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Identification of Indigenous Bacterial Strains from Thai Agricultural Fields for Potential Bioremediation of Carbofuran

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Abstract

This study explored the bioremediation potential of indigenous soil bacteria for carbofuran-contaminated agricultural areas. Twenty soil samples from regions with a history of pesticide use in Pathum Thani and Nakhon Pathom provinces, Thailand, were collected. Five bacterial strains capable of degrading carbofuran were isolated and subjected to cell morphology and biochemical analyses. Phylogenetic analysis of the 16S rRNA gene sequences identified these strains as related to *Pseudomonas* and *Stenotrophomonas* species. High-Performance Liquid Chromatography (HPLC) analysis confirmed the complete degradation of carbofuran within 3 days, reducing its concentration from 0.05 mg/mL to below detectable limits. These bacteria could use carbofuran as their sole carbon source, demonstrating their potential for bioremediation of contaminated soils. Given their resilience and ability to thrive in natural environmental conditions, these indigenous strains are well-suited for in-situ degradation of pollutants. The findings indicate that these isolated soil bacteria present a promising method for reducing the environmental risks associated with carbofuran contamination.

Keywords: *carbofuran; bioremediation; biodegradation; soil bacteria; indigenous bacteria*

1. Introduction

Carbofuran, also known as 2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate, is a highly toxic broad-spectrum carbamate chemical that has been widely used in agriculture as a pesticide to protect crops from insects in Thailand (Hinhumpatch, & Wattanachaiyingcharoen, 2023). It is a potent inhibitor of acetylcholinesterase, disrupting the nervous system of pests and leading to their death (Suma et al., 2022; Bano, & Musarrat, 2004). The continuous use of this compound poses significant health risks to humans and has detrimental effects on the environment. Carbofuran contamination of soil, water, and air can result from agricultural practices such as spray drift, runoff, and leaching from treated fields (Mishra et al., 2020). The environmental behavior of carbofuran is determined by various significant physicochemical properties, including its high solubility in water and its low partition coefficient between octanol and water. These characteristics suggest that carbofuran is highly mobile in water and possesses a limited potential for bioaccumulation within organisms. In contrast, its moderate soil adsorption coefficient implies that carbofuran can bind to soil particles, providing a degree of immobilization (Farahani et al., 2007; Ariffin, & Rahman, 2020). Although the use of carbofuran has been restricted in some countries due to its detrimental impacts on humans and ecosystems, it continues to be used in certain regions where alternative options are unavailable or not yet practical. This research explored the degradation of carbofuran by soil bacteria, presenting a potential solution to

mitigate the harmful effects of this pesticide on our environment.

In recent decades, Thailand's agricultural sector has undergone substantial growth and diversification, simultaneously presenting various challenges, particularly concerning worker and consumer health and safety (Laohaudomchok et al., 2021). Projected figures indicate that in the near future, Thailand will import more than 200,000 tons of pesticides each year, with carbamate or carbofuran constituting a significant portion of these imports (Ministry of Agriculture and Cooperatives, 2020). The increasing reliance on pesticides has become a major concern, as the global demand for Thai agricultural products intensifies, and the use of such chemicals to control pests becomes widespread. Consequently, questions arise regarding the long-term impact of pesticide use on the environment and human health (Laohaudomchok et al., 2021).

The persistence of carbofuran in the environment necessitates the development of sustainable and eco-friendly methods for its removal from contaminated areas. Bioremediation, which employs microorganisms to degrade and transform organic pollutants into innocuous substances, presents a promising strategy for addressing carbofuran contamination. Soil bacteria, in particular, have demonstrated high efficiency in detoxifying this pesticide under both aerobic and anaerobic conditions (Mishra et al., 2020). Bioremediation boasts several advantages over traditional remediation techniques, such as lower cost, minimal risk of secondary pollution, and the capacity to degrade pollutants into non-toxic compounds. Several studies have explored the biodegradation of carbofuran by soil bacteria, examining the isolation and characterization of carbofuran-degrading microorganisms, the factors influencing their activity, and the molecular mechanisms involved in the degradation process. These investigations have revealed that carbofuran-degrading bacteria encompass various taxonomic groups, including *Bacillus*, *Pseudomonas*, *Chryseobacterium*, *Novosphingobium*, *Paracoccus*, and *Agrobacterium* (Parekh et al., 1995; Karpouzas, Morgan, & Walker, 2000; Desaint et al., 2000; Slaoui et al., 2007; Yan et al., 2007; Peng et al., 2008; Park et al., 2022). However, the biodegradation pathways and genes involved in this process remain incompletely understood and warrant further research.

This study investigates the bioremediation of carbofuran using indigenous bacterial strains isolated from Thai agricultural soils. By exploring the potential of these bacteria to degrade carbofuran, we aim to contribute to the development of environmentally sustainable practices in managing pesticide pollution. The findings from this research are particularly valuable, as they demonstrate the effectiveness of using locally adapted microorganisms, a strategy that can be replicated in other regions by harnessing the bioremediation potential of native bacterial communities.

One key advantage of this approach is the use of indigenous bacteria, which are already adapted to the specific environmental conditions in Thailand. These bacteria have evolved to thrive in the local soil and climate, thereby enhancing their effectiveness in bioremediation efforts. By utilizing bacteria that are naturally suited to the environment, this method offers a more sustainable and efficient way to remediate carbofuran contamination. Furthermore, this approach is not limited to Thailand. Similar bioremediation strategies can be applied in other regions by isolating and employing local bacterial strains that are adapted to their respective environments. This flexibility makes bioremediation a globally applicable solution to pesticide pollution

2. Objective

This study aims to identify indigenous soil bacteria capable of degrading carbofuran and assess their potential for bioremediation of contaminated agricultural fields. The research focuses on isolating and characterizing effective bacterial strains to support future environmental cleanup efforts.

3. Materials and Methods

3.1 Soil Sample Collection and Isolation of Carbofuran-Degrading Bacteria

Twenty soil samples were collected from agricultural areas with a history of fertilizer or herbicide use in Pathum Thani and Nakhon Pathom provinces, Thailand. The soil samples were treated individually, and bacteria capable of utilizing carbofuran as their sole carbon source were isolated. To isolate carbofuran-degrading bacteria from soil, 1 g of soil sample was added to 50 mL of carbofuran-MS liquid culture medium (Sukplang, Thongmee, & Vela, 1999) containing $Na_2HPO_4.2H_2O$ 6.0 g/L, KH_2PO_4 3.0 g/L, NH_4Cl 1.0 g/L, $MgSO_4.7H_2O$ 0.2 g/L, $CaCl_2.2H_2O$ 0.1 g/L, and carbofuran 50 mg/L. The culture was incubated at 30°C with shaking at 100-200 rpm for 10 days. On days 1, 2, 3, 5, and 7 of incubation, 50 µl of culture medium was spread on carbofuran-MS solid culture medium and

incubated at 30°C. The growth of bacteria on the culture medium was observed, and each colony was isolated and purified on carbofuran-MS solid culture medium.

3.2 Carbofuran Degradation

Each bacterial isolate was added to carbofuran-MS solid culture medium and incubated at 30 °C for 24-48 hours. Cells were washed three times with 0.85% sodium chloride and adjusted to a McFarland standard number 0.5 (approximately 1.5 x 10^8 CFU/mL). One milliliter of the adjusted cells was added to mineral salt liquid medium with 50 mg/L carbofuran as the sole carbon source and incubated at 30 °C. The number of viable cells was counted, and the carbofuran concentration was measured using high performance liquid chromatography (HPLC) with a C18 column as the stationary phase and water: acetonitrile (60:40 by volume) as the mobile phase at a flow rate of 1 mL/min. The carbofuran concentration was measured using a UV spectrophotometer at a wavelength of 220 nm.

To confirm that the growth of bacteria was due to carbofuran as a carbon source, not nutrients inside the cells, a growth control was performed by adding carbofuran-degrading bacteria to MS liquid medium without carbofuran. The growth of bacteria was compared between culture media with and without carbofuran. An autodegradation control was also performed by measuring the carbofuran concentration in carbofuran-MS liquid medium without bacterial growth.

3.3 Morphological and Biochemical Test of the Isolates

Gram's stain was performed to observe the bacteria morphology and Gram's reaction. Preliminary biochemical tests, such as the oxidase test, catalase test, Triple Sugar Iron (TSI), glucose O/F test, and motility test were performed according to MacFaddin (2000).

3.4 16S rRNA Gene Sequencing and Phylogenetic Tree Construction

DNA extraction from each carbofuran-degrading bacterial isolate was conducted according to Weisburg et al., (1991) and Thongmee, & Sukplang (2015) using phenol-chloroform and CTAB methods, followed by amplification utilizing the universal 16S rRNA primer (5'AAGGAGGTGATCCAGCCGCA 3' and 5'AGAGTTTGATCCTGGCTCAG 3') with the Promega PCR Wizard kit (Promega Corp., Madison, WI). Subsequently, the DNA sequences were determined using ABI PRISM 3100 Genetic Big Dye Terminator Cycle Sequencing (BIOSERVICE UNIT, Bangkok, Thailand). Sequence alignment was performed using Clustal W (Hall, 2001). Upon completion of a multiple alignment, both ends were trimmed before generating a phylogram with MEGA 7.0.

Evolutionary history was determined using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). A bootstrap consensus tree, generated from 1000 replicates, was employed to visualize the evolutionary relationships among the analyzed taxa (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. The percentage of replicate trees where associated taxa clustered together in the bootstrap test (1000 replicates) was indicated adjacent to the branches (Felsenstein, 1985). Initial trees for the heuristic search were automatically generated using Neighbor-Join and BioNJ algorithms applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, followed by selecting the topology with the highest log likelihood value. The analysis involved 17 nucleotide sequences, covering codon positions of the 1st, 2nd, 3rd, and noncoding positions. Removal of positions containing gaps and missing data yielded a final dataset of 420 positions. Evolutionary analyses were conducted using MEGA7 (Kumar, Stecher, & Tamura, 2016).

4. Results

4.1 Isolation of Carbofuran-Degrading Bacteria from Soil in Agricultural Areas

From the 20 soil samples collected from agricultural areas with a history of fertilizer or herbicide use, 46 bacterial strains that were able to grow on carbofuran as a carbon source at a concentration of 0.05 mg/mL were isolated. Some of these strains used carbofuran as a source of carbon, while others were able to tolerate it. Five different isolates were further identified. The isolates 1-5 were named CF1, CF2, CF3, CF4, and CF5, respectively.

4.2 Carbofuran Degradation by Bacteria

The ability of the five different isolates to degrade carbofuran was evaluated by growing the isolates in MS liquid culture medium containing 0.05 mg/mL of carbofuran. The control set for detecting carbofuran in HPLC analyses is shown in Figure 1. The control set showed that the detected chromatogram peak corresponded with the amount of

carbofuran added to the medium. The autodegradation control set showed that there was no autodegradation of carbofuran during the 5-day experiment. Each bacterial strain was found to increase in number, and that the amount of carbofuran decreased until it was completely gone after three days (Figures 2-6) leaving only the chromatogram peak area of mineral salt, as seen in the MS medium control set. This result indicates that carbofuran was being used as a source of food and energy by bacteria. As shown in Table 1, bacterial growth was significantly reduced in medium without carbofuran (used as a control), indicating that the bacteria were using carbofuran as their primary source of carbon.

Table 1 The number of bacteria that grow in Mineral Salt (MS) medium without carbofuran and in MS medium containing carbofuran (0.05 mg/mL)

Isolate	Number of cells growing in MS medium without carbofuran (CFU/mL)				Number of cells growing in MS medium containing carbofuran (CFU/mL))			
	Day 0	Day 1	Day 2	Day 3	Day 0	Day 1	Day 2	Day 3
CF1	2.5×10^6	2.9×10^6	8.4×10^5	4.7×10^4	2.5×10^6	4.0×10^7	1.8×10^8	9.7×10^8
CF2	4.9×10^6	6.8×10^6	1.4×10^6	7.2×10^5	4.9×10^6	2.8×10^7	4.3×10^8	1.9×10^9
CF3	2.7×10^6	2.1×10^6	6.4×10^6	3.5×10^5	2.7×10^6	5.2×10^7	6.3×10^8	1.2×10^9
CF4	2.1×10^6	2.2×10^6	1.4×10^6	1.1×10^6	2.1×10^6	2.3×10^7	3.2×10^8	6.9×10^9
CF5	4.4×10^6	3.2×10^5	3.1×10^5	2.8×10^5	4.4×10^6	2.4×10^7	1.4×10^8	2.7×10^9

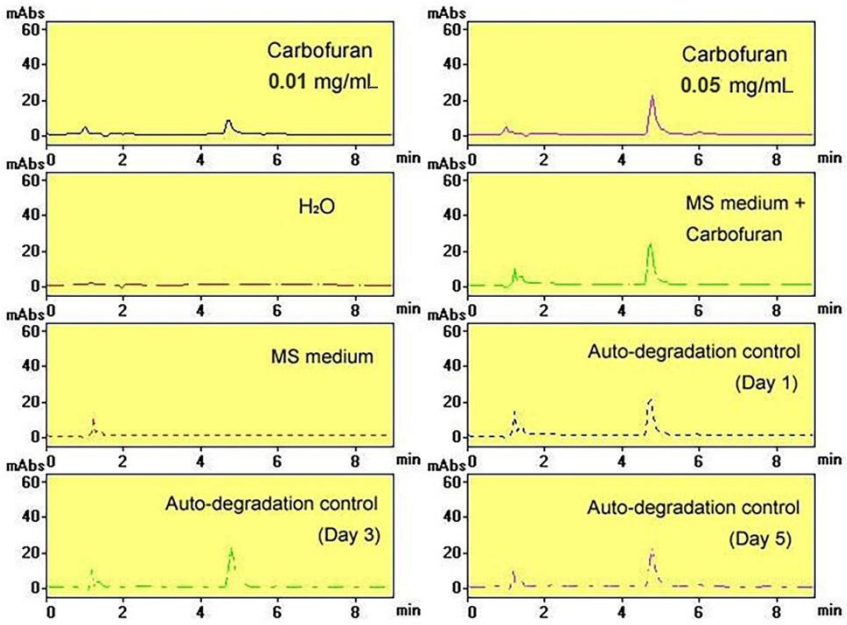

Figure 1 The control set for detecting carbofuran in HPLC analyses; the concentration of carbofuran in MS medium+carbofuran and autodegradation controls on day 1, day 3, and day 5 is 0.05 mg/mL each

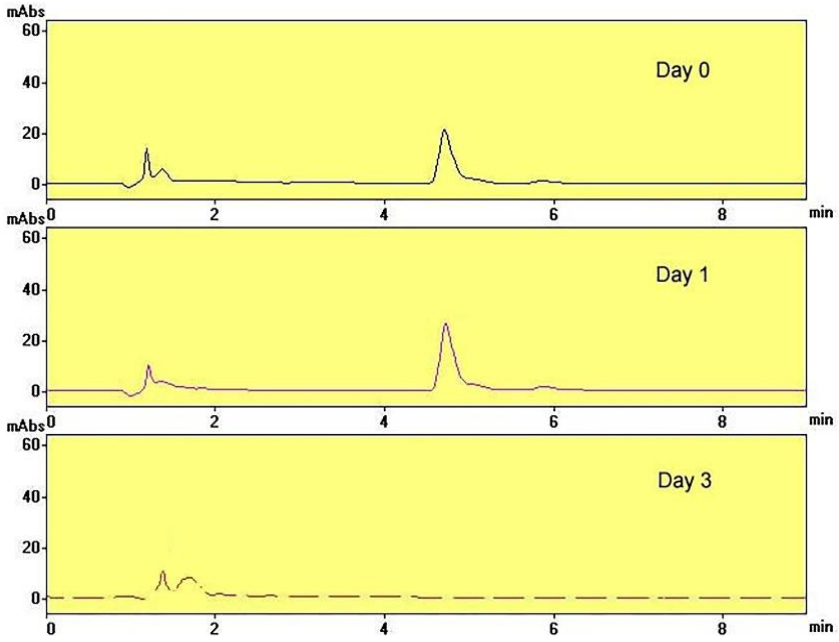

Figure 2 The decrease in the amount of carbofuran detected by HPLC in the CF1 strain culture; the concentration of carbofuran on day 0 was 0.05 mg/mL.

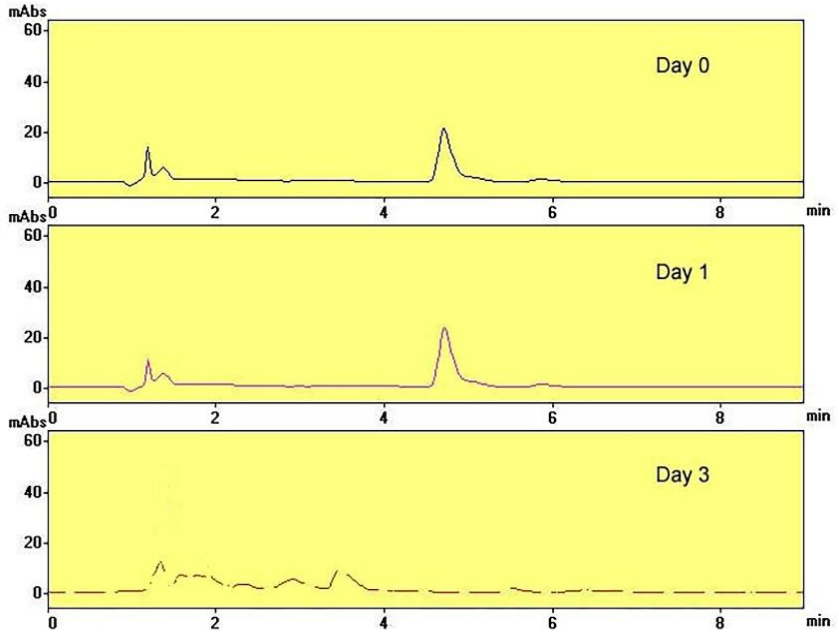

Figure 3 The decrease in the amount of carbofuran detected by HPLC in the CF2 strain culture; the concentration of carbofuran on day 0 was 0.05 mg/mL.

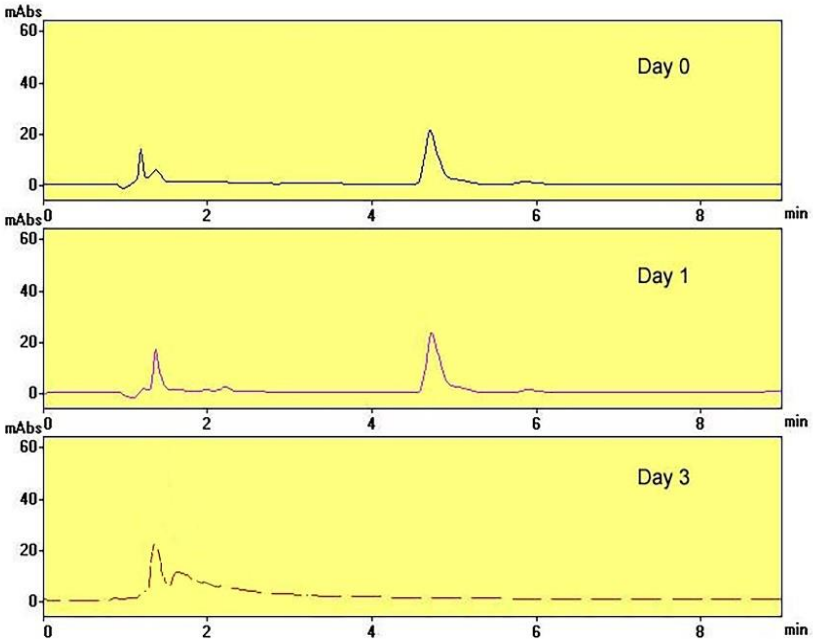

Figure 4 The decrease in the amount of carbofuran detected by HPLC in the CF3 strain culture; the concentration of carbofuran on day 0 was 0.05 mg/mL.

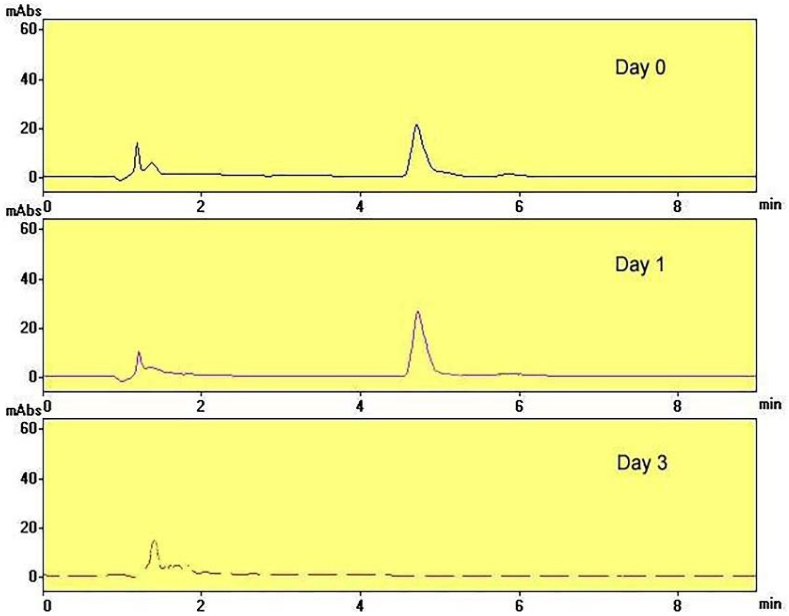

Figure 5 The decrease in the amount of carbofuran detected by HPLC in the CF4 strain culture; the concentration of carbofuran on day 0 was 0.05 mg/mL.

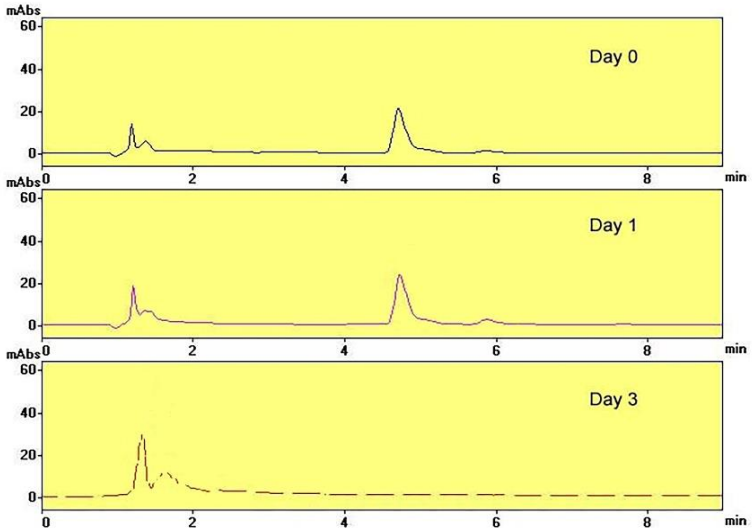

Figure 6 The decrease in the amount of carbofuran detected by HPLC in the CF5 strain culture; the concentration of carbofuran on day 0 was 0.05 mg/mL.

4.3 Cell Morphology and Biochemical Test

After performing cell morphology staining and biochemical testing, the isolates were identified as glucose non-fermenting Gram-negative bacilli. They were aerobic, oxidase-positive, motile bacteria, which showed oxidative metabolism results in Glucose O/F test.

4.4 16S rRNA Gene Sequencing and Phylogenetic Tree Construction

The PCR amplification of DNA from each isolated bacterial strain resulted in a PCR product of approximately 1.5 kilobase pairs. Subsequent multiple sequence alignment using ClustalW indicated that the isolated bacteria belong to the genera *Pseudomonas* and *Stenotrophomonas*, with the percent identities detailed in Table 2.

Table 2 Sequence Identity (pairwise alignment with highest percent identity)

Isolate	Taxon	Identities
Isolate1 (CF1)	*Pseudomonas psychrophila* (MT163428.1) *Pseudomonas fragi* (MG972898.1)	439/484 (91%)
Isolate2 (CF2)	*Pseudomonas putida* (MK878726.1) *Pseudomonas hutmensis* (NR165748.1) *Pseudomonas guariconensis* (MG674339.1) *Pseudomonas fulva* (KY511074.1) *Pseudomonas entomophila* (KX008299.1)	478/482 (99%)
Isolate3(CF3)	*Pseudomonas putida* (MK878726.1) *Pseudomonas hutmensis* (NR165748.1) *Pseudomonas guariconensis* (MG674339.1) *Pseudomonas fulva* (KY511074.1) *Pseudomonas plecoglossicida* (PP218104.1) *Pseudomonas entomophila* (KX008299.1)	474/481 (99%)
Isolate4 (CF4)	*Pseudomonas putida* (MK878726.1) *Pseudomonas hutmensis* (NR165748.1) *Pseudomonas guariconensis* (MG674339.1) *Pseudomonas fulva* (KY511074.1) *Pseudomonas entomophila* (KX008299.1)	478/482 (99%)
Isolate5 (CF5)	*Stenotrophomonas pavanii* (OQ692572.1) *Stenotrophomonas humi* (OP986541.1)	476/484 (98%)

As shown in Table 2, the alignment resulted in identical matches with multiple sequences upon BLAST analysis. This outcome can be attributed to the relatively short length of the nucleotide sequences, approximately 480 base pairs, after trimming. To enhance the discriminatory power of future analyses and provide more precise information, acquiring longer sequences is recommended. Longer sequences would facilitate better differentiation among closely related organisms, thus improving the accuracy and depth of our understanding.

4.5 Phylogenetic Tree Analysis

Phylogenetic analysis helps to classify the isolated bacteria and provides context for their relationship to other bacteria (Sawaengwong el al., 2023). Since isolates 1-4 (CF1-4) belong to the genus *Pseudomonas* and isolate 5 (CF5) belongs to the genus *Stenotrophomonas*, the 16s RNA sequences of *Pseudomonas* sp. and *Stenotrophomonas* sp. in the NCBI database were retrieved. The phylogenetic tree was constructed to analyze the evolutionary relationships of the isolated carbofuran-degrading bacteria and other related bacteria, as depicted in Figure 7. The 16s rRNA sequence of *Bacillus subtilis* was used as an outgroup.

5. Discussion

This study highlights the potential of indigenous soil bacteria in curing carbofuran contamination in agricultural areas of Thailand. By isolating and characterizing five carbofuran-degrading bacterial strains, primarily belonging to *Pseudomonas* and *Stenotrophomonas* genera, our research highlights the promising efficacy of native bacteria in degrading carbofuran as a sole carbon source. Notably, these bacteria were sourced from the top soil layer, where aerobic or facultative anaerobic bacteria predominantly thrive, owing to their superior growth and reproductive capabilities compared to anaerobic counterparts (Rajmohan, Chandrasekaran, & Varjani, 2020).

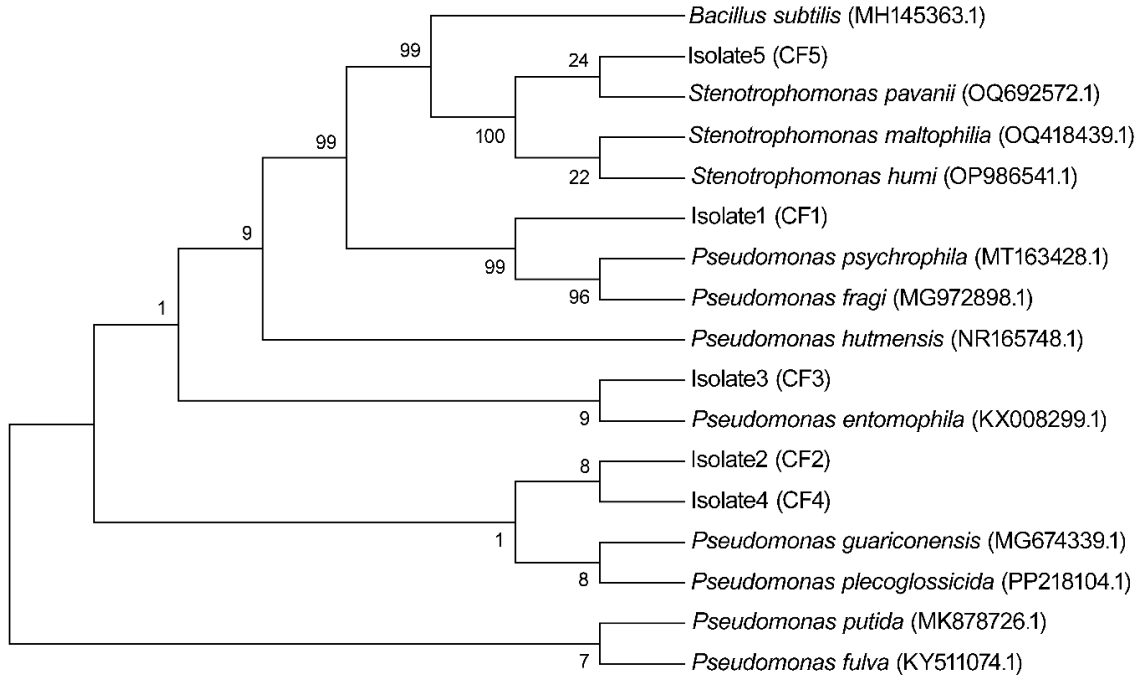

Figure 7 Molecular phylogenetic analysis by maximum likelihood method

Our findings illuminate the resilience and adaptability of indigenous bacteria to local environmental conditions, positioning them as viable candidates for in-situ bioremediation efforts. In line with prior research, our study emphasizes the pivotal role of native soil bacteria in mitigating environmental risks associated with pesticide contamination (Ariffin, & Rahman, 2020). The morphological and biochemical properties of the bacteria found in our study are consistent with those reported by Karpouzas et al., (2000), who categorized carbofuran-degrading bacteria using Restriction Fragment Length Polymorphism (RFLP) of the 16S rRNA gene and partial 16S rRNA gene sequencing. Their study identified 9 out of 23 isolates as *Pseudomonas*, a bacterial group commonly found in various environments, including soil.

While *Pseudomona*s dominated our isolates, the diversity of carbofuran-degrading bacteria, as demonstrated by studies like Onunga et al., (2015), suggests ample potential for tailored soil remediation strategies. Analyzing the relationships between carbofuran-degrading bacterial strains and related taxa offers valuable insights into potential synergies and optimization avenues for bioremediation efforts.

Given the limitations of the 16S nucleotide sequences and the low bootstrap values, we acknowledge that the phylogenetic analysis presented in this study has constraints in its ability to conclusively determine genetic relationships. However, the phylogenetic tree, though not definitive, provides a preliminary view of the evolutionary relationships among the isolated strains. This information, while requiring cautious interpretation, complements our findings and offers a foundational understanding that could guide future, more robust analyses.

The efficacy of bioremediation methods hinges on various factors, including nutrient availability and environmental conditions (Alori et al., 2022). Strategies like microbial immobilization and synergistic approaches combining microbial consortia and phytoremediation hold promise for improving carbofuran degradation efficiency in soil (Duc, 2022; Umar Mustapha et al., 2020). In addition, improved carbofuran degradation efficiency may be achieved by combining microbial consortia and phytoremediation methods. Sarapirom et al., (2022) demonstrated the effectiveness of bioaugmentation and phyto-remediation techniques in degrading paraquat in contaminated soil. This synergistic approach can be partly attributed to the improvement of soil conditions by plants, which enhances microbial activities. Processes such as root growth allow increased oxygen, water, and nutrients into deeper soil layers, ultimately creating favorable conditions for bacteria to break down specific compounds.

6. Conclusion

The demonstrated biodegradation capacity of indigenous soil bacteria presents a significant opportunity for the development of sustainable bioremediation strategies in carbofuran-contaminated agricultural sites. Future research endeavors should prioritize understanding the molecular mechanisms underlying degradation processes and optimizing environmental conditions to further enhance bioremediation efficacy. Such advancements are crucial for safeguarding agricultural and environmental health while ensuring the sustainable management of pesticide contamination.

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