

Original Research

Isolation, Characteristics, and Prospects of Using the *Ochrobactrum Intermedium* Strain in the Degradation of the Cypermethrin Pesticide

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Abstract

Background: Due to the constant and improper use of chemicals, including pesticides, many substances, and their degradation products can accumulate in the soil and negatively affect its organisms. **Methods:** In this study, morphological methods, Gram-staining, and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) methods were used to isolate bacteria from agricultural soils, while genetic identification was conducted using 16S rRNA. The density of bacteria was determined using the spectrophotometric method, and the residual amount of cypermethrin was determined and analyzed using Gas chromatography-mass spectrometry (GC-MS) methods. **Results:** Nine isolates were obtained from various agricultural soils. Isolate No. 3 showed the greatest effectiveness against cypermethrin and was selected for further research. Isolate No. 3 was identified as the *Ochrobactrum intermedium* strain PDB-3 and was registered in the National Center for Biotechnology Information (NCBI) database (GenBank: OL587509.1). Using this strain, the influence of various external factors on the degradation of cypermethrin was studied. This bacterium demonstrated 100% degradation of cypermethrin in 20 days under optimal conditions (temperature: 30 °C; optical density (OD) = 0.2; cypermethrin concentration: 80 ± 0.02 mg/kg). In addition, PDB-3 changed the original structure of cypermethrin into various intermediate metabolites, such as 2-hydroxy-3-phenoxy benzeneacetonitrile, 3-phenoxybenzaldehyde, 3-phenoxybenzaldehyde, methyl stearate, anethol, citral, and phenol. **Conclusions:** The results obtained using PDB-3 provide the basis for large-scale field trials on the bioremediation of cypermethrin-contaminated soils.

Keywords: *Ochrobactrum intermedium*; cypermethrin; degradation; 16S rRNA; GC-MS

1. Introduction

Pyrethroids are insecticides that contain an ester bond formed by an alcohol and an acid [1]. These insecticides are synthetic derivatives of pyrethrin compounds produced by chrysanthemum plants [2]. This group of pesticides has been used in agriculture for more than 40 years and is widely used in forestry, horticulture, healthcare, home care, and for protecting textiles and buildings [3,4]. A complete or partial ban on using organochlorine and organophosphate pesticides contributes to the wider use of pyrethroids in agriculture and households, accounting for about 25% of the global pesticide market [5,6].

Cypermethrin ((±)-α-cyano-3-phenoxybenzyl (±)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate) is a synthetic pyrethroid commonly used against agricultural and household pests. Cypermethrin and other pyrethroids are used worldwide to protect crops from harmful insects [7,8]. Originally synthesized in 1974, cypermethrin is a toxic chemical first marketed as an agricultural and public health insecticide in 1977. Due to the low vapor pressure and Henry's law constant, it cannot evaporate in nature [9]. Hence, the

US Environmental Protection Agency has also classified cypermethrin as a possible human carcinogen [9]. Cypermethrin is a non-polar compound with low water solubility and, therefore, adsorbs soil particles, while intermediate metabolites are mobile in the soil. Further, 3-phenobenzoic acid is a toxic breakdown intermediate of cypermethrin and other pyrethroids that is an endocrine disruptor [10]. The persistence of cypermethrin in the environment varies depending on the physicochemical conditions of the soil [11,12]. Depending on the properties of the soil, the half-life of cypermethrin in the environment ranges from 14.6 to 76.2 days [12]. Microorganisms help reduce the concentration of cypermethrin in the natural environment [7]. However, because of existing metabolic pathways, microorganisms can degrade cypermethrin. Initially, hydrolysis of the ester bond occurs [13–16], forming alcohol and acid, then the alcohol-containing metabolite is converted into 3-phenobenzoic acid [7]. The decomposition of cypermethrin in soil involves various pure and mixed cultures of bacteria [13,17]. Some bacterial isolates effectively degraded various concentrations of cypermethrin [7,18]. Microbial strains of the genera *Bacillus*,



Brevibacillus, *Pseudomonas*, *Acinetobacter*, *Aspergillus*, *Candida*, *Roultella*, *Micrococcus*, *Ochrobactrum*, *Serratia*, and *Cunninghamella* can be used in the decomposition of cypermethrin and other peritroids [13,19–22].

2. Materials and Methods

2.1 Collection of Soil Samples

Soil samples were collected from various farm sites (where multiple plants were planted) in the Samarkand region of the Republic of Uzbekistan—where pesticides (chlorpyrifos and cypermethrin) have been used for many years. Soil samples were taken from depths of 0–20 cm using the envelope method and placed in sterile bags. Under laboratory conditions, the soil was cleared of all additional parts (large stones, plant debris, etc.) and filtered through a stainless-steel sieve with a diameter of 2 mm.

2.2 Chemicals and Media

The cypermethrin standard (purity 97), acetone, and all other substances and reagents were chemically pure and commercially available. Stock solutions (40 ± 0.05 mg/kg, 80 ± 0.02 mg/kg) were prepared using acetone. A Mettler Toledo AG pH meter (8603, Mettler Toledo, Schwerzenbach, Switzerland) was used. The following nutrient media were used to grow the microorganisms: MPA, MPB (Hi-Media Laboratories Private Limited, Maharashtra, India), mineral salt medium (MSM) (pH 6.8–7.0), containing (g/L) K_2HPO_4 : 1.5, KH_2PO_4 : 0.5, NaCl: 0.5, $(NH_4)_2SO_4$: 0.5, $MgSO_4 \cdot 7H_2O$: 0.2, and 1 mL of trace element solution. The microelements solution consisted of (g/L) H_3BO_3 : 5.0, $Na_2MoO_4 \cdot 2H_2O$: 5.0, $MnSO_4 \cdot 4H_2O$: 3.0, KI: 0.5, NaBr: 0.5, $ZnSO_4 \cdot 7H_2O$: 0.2, and $Al_2(SO_4)_3 \cdot 18H_2O$: 0.3.

2.3 Search, Isolation, and Selection of Microorganisms Resistant to Cypermethrin

The collected soil samples were mixed and additionally contaminated with cypermethrin, then kept in a thermostat at 30 °C for 2 months. After a month, 10 g of soil was added to Erlenmeyer flasks containing nutrient broth with cypermethrin and incubated on a rotary shaker at 30 °C with a rotation speed of 150 rpm for 48 hours. On day 5, appropriate dilutions were prepared from the enrichment culture and added to agar + cypermethrin plates. The grown individual colonies were subcultured on MSM plates supplemented with higher concentrations of cypermethrin.

2.4 Identification and Characterization of Test Bacteria

Identification of bacteria was carried out by studying morphological and cultural properties, Gram-staining, and Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis. The 16S rRNA method determined the nucleotide sequences to ensure a more accurate identification.

First, a nutrient broth was prepared, and a bacterial culture was added. Then, the broth was grown at 30 °C for

12 hours. Next, bacterial DNA was isolated from the bacterial culture grown in 10 mL nutrient broth using the RIBO-prep (InterLabServis, Moscow, Russia) reagent kit. DNA extraction was performed according to the protocol provided in the kit. Extracted DNA samples were analyzed on 0.9% agarose and a spectrophotometer before storing at –20 °C. PCR was used to amplify the 16S rRNA gene, which had been selected for molecular genetic identification of bacterial cultures. The following primers were used to amplify the 16S gene: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3' [23]. The total volume of the amplification reaction was 20 μ L, and a ready-made lyophilized kit of PCR core (Isogene, Russia) was used. Primers (2 μ L at 5 pmol/ μ L) were added alongside 2.5 μ L free nucleotides and 2 μ L of DNA at 20 ng/ μ L. Enzymes and free nucleotides were acquired in a ready-made kit and placed separately in each test tube in a lyophilized form. Then, 10 μ L of buffer and up to 20 μ L of deionized water were added. The reaction program was performed using the following conditions: initial denaturation at 95 °C for 5 minutes, then 35 cycles of 95 °C for 20 seconds, 57 °C for 20 seconds, and 72 °C for 40 seconds before a final denaturation step of 72 °C for 3 minutes. The PCR product was analyzed using electrophoresis on a 2% agarose gel. The PCR product was purified using the GFX™ PCR DNA and Gel Band Purification kit (Thermo Fisher, Waltham, MA, USA) and its concentration was measured on a Qubit 2.0 (Invitrogen, Carlsbad, CA, USA) apparatus. The purified PCR product was subjected to a sequencing reaction using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher). The sequencing product was purified using the BigDye X Terminator Purification kit and loaded into the sequencer. Sequencing reactions and sequencing product purification procedures were performed according to kit instructions. The resulting sequence product was processed in the Codon Code Aligner software and compared (BLAST) to the National Center for Biotechnology Information (NCBI) database. The resulting sequence was deposited in the international NCBI database and registered with the corresponding number (GenBank: OL587509.1).

2.5 Influence of External Factors (Temperature, Optical Density of PDB-3, and Pesticide Concentration) on the Decomposition of Cypermethrin

The PDB-3 strain was introduced into soil containing 80 ± 0.02 mg/L cypermethrin at various temperatures (25, 30, 35, 40 °C) and PDB-3 optical densities (optical densities (ODs) = 0.05 ± 0.02 ; 0.1 ± 0.04 ; 0.15 ± 0.05 ; 0.2 ± 0.05). To study the effect of the initial pesticide concentration, cypermethrin was added in the following concentrations: 80 ± 0.02 , 120 ± 0.4 , 160 ± 0.01 , and 200 ± 0.05 mg/L. The residual cypermethrin concentration was determined by Gas chromatography-mass spectrometry (GC–MS).

2.6 Addition of a Test Culture to the Soil

A test culture was grown for 24 hours at a ratio of 50:1 and added to sterile soil contaminated with cypermethrin. The cell titer of the culture fluid was 10^8 cells/mL. Every 3 days, the soil was rinsed with sterile distilled water.

2.7 Biodegradation of Cypermethrin in the Soil

Studies on the biodegradation of cypermethrin by the isolated culture were conducted using sterile soils. An initial cypermethrin concentration of 80 ± 0.02 mg/kg was added to soil samples (100 g) using an acetone-based cypermethrin solution. Initially, the prepared solution was added to a small part (15–20 g) of the soil, which was then mixed with the remaining soil after the solvent had evaporated. Soil samples were inoculated and incubated in a thermostat at 30 °C. The tests were carried out in triplicate. The control was uninoculated sterile soil with cypermethrin. The duration of the experiment was 20 days.

2.8 GC–MS Analysis of Cypermethrin Biodegradation

Cypermethrin concentrations were determined using an Agilent 8890B gas chromatograph mass spectrometry with split and splitless evaporators, which was used in conjunction with an Agilent 5977B series GC/MS in SIM, SCAN, and electron impact ionization (EI) modes. Analysis conditions: Gas chromatography analysis parameters. Analytical column HP: 5 ms; Ultra Inert: 30 m • 250 μ m • 0.25 μ m. Injection volume of 1 μ L; Splitless injection mode. Evaporator temperature 280 °C UI liner, splitless, single throat, fiberglass Sputtered Gasket Gold plated, The evaporator temperature was 280 °C. Hydrogen was used as the carrier gas Hydrogen, and a constant flow, of 1.2 mL/min. The thermostat program was 60 °C for 1 minute, then 40 °C/min to 170 °C, then 10 °C/min to 310 °C, then hold for 2 minutes. The temperature in the transport line was 280 °C. Data collection mode: SIM, SCAN. Gain factor 1.00. Source temperature: 250 °C. Quadrupole temperature: 150 °C.

2.9 Statistical Analyses

Statistical analysis and exponential curve fitting were performed using Origin 8.6 software (Microcal Software Inc., Northampton, MA, USA). Results were expressed as the mean \pm SE. One-way ANOVA was performed to determine the statistical significance of the results.

3. Results

3.1 Search, Isolation, and Selection of Cypermethrin-Resistant Microorganisms

To isolate microorganisms resistant to the insecticide cypermethrin, we used soil from various agricultural plots in the Samarkand region, which had been treated with pesticides chlorpyrifos, cypermethrin, and others for many years. Under laboratory conditions, soil samples were also

contaminated with cypermethrin at 40 ± 0.05 mg/kg of soil. The treated samples were kept in a thermostat for 2 months at 30 °C to allow the microorganisms to adapt to the pesticide. Then, the samples (10 g of soil) were added to Erlenmeyer flasks containing nutrient broth with cypermethrin and incubated on a rotary shaker at 150 rpm at 30 °C for 48 hours. As a result of such passages, microorganisms resistant to these pesticide concentrations were isolated. Nine isolated microorganisms could grow and multiply on a medium containing cypermethrin. Next, microorganisms were selected among the isolated microorganisms to determine the decomposition of cypermethrin in the soil.

The results showed that isolate 3 was the most effective at degrading cypermethrin. This microorganism was isolated from soil brought from an orchard. In further studies, this isolate was selected to determine the effective degradation of high concentrations of cypermethrin.

3.2 Identification and Characterization of Test Bacteria

Under laboratory conditions, morphological and cultural properties, some physiological and biochemical properties, and the movements of living cells were studied under a microscope. The cells are rod-shaped, size 1.2–2.0 μ m, solitary, and on MPA, they form smooth convex, shiny colonies, are white–gray in color, 2–2.5 mm in diameter, do not form spores, and have a pH of 6.9–7.2. Gram-staining indicated that this strain is a Gram-negative bacterium (Fig. 1). MALDI–TOF MS analysis was also performed.

The obtained results tentatively suggest that this strain is *Ochrobactrum intermedium*. To ensure a more accurate identification, the 16S rRNA nucleotide sequence was determined. The results show 100% similarity to the species *Ochrobactrum intermedium* (Fig. 2).

The 16S rRNA gene sequence data of *Ochrobactrum intermedium* strain PDB-3 were processed using UniGene Ver35 and Chromas software, and the low-confidence peaks were removed. Then, the DNA sequences were analyzed against the NCBI nucleotide database using BLAST. Highly similar DNA sequences were aligned using the MegaX program and the ClustalW algorithm. A phylogenetic tree was constructed using the Neighbor-Joining algorithm in the MegaX program.

Thus, strain PDB-3 *Ochrobactrum intermedium* showed very close similarity (100%) to strains of *Ochrobactrum intermedium*. This indicates that the strain under study belongs to the phylum *Ochrobactrum intermedium*. The strain was registered in the NCBI database: GenBank: OL587509.1.

3.3 Influence of External Factors (Temperature, pH, Optical Density of PDB-3, and Pesticide Concentration) on the Degradation of Cypermethrin

The influence of external factors on cypermethrin decomposition was studied under laboratory conditions.

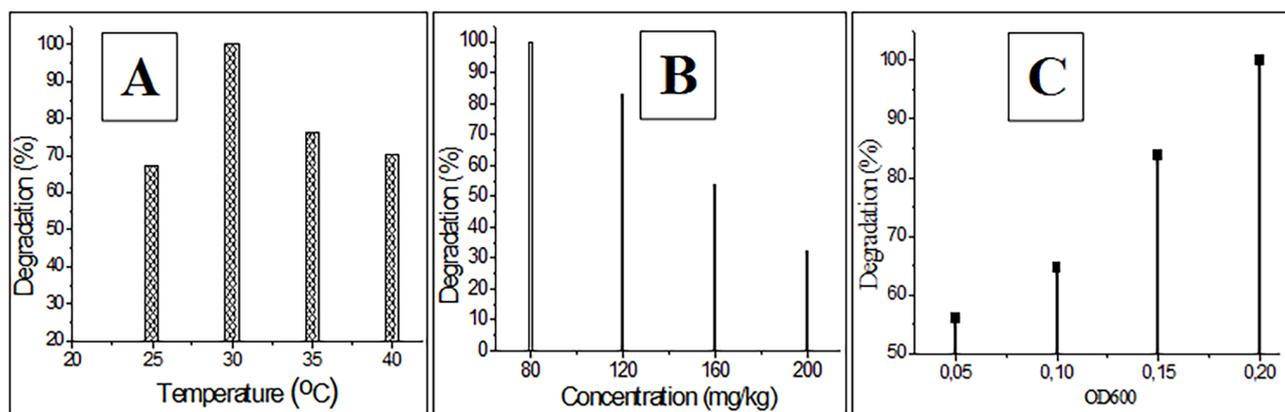


Fig. 3. Determination of the influence of some external factors on the degradation of cypermethrin. (A) Temperature. (B) Cypermethrin concentration. (C) Optical density.

trophotometer [24–26]. Decomposition proceeded slower at the lower OD values. The results obtained confirm that an increase in microbial mass has a positive effect on the decomposition process. All studies were carried out in triplicate. At the end of the experiment, the residual concentration of cypermethrin was determined by GC–MS. Our next step was to determine the effect of the initial concentration of cypermethrin on the biodegradation process. Strain PDB-3 was actively multiplied in a medium supplemented with cypermethrin at a concentration of up to 80 mg/kg and was completely degraded. Based on this, the concentration was increased to the following values: 80 ± 0.02 , 120 ± 0.4 , 160 ± 0.01 , and 200 ± 0.05 mg/L (Fig. 3C). It has been established that as the concentration increased, the speed of degradation process reduced.

The potential for maximum microbial degradation of pesticides depends entirely on optimal environmental conditions, such as initial concentration [27], pH, temperature, additional energy sources, and inoculum concentration [28]. Biotechnological processes based on microorganisms are usually influenced by environmental factors [29,30]. *Lysinibacillus cresolivorans* strain HIS7 was isolated from contaminated soil. Thus, the degradation process of cypermethrin was studied using this strain under the influence of various environmental factors. The optimal parameters for the biodegradation of cypermethrin were previously established as an incubation period of 8 days, inoculation volume of 3 mL, temperature of 35 °C, pH 7, and an additional source of carbon and nitrogen, which increased decomposition from 57.7% to 86.9%. In soil, the HIS7 strain degraded cypermethrin to 93.1% within 42 days [31].

3.4 Biodegradation of Cypermethrin in Soil

Studies on the biodegradation of cypermethrin were carried out in sterile soils. The soil was dried in laboratory conditions, and all unnecessary parts were removed, e.g., large stones, plant debris, etc. Then, it was sterilized in an autoclave at 121 °C for 45 minutes. Cypermethrin solutions

were prepared using acetone and added to a small part of the soil (approximately 15–20% of the total soil mass); the soil was thoroughly mixed with the cypermethrin solution (80 ± 0.02 mg/kg) and then mixed with the remaining soil. Subsequently, the cultural liquid of the PDB-3 strain ($OD = 0.2 \pm 0.05$) was added to the soil. The studies were carried out in triplicate. The experiments lasted 20 days. Humidity plays an important role in the reproduction and development of microorganisms because when humidity decreases, the growth and reproduction of microorganisms decrease, negatively affecting the biodegradation process. Therefore, to maintain humidity (about 60–70%), the test soil samples were rinsed with sterile distilled water every 3 days. Soil samples were collected every 4 days for chromatographic analysis. The GC–MS results show the presence of cypermethrin as a peak appeared in the soil samples at a retention time of 19.357 min (Fig. 4).

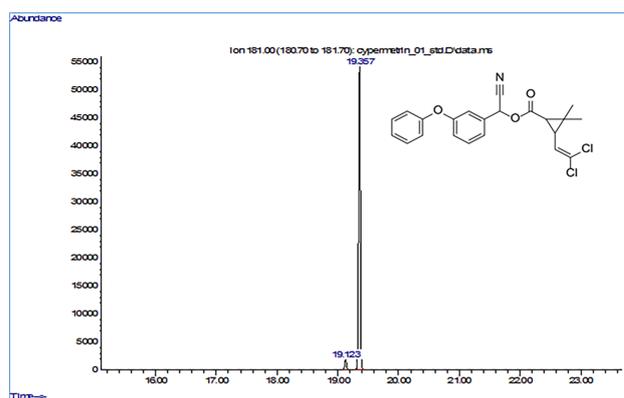


Fig. 4. Initial Gas chromatography-mass spectrometry (GC–MS) chromatogram of cypermethrin.

Thereafter, this peak gradually decreased and was undetectable in the soil samples on day 20. In the control sample, virtually no changes were observed during the exper-

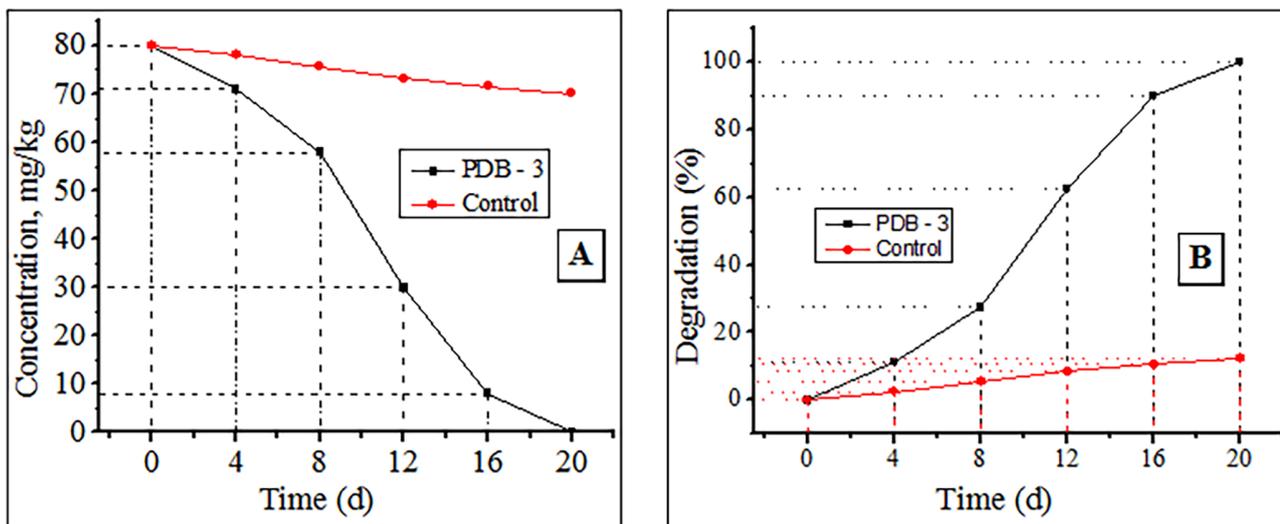


Fig. 5. Cypermethrin degradation using PDB-3. (A) Decrease in initial concentration. (B) Degradation percentage.

iment. Based on the chromatographical analyses, the decrease in the initial concentration of cypermethrin (Fig. 5A) and the percentage of degradation (Fig. 5B) were calculated.

Fig. 5 shows that strain PDB-3 successfully decomposes 80 ± 0.02 mg/kg of cypermethrin in the soil compared to the control sample. Initially, the decomposition rate was somewhat slower, although an increase in microbial biomass in the soil environment, starting from day 8, accelerated the degradation of cypermethrin. The percentage of cypermethrin degradation on day 4 was $11.25 \pm 0.03\%$; on day 8: $27.5 \pm 0.05\%$; on day 12: $62.5 \pm 0.05\%$; on day 16: $90 \pm 0.02\%$. Cypermethrin residues were not detected in our soil samples on day 20, which confirms that the initial concentration was completely degraded. Comparatively, the degradation percentage was $12.3 \pm 0.05\%$ in the non-inoculated control soil.

4. Discussion

Cypermethrin has been widely used in agriculture and households since the late 1980s [7]. However, due to its lower bioavailability, it persists in soil for a long time; its half-life ranges from 100 to 200 days [32]. Many studies examining the toxicity and persistence of cypermethrin indicate the need to develop methods for removing it and its metabolites from the environment. Biodegradation based on the use of microorganisms is the preferred method for breaking down pesticides into simple inorganic chemicals since it is environmentally friendly and cost-effective [33]. Numerous studies have been conducted on isolating, characterizing, and selecting pesticide-degrading microorganisms [34,35]. In our study, strain PDB-3 was isolated and identified from soils exposed to pesticides. Morphological and cultural studies and MALDI-TOF MS and 16S rRNA analyses showed that this strain belongs to the

bacterial species *Ochrobactrum intermedium*. This type of bacteria was first described in 1998 [36]. Based on recent genome comparison studies, the genus *Ochrobactrum* has been reclassified, and its species are included in the genus *Brucella* [37,38]. *Ochrobactrum intermedium* degrades many pollutants [39], including pesticides [40–42]. For example, *Ochrobactrum intermedium* SP9 degraded the pesticide cypermethrin to 69.1% within 8 days [43]. *Ochrobactrum anthropi* has been used to degrade 2,4-dichlorophenoxyacetic acid (2,4-D) [44]; *Ochrobactrum anthropi* strain L1-W successfully degraded air pollutant Di-2-ethylhexyl phthalate (DEHP) [45]. The effectiveness of bacteria in decomposing pesticides depends on the following environmental factors: pH and temperature [33], initial concentration of pesticide [46,47], optical density of microorganisms, etc. We conducted studies to determine the influence of temperature, initial pesticide concentration, and optical density of the PDB-3 strain. The optimal parameters for degrading cypermethrin using the PDB-3 strain were established: temperature: 30 °C; time: 20 days; concentration: 80 mg/kg; OD: 0.2. It was shown that changing these parameters negatively affects the bacterial growth phases (soil microbial analysis showed a decrease in bacterial colonies under such conditions). This, in turn, slows down the degradation process. Our results confirm the data from previous studies on the biodegradation of the pyrethroid insecticide fenvalerate by the strain *Stenotrophomonas sp.* ZS-S-01. It was inactive at temperatures below 25 °C or above 35 °C, at which significant cell death was observed and degradations of 46.5% and 56.6%, respectively, were achieved [35]. The degradation percentage was 99% at a temperature of 30 °C, which is the optimal temperature for ZS-S-01 [35]. It is also reported that the initial pesticide concentration plays a significant role in the degradation process. Various concentrations of cyper-

stearate. Additionally, 2-hydroxy-3-phenoxy benzeneacetoneitrile (16.778 min), 3-phenoxybenzaldehyde (16.946 min), and 3-phenoxybenzaldehyde (17.775 min) were observed. Similar studies have confirmed that cypermethrin is transformed into 3-phenoxybenzaldehyde and α -hydroxy-3-phenoxybenzeneacetoneitrile by the *Streptomyces aureus* strain HP-S-01. In laboratory conditions, this strain can utilize cypermethrin up to 80.5% and 3-PBA up to 73.1% (initial dose: 50 mg/kg) within 10 days. *Streptomyces* sp. converted β -CP into 2-(4-hydroxyphenyl)benzoate-methyl-ester, 3,5-dihydroxybenzoic acid, 3,5-dimethoxyphenyl, 4-hydroxy-3-methoxybenzoic acid, and phenol using the enzyme monooxygenase [12].

5. Conclusions

In this work, the *Ochrobactrum intermedium* PDB-3 strain was isolated from agricultural soils contaminated with the pesticide cypermethrin. This strain completely degraded the initial amount of cypermethrin. Moreover, various intermediate metabolites were formed due to the decomposition of this pesticide. The obtained results serve as the basis for conducting field studies on the bioremediation of soils contaminated with cypermethrin.

Availability of Data and Materials

All experiments and results obtained during this study are presented in this article. Additional information is available on request from the corresponding author.

Author Contributions

DK participated in the isolation, selection, and inoculation of bacteria in the soil. DK, LZ, and AM participated in the identification of bacteria. DK and RE carried out GC-MS analyses of the soil, and NL and TK analyzed the results and worked with figures. All authors of the article bear equal responsibility for the results obtained in this study. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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