazine application for at least 2 years to determine the degradation effect of IM-FN on atrazine in the soil. The atrazine solution was added to soil and mixed well with the concentration of atrazine in soil adjusted to 20 mg/kg and the water content adjusted to 20% with sterile water to obtain the contaminated soil sample. A total of four experimental treatments were prepared with: (1) soil spiked with 20 mg/kg atrazine; (2) soil spiked with 20 mg/kg atrazine and FN (1.6×10^8 cfu/g dry soil); (3) soil spiked with 20 mg/kg atrazine and IM-FN (1.6×10^8 cfu/g dry soil); and (4) soil spiked with 20 mg/kg atrazine and IM. Each treatment was incubated at 25°C with 20 g soil samples collected in 0, 1, 3, 7, 14, 21, 28, and 35 days to determine the contents of atrazine in soil.

2.12. Detection of atrazine in soil

To determine the residue of atrazine, a total of 20 g of the soil sample were added with 50 ml of acetonitrile and 10 ml of water in a 250 ml Erlenmeyer flask, shaken for 40 min at 30°C, and the filtrate was obtained by vacuum filtration and transferred to a 100 ml stopper cylinder containing ~8 g of NaCl. The stopper cylinder was shaken up and down 100 times and allowed to stand for 30 min. Again, the stopper cylinder was shaken 150 times and kept still for another 1 h. The

top 25 ml of acetonitrile solution was removed and the extracts were evaporated to nearly completely dry at 40°C under reduced pressure using a rotary evaporator. Finally, the evaporated residues were mixed with 1 ml acetonitrile by vortexing for 1 min and then filtered through a PTFE filter (0.22 μm) for HPLC analysis.

2.13. Effect of immobilized bacterial mixture on the growth of atrazine-sensitive crops

Three soil treatments were prepared to investigate the alleviation effect of IM-FN on the phytotoxicity of atrazine, including soil sample without atrazine (CK), soil sample with atrazine at concentration of 0.1 mg/kg (A), and soil sample with both atrazine at concentration of 0.1 mg/kg and IM-FN (1.6×10^8 cfu/g soil), incubated for 7 days. The seeds of wheat and soybean were soaked in water, germinated, and selected and sown in each treatment of soil with 10 seeds per pot. The seedlings were grown at 25°C under constant light with the seedling emergence percentage measured on day 3 and the plant height measured every 15 days.

2.14. Data analysis

Results of the measurements were given as the average ± standard deviation (SD) of each treatment. The one-way analysis of variance (ANOVA) was performed with DPS software version 7.05 to determine the significant differences between treatments based on a p value of 0.05. All experiments were performed in three biological replicates.

3. Results

3.1. Selection of carrier materials

The effective viable bacterial cell numbers grown with rice straw powder, rice husk, wheat bran, corn straw powder, waste fungal substrate, and vermiculite stored under varied conditions are shown in Table 2. The numbers of viable cells in these carrier materials were in the following order: rice straw powder > rice husk > wheat bran > corn straw powder > abandoned fungus substrate > vermiculite. From 10 to 90 days, varied numbers of adsorbed bacteria were revealed under two culture temperatures, showing higher bacterial growth rate at room temperature (25 ± 5°C) than that of 4°C in 90 days. Based on these results, the top three types of carrier materials (i.e., rice straw powder, rice husk, and wheat bran) with the highest viable cell numbers at room temperature (25 ± 5°C) were selected as the immobilizing materials in this study.

3.2. Determination of the optimal ratios of carrier materials

The degradation of atrazine by carrier materials (i.e., rice straw powder, rice husk, and wheat bran) in four ratios and bacteria is shown in Figure 1. The results showed that the carrier materials alone could adsorb atrazine in the culture medium with varied adsorption capacities. In particular, the highest adsorption efficiencies of atrazine in the culture medium were obtained at 8.65 and 7.49% based on the ratio of these three types of carrier materials (i.e., rice straw powder, rice husk, and wheat bran) at 1:1:1 and 1:2:1, respectively, in comparison with those of 1:1:2 (4.68%) and 2:1:1 (6.31%). With the FN added to the inorganic salt culture medium containing the carrier materials at the ratio 1:1:1 and atrazine (7.50 mg/L), the degradation percentage of atrazine reached the highest 83.29% in 9 days. These results suggested that the highest compatibility was achieved between the carrier materials with rice straw powder, rice husk, and wheat bran mixed in the ratio of 1:1:1 to obtain the highest degradation percentage of atrazine.

3.3. Adsorption kinetics and isotherms of immobilized carrier materials

3.3.1. Adsorption kinetics of immobilized carrier materials

In order to explore the adsorption mechanism and absorption percentage of atrazine by the immobilized carrier materials, both pseudo-first-order kinetics and pseudo-second-order kinetics models

TABLE 2 Effective number of living bacterial cells (×10^6 cfu/g) grown with different immobilizing materials at different storage temperature and time.

Material	Storage temperature	2 h	10 days	20 days	40 days	60 days	90 days
Rice straw powder	4°C	52	283	4,064	3,552	2,941	1,542
	25 ± 5°C	58	422	3,520	3,782	3,111	2,893
Corn straw powder	4°C	6.5	204	640	124.3	62.1	30.1
	25 ± 5°C	6.3	35.3	236	149	121.3	102.3
Vermiculite	4°C	0.8	5.8	1.2	1.8	–	–
	25 ± 5°C	1.0	70.3	19.8	2.8	0.8	–
Wheat bran	4°C	12	42.4	328	271	254	142
	25 ± 5°C	29	66.7	803	669	304	203
Rice husk	4°C	53	206	4,650	3,200	2,565	1,310
	25 ± 5°C	47.7	355	3,000	3,350	3,030	2,779
Waste fungal substrate	4°C	5.3	9.8	11.7	1.49	0.41	–
	25 ± 5°C	5.6	11.7	12.9	11.1	9.6	9.2

Symbol "–" indicates data not detected.

were used to fit the adsorption results (Figure 2). As shown in Figure 2A, the rapid adsorption occurred in the first 4 h and the pesticide removal percentage reached about 90%. Then, the adsorption was slowed down for about 8 h, and finally reached the predetermined adsorption equilibrium in 24 h. During the rapid adsorption, the adsorption percentage of atrazine by immobilized carrier materials was evidently higher than that of the composite of carrier materials. The maximum adsorption capacities of atrazine by the composite of M and IM reached 2801.12 mg/kg and 3802.281 mg/kg, respectively. The fitting parameters of pseudo-first-order kinetics model ($R^2 = 0.98$ and 0.98) of the adsorption of atrazine by IM and M were lower than those of pseudo-second-order kinetics model ($R^2 = 0.99$ and 0.99; Figure 2; Table 3). These results suggested that the pseudo-second-order kinetics model was more suitable for describing the adsorption kinetics of atrazine by M and IM, while the chemical adsorption was probably the main adsorption mode of atrazine by IM and M (Figure 2B).

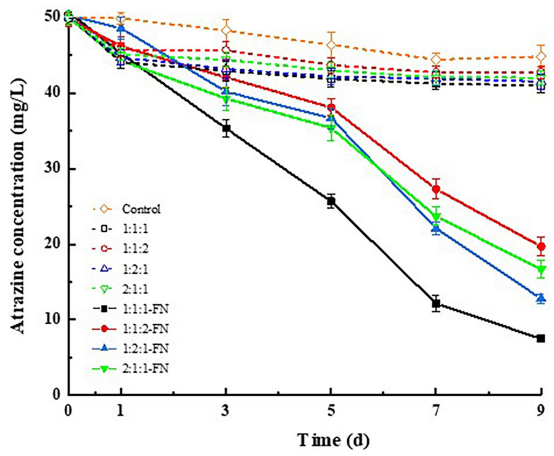

FIGURE 1
Degradation effect of atrazine by three types of carrier materials (i.e., rice straw powder, rice husk, and wheat bran) at varied ratios with or without the bacterial suspensions (FN).

3.3.2. Adsorption isotherms of immobilized carrier materials

The adsorption isotherms of atrazine on the IM at different temperatures are shown in Figure 3. The fitting parameters and coefficients were determined by both Freundlich and Langmuir models (Table 4). The results showed that the Langmuir model fitted better the adsorption process of atrazine on IM and M than the Freundlich model, as indicated by R^2 value greater than 0.97 and the decreased nonlinear coefficient (n) with the increase of temperature. Therefore, the Langmuir equation was more suitable for describing the adsorption of atrazine than the Freundlich equation. With the increased concentration of atrazine, the adsorption of atrazine on IM and M was increased rapidly, and then reached a stabilized period. The results showed that different temperatures also affected the adsorption capacity of IM and M of atrazine. With the increase of temperature from 20 to 30°C, the maximum adsorption capacities of atrazine by IM and M were increased from 2994.77 to 3648.42 mg/kg and from 2668.74 to 3190.55 mg/kg, respectively, with the adsorption capacity of IM constantly greater than those of M at different temperatures.

3.4. Morphological observations of immobilized bacterial mixture using scanning electron microscopy

The morphological features based on SEM observations of the various types are presented in Figure 4. Compared with the carrier materials (Figure 4A), the bacterial suspensions were directly adsorbed on the relatively wrinkled surfaces and the bacteria formed small bubbles (Figure 4C). The carrier materials immobilized with sodium alginate-$CaCl_2$ showed dense outer surface with cracks, micropores, and ridges (Figure 4B). Careful examination of cracks and internal structures of the IM-FN revealed bacterial cells embedded in cellulose tubes in the carrier materials (Figure 4D). In our study, rice straw powder, wheat bran, and rice husk with dense fiber structure were used as additives. The SEM images showed that the rice straw powder, wheat bran, and rice hull embedded with

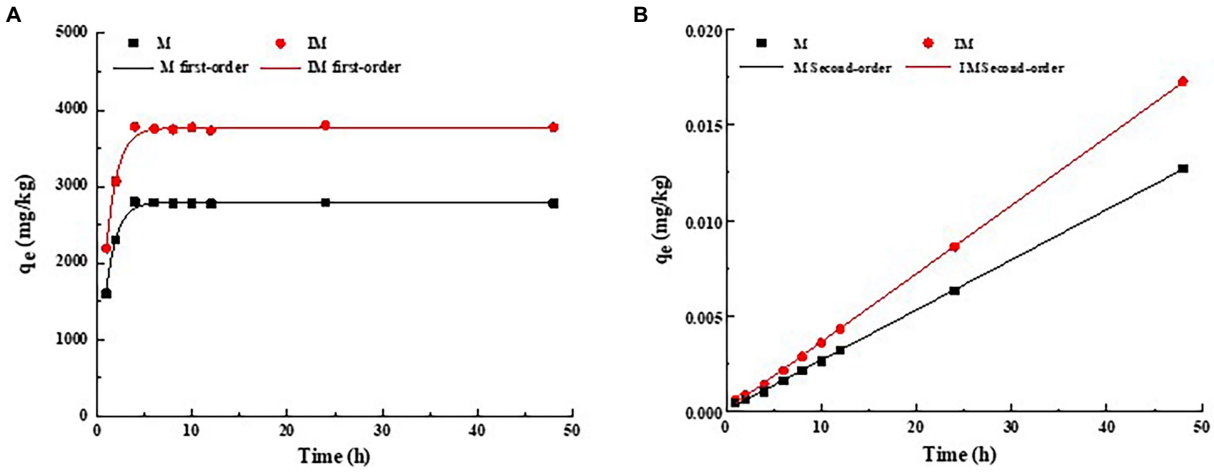

FIGURE 2
Adsorption pseudo-first-order kinetics **(A)** and pseudo-second-order kinetics plots fitting of t/qt **(B)** of atrazine on carrier materials (M) and immobilized carrier materials (IM).

TABLE 3 Atrazine adsorption kinetics parameters described on carrier materials (M) and immobilized carrier materials (IM) by the pseudo-first-order kinetics model (R^2=0.98) and pseudo-second-order kinetics model (R^2=0.99).

Sample	First-order kinetics			Second-order kinetics		
	q_e	K_1	R^2	q_e	K_2	R^2
M	2794.14 ± 15.07	0.88 ± 0.04	0.98	2801.1 ± 2.28	$(29.43 \pm 16.59) \times 10^{-4}$	0.99
IM	3764.97 ± 11.68	0.87 ± 0.05	0.98	3802.28 ± 1.63	$(22.38 \pm 10.23) \times 10^{-4}$	0.99

q_e represents the amount of atrazine (mg/kg) removed at equilibrium; k_1 and k_2 represent the first-order and second-order adsorption percentage constants (1/h), respectively. R^2 represents the fitting parameters of each model.

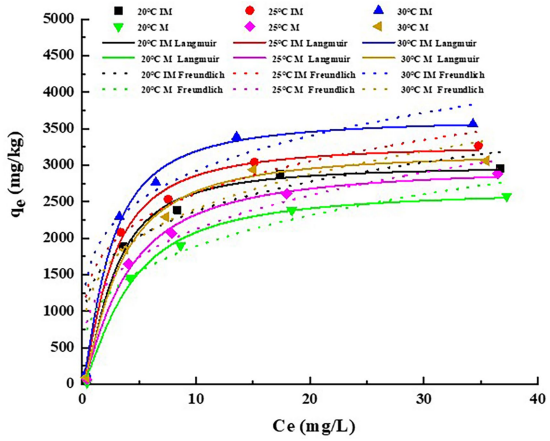

FIGURE 3
Adsorption isotherms plots of atrazine on carrier materials (M) and immobilized carrier materials (IM). q_e represents the adsorption capacity of M and IM in equilibrium at different temperatures.

sodium alginate contained numerous cellulose tubes, which increased the surface area and porosity, and were conducive to the adhesion of bacteria.

3.5. Characteristics of atrazine degradation by immobilized bacterial mixture

The effects of varied culture conditions (i.e., pH level, cell concentration of bacteria, temperature, and atrazine concentration) on the degradation of atrazine by IM-FN and FN were investigated to evaluate the practical application potential of IM-FN in the degradation of atrazine. The results showed that the degradation efficiency of atrazine was gradually increased with the increase of bacterial cell concentration (Figure 5A). At the cell concentration of 4.0×10^7 cfu/ml, the degradation efficiency of atrazine by FN and IM-FN were 67.23 and 70.76%, respectively. As the cell concentration was increased to 1.6×10^8 cfu/ml, the highest degradation efficiencies of atrazine by FN and IM-FN reached 89.81 and 96.00% in 9 days, respectively. As the cell concentration was increased to 2.0×10^8 cfu/ml, the degradation efficiency of atrazine by IM-FN was decreased slightly.

Different concentrations of atrazine of 10–200 mg/L significantly affected the degradation of atrazine by FN and IM-FN (Figure 5B). The results showed that the highest degradation efficiency was obtained when the concentration of atrazine was 50 mg/L. In particular, when the concentration of atrazine was 10 mg/L, the degradation efficiencies of atrazine by FN and IM-FN were 41.43 and 46.83%, respectively. With the increase of atrazine concentration, the degradation efficiencies of atrazine by FN and IM-FN were gradually increased. When the concentration of atrazine was increased to 50 mg/L, the degradation efficiency of atrazine by FN and IM-FN reached the highest of 86.13 and 95.70%, respectively, in 9 days. With the increase of atrazine substrate concentration from 10 to 200 mg/L, the degradation efficiency of atrazine by IM-FN was significantly higher than those of FN.

The effects of temperature on the degradation of atrazine are shown in Figure 5C. The highest degradation efficiencies of atrazine were obtained at 92.72 and 98.18% for FN and IM-FN, respectively, at 30°C in 9 days. As the temperature was increased from 20 to 30°C, the degradation efficiencies of atrazine by both IM-FN and FN were increased. As the temperature was increased from 30 to 40°C, the degradation of atrazine was obviously inhibited with the degradation efficiencies of FN and IM-FN obtained at only 40.81 and 68.49%, respectively, at 40°C. Our results revealed poor resistance of FN to temperature in the environment, i.e., when the temperature was higher than 30°C, the atrazine degradation ability of the FN was significantly lower than that of the IM-FN. The effect of pH level also affected the degradation of atrazine by IM-FN and FN (Figure 5D). The highest degradation efficiencies of atrazine by both FN and IM-FN were obtained at pH 7. At pH 9.0, the degradation efficiencies of atrazine by FN and IM-FN were 92.98 and 98.23%, respectively, in 9 days. At pH 6.0, the inhibition of atrazine degradation by FN was higher than that by IM-FN, showing the degradation efficiencies of 67.36 and 85.35%, respectively.

3.6. The Box–Behnken analyses

The Box–Behnken design-response surface methodology was used to determine the key factors that affected the degradation percentage of atrazine by the FN and IM-FN. The experiments were performed in minimal salt medium supplemented with atrazine for 9 days. The degradation percentage of atrazine ranging from 65.92 to 98.59% were optimized by three crucial factors, i.e., pH level, initial concentration of atrazine, and temperature. The results of the variance analysis with the best optimization model of atrazine biodegradation are provided in Supplementary Table 3. The regression equation was obtained to represent the atrazine degradation percentage Y with three influencing factors A (pH level), B (temperature), and C (initial concentration of atrazine).

$$Y = 97.46 + 0.2475\,A + 0.4663\,B + 1.05\,C - 0.33\,AB + 0.98\,AC - 1.05\,BC - 12.45\,A^2 - 14.54\,B^2 - 15.31\,C^2$$

The magnitude of *F*-value represented the order of influence of different factors on the response value, i.e., A = 0.37, B = 1.32, and

TABLE 4 Atrazine adsorption parameters of the isotherms on carrier materials (M) and immobilized carrier materials (IM) described by the Freundlich and Langmuir equations.

Sample	Langmuir equation				Freundlich equation		
	T/*K*	q_e	*K*	R^2	K_f	*n*	R^2
M	293	2668.74 ± 157.77	0.15 ± 0.061	0.99	962.24 ± 12.57	0.29 ± 0.004	0.88
	298	2994.88 ± 176.85	0.18 ± 0.061	0.99	1104.85 ± 13.10	0.28 ± 0.004	0.89
	303	3190.55 ± 184.80	0.20 ± 0.08	0.99	1320.78 ± 16.03	0.26 ± 0.04	0.86
IM	293	2994.77 ± 148.38	0.22 ± 0.098	0.98	1404.24 ± 17.67	0.23 ± 0.004	0.81
	298	3298.78 ± 200.15	0.28 ± 0.114	0.98	1550.20 ± 17.45	0.23 ± 0.004	0.84
	303	3648.42 ± 154.27	0.30 ± 0.085	0.99	1763.04 ± 19.40	0.22 ± 0.04	0.83

q_e represents the adsorption amount at equilibrium; K_f represents the Freundlich affinity coefficient [(mg/kg)/(mg/L)]; *n* is the Freundlich constant; *K* represents the adsorption affinity coefficient (L/kg). R^2 represents the fitting parameters of each model.

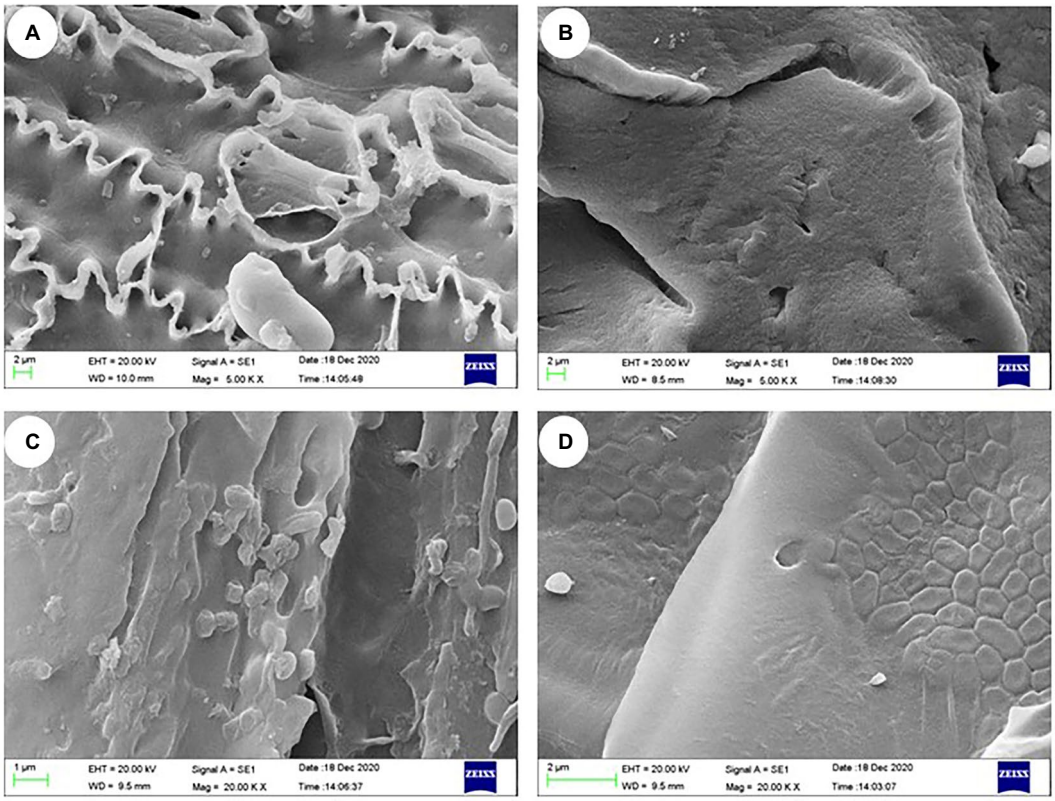

FIGURE 4
Scanning electron microscope observations of carrier materials **(A)**, immobilized carrier materials **(B)**, carrier materials containing bacterial suspensions **(C)**, and immobilized bacterial mixture (IM-FN) **(D)**.

C = 6.67, respectively, indicating that the ability of the bacteria to degrade atrazine was influenced by these three factors in the following order: initial concentration of atrazine (C) > temperature (B) > pH level (A) (Supplementary Table 4). The contour plot and the 3D response surface plot showing the optimized culture conditions were evaluated (Supplementary Figure 1). These plots revealed the greater effect of the initial concentration of atrazine and pH level on the degradation of atrazine by IM-FN. The most influential degradation conditions for atrazine degradation by IM-FN were predicted to be pH 9.15, temperature 30.03°C, and initial concentration of atrazine 62.17 mg/kg, with the highest atrazine degradation percentage obtained at 98.19% based on the optimization using the response surface analysis. These predicted conditions were validated by the degradation percentage of atrazine at 98.19 ± 0.5%, indicating that it was appropriate to use the response surface analysis to predict these three factors on the degradation of atrazine by IM-FN.

3.7. Stability and reusability of immobilized bacterial mixture

The stability of IM-FN was investigated due to its importance in improving the degradation percentage of atrazine (Figure 6A). The results showed that the IM-FN maintained its initial degradation

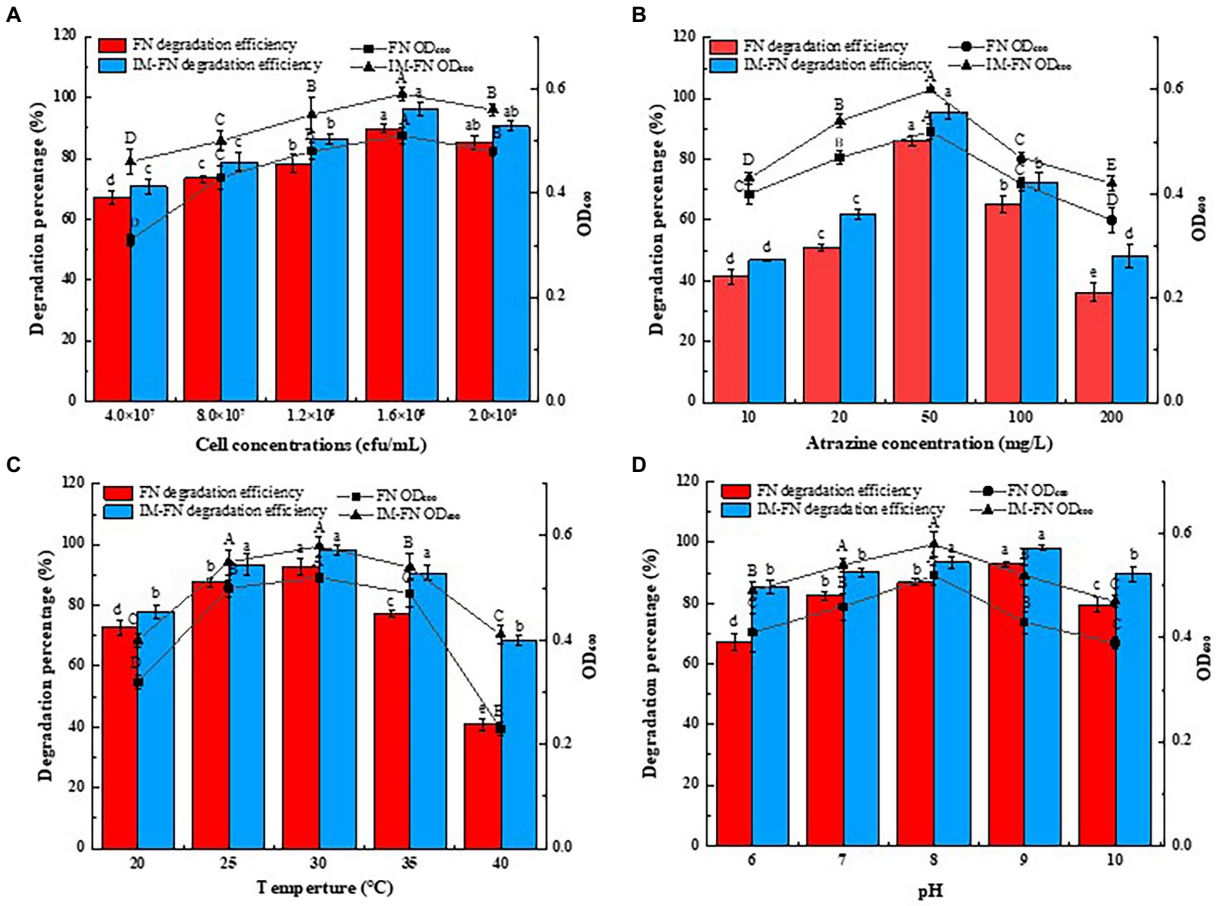

FIGURE 5
Effects of culture conditions, i.e., bacteria concentration **(A)**, atrazine concentration **(B)**, temperature **(C)**, and pH level **(D)** on degradation of atrazine by bacterial suspensions (FN) and immobilized bacterial mixture (IM-FN). The lowercase letters above the bars indicate significant differences between different treatments at the level of $p<0.05$; the uppercase letters indicate significant differences between different treatments at the level of $p<0.01$. Error bars represent standard deviation of triplicate samples.

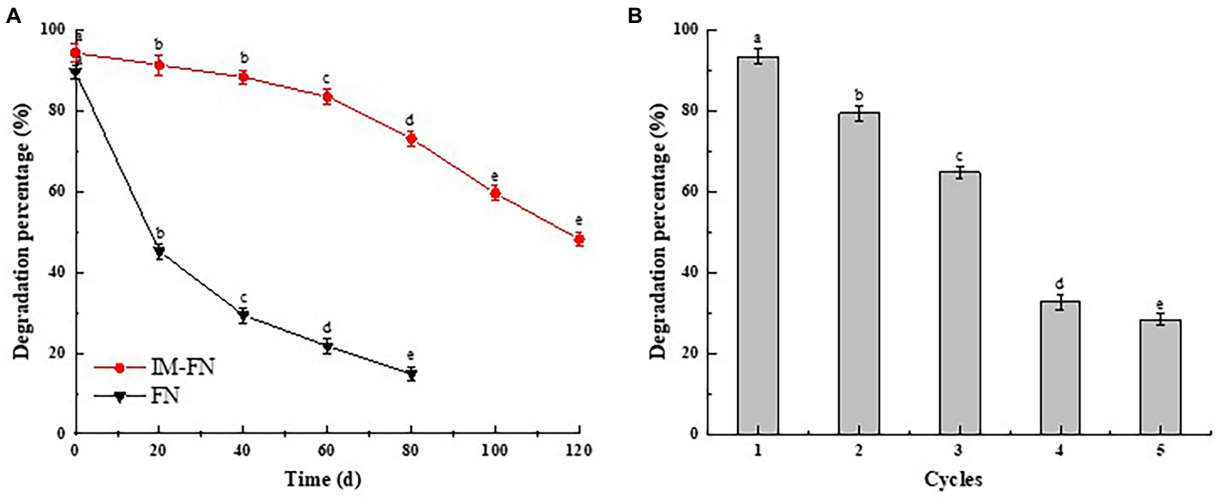

FIGURE 6
The storage stability **(A)** and reusability **(B)** of immobilized bacterial mixture (IM-FN). Different letters (a, b, c, and d) indicate significant difference between the same treatment at different times based on Duncan's significant difference test ($p\leq0.05$).

efficiency of 88.25% after being stored at room temperature (25°C) for 40 days, whereas the degradation efficiency of atrazine by FN decreased to 29.36%. In 80 days of storage, the degradation efficiency of atrazine by IM-FN could still reach 73.02%, whereas the degradation of atrazine was not detected by the FN, indicating the high stability of the IM-FN with high degradation efficiency maintained.

The reusability, i.e., the effect of repeated use of IM-FN on atrazine degradation, is shown in Figure 6B. With the increased times of reuse of IM-FN, the degradation percentage of atrazine was gradually decreased. In the third cycle, the degradation efficiency of atrazine still reached 64.80%, whereas in the fourth cycle, the degradation percentage of atrazine was significantly decreased to 32.7%, while the IM-FN began to swell and partially break. These results showed that the IM-FN could be used 2–3 times. To summarize, these results revealed higher reusability and stability of IM-FN than those of FN, providing a potentially feasible bioremediation strategy of the atrazine-polluted soil environments.

3.8. Soil colonization by immobilized bacterial mixture

The specificity of strains FH-1 and NJ-1 in FN and IM-FN was detected by PCR using the total DNA of soil samples as template. The results showed that no amplification of the target genes (i.e., Zn^{2+}-dependent hydrolase gene *PydC* and esterase gene *estD*) was detected in the total DNA of untreated soil samples (Supplementary Figure 2), while both target genes were amplified in the total DNA of soils supplemented with either FN or IM-FN, respectively (Supplementary Figure 3), indicating the high specificity of strains FH-1 and NJ-1 in soils, and the real-time PCR system based on these two genes could be used to quantify the colonization dynamics of strains FH-1 and NJ-1 in these soil samples. The copy numbers of *PydC* and *estD* genes of strains FH-1 and NJ-1 in the soil treated with FN were significantly higher than those of the IM-FN on the third day, while the copy numbers of these genes of both strains were gradually decreased in 7 days (Figures 7A,B). These results indicated that strains FH-1 and NJ-1 in FN could colonize the soil and become the dominant strains within a short period of time (i.e., less than 3 days), though this effect was not maintained. Furthermore, the detection percentage of target genes of these two bacterial strains in soil treated with IM-FN was significantly lower than that of FN in 3 days. However, the copy numbers of both genes were significantly higher than those of the FN after 7 days, and this effect was maintained until 21 days. These results suggested that IM-FN could enhance the duration of the distribution of both strains in the soil.

3.9. Degradation of atrazine by immobilized bacterial mixture in contaminated soils with different treatments

As shown in Figure 8, the degradation of atrazine in soil under different treatments of atrazine, IM, FN, and IM-FN followed the first-order equations ($R^2 > 0.93$) of c = 20.884e − 0.023t, c = 20.515e − 0.025t, c = 20.889e − 0.035t, and c = 24.824e − 0.087t, with the degradation half-life of atrazine under the laboratory conditions of 30.13, 27.72, 19.80, and 7.96 days, respectively. These results showed that the introduction of exogenous atrazine-degrading bacteria and IM-FN into the soil significantly promoted the dissipation of atrazine and shortened the half-life of atrazine. In 35 days, the degradation efficiencies of atrazine by IM-FN and FN in soil reached 96.84 and 73.97%, respectively, Our study showed that in 35 days, the degradation efficiency of atrazine in soil by FN reached only 73.97%, suggesting that the degradation of atrazine by direct spraying of bacterial suspension could be easily affected by the uncertain characteristics of soil matrix. However, compared with the FN, the IM-FN showed high applicability in the atrazine-polluted soils and increased degradation efficiency by 22.87% in 35 days, evidently indicating that the combination of atrazine-degrading bacteria and immobilizing materials could more effectively remediate the atrazine-contaminated soil environments.

3.10. Effect of immobilized bacterial mixture on the growth of wheat and soybean plants

The application of IM-FN in soil showed significant effects on seedling emergence and plant height of both wheat and soybean (Figures 9A,B). With the application of both atrazine at a concentration

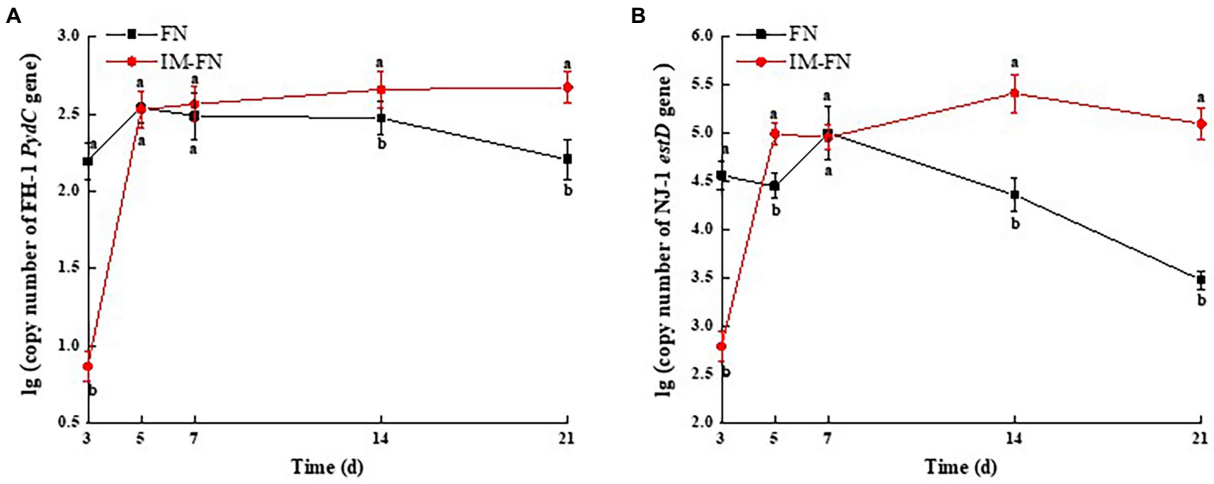

FIGURE 7
Dynamics of the colonization of strains *Klebsiella variicola* FH-1 and *Arthrobacter* sp. NJ-1 in bacterial suspensions (FN) and immobilized bacterial mixture (IM-FN) in soil for 3, 5, 7, 14, and 21 days. **(A)** The copy number of the *PydC* gene of strain FH-1 in the soils of bacterial suspensions (FN) and immobilized bacterial mixture (IM-FN) at different times. **(B)** The copy number of *estD* gene of strain NJ-1 in the soils of bacterial suspensions (FN) and immobilized bacterial mixture (IM-FN) at different time. Letters a and b indicate significant difference between the same treatment at different times based on Duncan's significant differences test ($p \leq 0.05$).

of 0.10 mg/kg and 10 g of IM-FN in soil, the seedling emergence percentage of wheat and soybean were 76.00 and 88.00%, and the plant heights were increased by 14.99 and 64.74%, respectively, compared with the group in the soil without IM-FN. These results indicated that the addition of IM-FN could promote the degradation of residual atrazine in the soil to reduce the harmful effects of the phytotoxicity of atrazine on wheat and soybean plants.

4. Discussion

4.1. Adsorption effect of immobilized carrier materials on atrazine

Our results showed that in 4 h, the atrazine was quickly adsorbed by both IM and M. This was probably because the high concentration of atrazine at the interface between both M and IM and the bacterial solution promoted a strong driving force of mass transfer, ultimately causing atrazine to quickly occupy the adsorption sites, which allowed the rapid physical adsorption of atrazine in a short time. These results were consistent with those reported previously (Ren et al., 2022). With the slow saturation of the active sites, the resistance was gradually increased, which was due to the fact that the immobilization technology improved the mechanical strength and chemical stability of the carrier materials at different degrees and the pore structure of the carrier materials, ultimately enhancing the total adsorption capacity of atrazine (Yu et al., 2018). Furthermore, the pore volume of M was smaller and atrazine could reach the adsorption equilibrium quickly, though the adsorption capacity was relatively limited, which as consistent with the results reported previously (Hu et al., 2021). Overall, our results revealed a stronger ability to adsorb atrazine by IM than M.

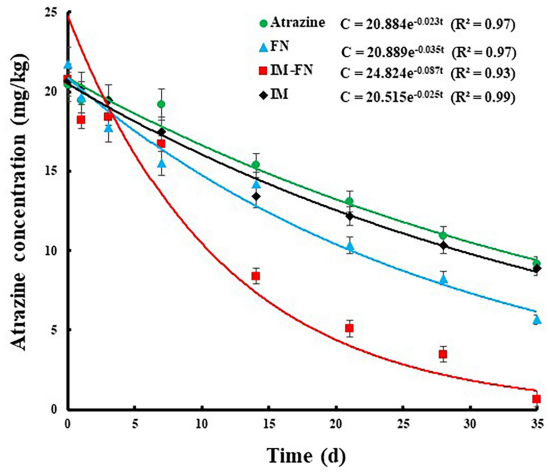

FIGURE 8
Degradation effect of atrazine in soil treated with atrazine, bacterial suspensions (FN), immobilized bacterial mixture (IM-FN), and immobilized carrier materials (IM).

Both Langmuir and Freundlich models were used to simulate the adsorption process of atrazine, showing that Langmuir model was more consistent and suitable than Freundlich model to describe the adsorption process. The results showed that the temperature increase in a certain range could enhance the adsorption capacity of atrazine by carrier materials. Studies have shown that the adsorption of atrazine by IM and M is mainly the monolayer adsorption of the chemical adsorption (Macías-García et al., 2017). With the adsorption capacity of atrazine by IM constantly higher than that of M and increased with the increase of temperature. This was probably because that the increase of temperature caused a positive effect of carrier materials on the adsorption of atrazine, indicating that the adsorption of atrazine was an endothermic process. Furthermore, the increase of pore size of composite carrier materials at high temperature was probably one of the reasons for the increased adsorption capacity of adsorbent, as reported previously (Ali et al., 2022).

4.2. Scanning electron microscopy observation of immobilized bacterial mixture

Compared with other types of carrier materials such as polyvinyl alcohol used in bioremediation technology, alginate generally shows

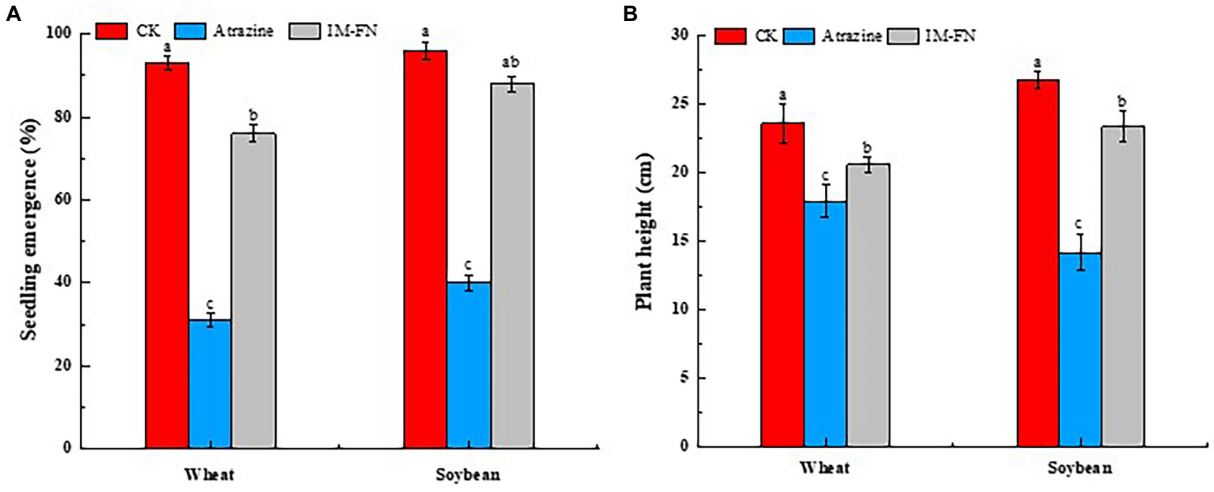

FIGURE 9
Seedling emergence percentage in 3 days **(A)** and plant height in 15 days **(B)** of wheat and soybean plants grown in soils treated with immobilized bacterial mixture (IM-FN). Different letters (a, b, and c) indicate significant differences between different treatments at the level of $p<0.05$.

smaller mass transfer resistance (Zhang et al., 2022). The carboxyl group of sodium alginate could exchange with Ca^{2+} and cross-link to form calcium alginate beads. Studies have shown that the SEM observations suggested that adding specific additives to sodium alginate beads was beneficial to improve the biosorption performance of alginate beads (Jampala et al., 2016), with straw, sawdust, and sugarcane generally used as additives (de Castro et al., 2001). Studies have shown that hollow cellulose tubes contained in wheat bran contribute to the diffusion of both oxygen and substrate, ultimately maintaining the microbial activity (Li Y. et al., 2022). The bacterial growth in storage strongly supported the hypothesis that IM and its organic components could provide abundant carbon sources for bacterial colonization. The appearance of voids and folds on the outer surface of microspheres was beneficial to the adsorption of pollutants and the transfer of oxygen and nutrients from IM-FN, which enhanced the growth of microorganisms, as reported previously (Mannacharaju et al., 2021). Studies have shown that the extremely wrinkled structure provides a natural shelter for bacteria to inhabit, which could protect the selected bacteria from the direct competition with other bacteria and the threat of atrazine (Wei et al., 2020). Therefore, these studies suggested that the wrinkled structure could improve the carrier porosity and specific surface area, thus reducing the diffusion resistance and facilitating the transportation of oxygen and nutrients, ultimately accelerating the degradation of atrazine.

4.3. Effects of different culture conditions on degradation of atrazine by immobilized bacterial mixture and the optimization of culture conditions

The biodegradation of atrazine by FN and IM-FN was affected by pH level, cell concentration of bacteria, temperature, and atrazine concentration. Among these factors, the initial atrazine concentration was a particularly important factor based on the results of optimization experiments. Although atrazine could be effectively degraded by both FN and IM-FN, atrazine has been revealed with certain toxicity. With the increase of atrazine concentration, its degradation activity was significantly decreased. However, compared with FN, the IM-FN could still maintain a high degradation efficiency under long storage, probably due to the fact that the IM provided a relatively stable microenvironment for the bacteria, while this structure with a tight outer layer and a gradually loose inner surface provided a strong buffer capacity for alleviating the atrazine toxicity, thus protecting the immobilized microorganisms from being inactivated. These results were consistent with those previously reported (Khosla et al., 2017). Therefore, with the diffusion of atrazine to IM-FN, the concentration of atrazine was gradually decreased, slowing down the stress of atrazine on the atrazine-degrading bacteria. The temperature fluctuation over a certain range may increase or decrease the removal percentage of atrazine by affecting the biological activity of bacteria, thus reducing the adsorption of atrazine on cells. The low temperature is generally not conducive to the growth of bacteria, while under high temperature, the bacteria grow fast and quickly entered the decline stage with the enzymes in the bacteria cells inactivated, resulting in the decreased metabolic capacity and growth inhibition of the bacteria (Qi et al., 2020). The IM-FN still maintained a relatively high degradation efficiency at high temperature, probably due to the protection of immobilizing materials, which minimized or tolerated the influence of temperature changes (Li et al., 2020). Proper bacterial inoculation was beneficial to the degradation of atrazine. With the small inoculation, the bacteria grew slowly with low cell vitality, whereas the large inoculation shortened the growth cycle of the bacteria, resulting in insufficient substrate for bacterial growth and metabolism (Cai et al., 2021). The pH level also affected the degradation of atrazine by FN and IM-FN. The results showed that the IM-FN could degrade atrazine under a wide range of pH levels, i.e., 6.0–10.0, with higher degradation efficiencies obtained in neutral or alkaline conditions than those in acidic conditions. Furthermore, the degradation efficiency of atrazine by FN under highly acidic or alkaline conditions was significantly lower than that of IM-FN, which was probably due to the protection of the bacteria by the IM, providing a stable environment for microorganisms and to keep their cell activity, thus playing an important role even in the adverse environmental conditions (Jiang et al., 2022). In our study, the response surface methodology was used to optimize the culture conditions of atrazine degradation by IM-FN. The Box–Behnken model is commonly used to determine the relationship between responses and variables and to calculate the optimal responses (Abdollahi et al., 2012). For example, the Box–Behnken design-RSM was used to optimize the culture conditions of *Bacillus* sp. FA3 in the degradation of fipronil (Bhatt et al., 2021). Indeed, there are many factors that affect the degradation of atrazine. The three factors chosen in our study are the most influential effect on the degradation of atrazine (Guo et al., 2022). Furthermore, the selection of these three factors is verified by the findings revealed in our study, i.e., results presented on Figure 5A showed that the bacterial inoculation amount had trivial effect on the degradation of atrazine. Therefore, we chose these three factors (i.e., temperature, pH level, and initial concentration of atrazine) to optimize the degradation conditions.

4.4. Stability and reusability of immobilized bacterial mixture

Our studies revealed higher reusability and stability in IM-FN than those of FN, mainly because the IM and sodium alginate provided sufficient space for bacterial growth (Liu et al., 2012), which, in turn, improved the mechanical properties and stability of the fixed materials, as reported previously (Chen et al., 2020). Furthermore, our studies revealed that the immobilization system showed high durability and compatibility with free cells in water, which improved the adsorption capacity and biodegradation capacity of IM-FN, delayed the contact between microbial cells and the environment, and protected them from adverse environment (Zhang et al., 2020). The IM-FN achieved strong reusability, indicating that it was feasible to remediate the polluted environment by this methodology. Furthermore, compared with free cells, one of the advantages of using immobilized cells to remove atrazine was the reusability of the materials for multiple rounds of application. Meanwhile, the recycling of IM-FN could reduce the cost of manufacturing transportation materials and IM-FN and further promote the popularization and application of this technology.

4.5. Colonization dynamics of strain FH-1 and NJ-1 of immobilized bacterial mixture in soils

Previous studies showed that the total copy number of both bacterial strains of *Pseudomonas protegens* FD6 and *Bacillus subtilis* NCD-2 in the soil was gradually reduced with time in 14 days after inoculation as detected by qRT-PCR (Zhang Q. X. et al., 2021). These results were consistent with the finding revealed in our study, indicating that most

of the exogenously added bacteria could colonize the soil for a long time. Surprisingly, our results revealed the improved colonization of IM-FN in soil, which was probably due to the gradual release of the microorganisms, whereas the FN of strains FH-1 and NJ-1 could not persistently colonize the soil. These results were consistent with those previously reported (Dos-Santos et al., 2020). The ability of atrazine-degrading bacteria to persistently colonize the soil was an important factor determining their degradation effect. For example, studies have shown that *Mycolicibacterium* sp. Pyr9 colonized the soil for a long time and significantly enhanced the degradation and reduced the content and accumulation of pyrene in white clover (Yang et al., 2021). The joint bioremediation effects of bensulfuron-methyl-degrading strain *Hansschlegelia zhihuaiae* S113 and arbuscular mycorrhizal fungi on bensulfuron-methyl contaminated soil were evaluated (Qian et al., 2022). The results showed that arbuscular mycorrhizal fungi enhanced the colonization of strain S113 in maize rhizosphere and the coexistence of arbuscular mycorrhizal fungi in rhizosphere soil, while strain S113 could remove 3 mg/kg BSM from corn rhizosphere soil within 12 days. Our results suggested that IM-FN could effectively enhance the colonization of strains FH-1 and NJ-1 in soil and promote the degradation ability of atrazine by FN.

4.6. Remediation of atrazine-contaminated soil and the enhanced growth of sensitive crops by immobilized bacterial mixture

Studies have shown that the FN introduced into atrazine-contaminated soil is generally vulnerable with poor adaptability due to the influence of soil characteristics and competition with the indigenous communities (Zhu C. Y. et al., 2021). Our results show that the removal efficiency of immobilized microorganisms in soil is higher than that of free cells, suggesting that the IM-FN showed significant potential in remediating the atrazine-polluted soil environment. Li J. Y. et al. (2022) have isolated a novel bacterial strain, *Stenotrophomonas acidophilia* Y4B, to degrade glyphosate and its main metabolite, aminomethylphosphonic acid (AMPA). Strain Y4B degraded glyphosate in a wide concentration range (50–800 mg/L) to achieve high degradation efficiency over 98% in 72 h. Furthermore, strain Y4B showed strong competitiveness to significantly accelerate the degradation rate of glyphosate in both sterile and nonsterile soils to 71.93 and 89.81% (about 400 mg/kg), respectively. Moreover, the immobilized cells of Y4B showed a higher degradation effect on glyphosate than the free bacterial cells. Carrier materials used for microbial immobilization can play a buffering role between soil environment and microorganisms, and protect microbial cells from the harsh and changeable conditions of soil matrix (Girijan and Kumar, 2019). Walha et al. (2022) have comprehensively investigated the application of biochar as a type of carrier material to immobilize metribuzin-degrading bacterial colonies composed of four bacterial strains, which are used to repair metribuzin-contaminated soil and restore soil bacterial communities. The results revealed significantly higher metribuzin remediation in the bacterial alliance immobilized on biochar compared with the soil enhanced by the immobilized bacterial alliance. Furthermore, compared with the degradation rate of 0.010 Kd^{-1} and the half-life of 68 days in the treatment with non-immobilized bacteria alliance, the immobilization of MB3R on biochar resulted in significantly higher MB degradation rate (0.017 Kd^{-1}) and shortened half-life (40 days). These alleviation effects of IM-FN were validated by the enhanced growth of wheat and soybean plants grown in soils treated with both atrazine and IM-FN. These results revealed the deleterious effects of atrazine on the physiological activities in soybean and wheat plants, while the alleviation effects of IM-FN on the phytotoxicity of atrazine could be explained in two ways. First, the IM-FN improved the environmental conditions of two atrazine-sensitive crops by removing atrazine in soil or reducing its concentration. Second, the IM-FN played an important role in promoting plant growth and antioxidant activity, thus enhancing the resistance of plants to atrazine. These results were consistent with those previously reported (Pan et al., 2017).

5. Conclusion

In this study, we used rice straw powder, rice husk, and wheat bran with the ratio of 1:1:1 and bacterial strains FH-1 and NJ-1 for immobilization with sodium alginate. Our results showed that IM improved the adsorption capacity for atrazine. The degradation performance of IM-FN was further optimized using the Box–Behnken method. Our results showed that compared with FN, the IM-FN showed not only high degradation ability, but also improved stability and reusability as well as enhanced soil colonization ability, indicating that the IM-FN could improve the soil colonization and increase the colonization time of strains FH-1 and NJ-1, ultimately promoting their atrazine degradation ability and the agricultural application of both bacterial strains in remediating of atrazine-polluted soil environments. Furthermore, the immobilization of both bacterial strains significantly improved the degradation ability of atrazine. Compared with FN, the IM-FN significantly accelerated the degradation of atrazine in soil. Moreover, the alleviation effects of IM-FN on the phytotoxicity of atrazine were verified by the enhanced growth of atrazine-sensitive crop plants, showing significant potential of an effective bioremediation technique for the treatment of atrazine-polluted soil environments. In particular, the results of this study are helpful to optimize the bioremediation of atrazine-polluted environments by immobilized microorganism technology, provide an improved understanding of the removal mechanism of atrazine by the immobilized microorganism technology, and effectively treat micro-pollutants in agricultural soils. It is noted that in the future research, it would be important to investigate the influence of immobilized bacteria on the structural composition of the microbiota and the functions of microorganisms in atrazine-contaminated soils and to provide new insights for the practical application of functional microorganisms in atrazine soil remediation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

ZP and YW made the experiences in the article. ZP, YW, and QZ wrote the manuscript. XL, XX, and YT performed the statistical analysis. SL and HZ contributed to the design of the study and corrected the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1056264/full#supplementary-material

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