International Journal of Agricultural Technology 2024 Vol. 20(6):2379-2392
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Isolation and identification of endosulfan-degrading bacteria from agriculture land: A potential bioremediation approach

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Kumari, A., Kumar, T., Kumar, A., Kumar, A. and Deokant (2024). Isolation and identification of endosulfan-degrading bacteria from agriculture land: A potential bioremediation approach. International journal of Agricultural Technology, 20(6):2379-2392.

Abstract The findings revealed the presence of diverse bacterial populations capable of degrading endosulfan in the sampled soil. In this investigation, there are found bacterial species *Acinetobacter oryzae*, *Flavobacterium*, and *Bacillus* sp. which identified as potent agents for insecticide degradation. Bioremediation emerges as a compelling strategy due to its inherent eco-friendliness, cost-effectiveness, and efficiency in detoxifying pesticide-contaminated environments.

Keywords: Pesticides, Bioremediation, Acinetobacter, Flavobacterium, Bacillus

Introduction

Endosulfan is a highly toxic and persistent organochlorine pesticide that has been widely used in agriculture to control various pests, including insects, mites, and nematodes (Yadav and Devi, 2017). It was first introduced in the 1950s and gained popularity due to its effectiveness against a broad range of agricultural pests. it is chemically classified as a chlorinated hydrocarbon and is known for its long environmental half-life, which means it can persist in the environment for an extended period (Vivekanandhan and Duraisamy, 2012; Marčić *et al.,* 2011).

Itis notorious for its long-term persistence in soil, water, and sediments. It can remain in the environment for several years after application, leading to the risk of accumulation and ongoing exposure. Endosulfan can accumulate in the tissues of plants and animals. This process is known as bioaccumulation, and it can occur throughout the food. When organisms at lower trophic levels (e.g., plants or small aquatic organisms) are exposed to Endosulfan, the pesticide can accumulate in their tissues (Kuvarega and Taru, 2011). Predators higher up the food chain may then consume these contaminated organisms, leading to higher levels of Endosulfan in their bodies. While Endosulfan is effective against target pests,

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it can also harm non-target species, including beneficial insects, birds, fish, and mammals (Sánchez-Bayo, 2012). This widespread toxicity is a significant environmental concern. Endosulfan is known to leach into groundwater and contaminate surface waters such as rivers, lakes, and ponds. This contamination can have detrimental effects on aquatic ecosystems, leading to fish kills and the disruption of aquatic food webs (Singh *et al.,* 2007). Endosulfan exposure can also pose risks to human health. Recognizing the environmental and health risks associated with Endosulfan, many countries have taken regulatory actions to limit or ban its use. The Stockholm Convention on Persistent Organic Pollutants (POPs) added Endosulfan to its list of banned or restricted chemicals in 2011, further emphasizing the need for alternative pest control methods (Hagen and Walls, 2005). Due to its persistence, toxicity, and potential to cause harm to both the environment and human health, there is growing interest in finding effective methods for the remediation of Endosulfan-contaminated sites. Bioremediation, using microorganisms like bacteria to degrade Endosulfan, is one such approach that holds promise in mitigating the environmental impact of this pesticide (Senthil Kumar *et al.,* 2018). The need for bioremediation strategies and the role of bacteria in degrading pollutants are closely connected, as bioremediation relies on the natural capabilities of microorganisms, including bacteria, to break down and detoxify various pollutants in the environment. Pollution of air, soil, and water is a widespread and significant global problem (Shahi Khalaf Ansar, *et al.,* 2022). Traditional methods for remediating polluted sites, such as excavation and incineration, can be expensive and environmentally disruptive. Bioremediation offers a cost-effective and environmentally friendly alternative for cleaning up contaminated areas. A diverse group of bacteria, including members of the genera *Alcaligenes, Flavobacterium, Pseudomonas* and *Rhodococcus,* metabolize pesticides (Aislabie and Lloyd-Jones, 1995).

The isolation of indigenous bacteria capable ofmetabolising certain pesticides has received a lot of attention and is seen as an efficient method for pesticide bioremediation (Rahman *et al.,* 2018). These bacteria are often referred to as "pollutant-degrading bacteria" or "biodegraders." Biodegradation is the process by which microorganisms, including bacteria, break down pollutants into simpler, non-toxic compounds (Eskander and Saleh, 2017). Bacteria achieve this through enzymatic reactions that transform complex pollutants into smaller molecules that are easier to assimilate into their cellular processes. Bacteria are highly adaptable and can evolve over time to become more efficient at degrading specific pollutants. This adaptability is essential for dealing with complex and diverse pollutant mixtures found in contaminated environments (Escher *et al.,* 2020). Bacteria can target a wide range of pollutants, including organic compounds, hydrocarbons, heavy metals, and pesticides. Therefore, they are versatile tools for remediating various types of pollution. In some cases, multiple

bacterial species can work together in a synergistic manner to degrade complex pollutants. This can enhance the efficiency of bioremediation processes. They do not involve the transportation of contaminated materials and do not generate additional waste. Bioremediation strategies leverage the natural abilities of bacteria and other microorganisms to clean up polluted environments efficiently and cost-effectively. The present investigation was aimed to isolate and identify insecticides degrading bacteria from agriculture contaminated soil.

Materials and methods

Collection of soil sample

Soil sampling was conducted in the agricultural fields situated within the Samastipur district of Bihar, characterized by geographic coordinates at a longitude of 85.8044 and a latitude of 25.71526 (Figure 1). These fields are of particular interest due to the prevalent use of Endosulfan as an insecticide by local farmers on their cultivated crops. The collection process was carried out meticulously, with each soil sample being collected in duplicate to ensure the accuracy and reliability of the results. After gathering the soil samples from various locations within the district, they were carefully placed in sealed containers, typically jars, to preserve their integrity during transportation to the laboratory for further analysis and testing.

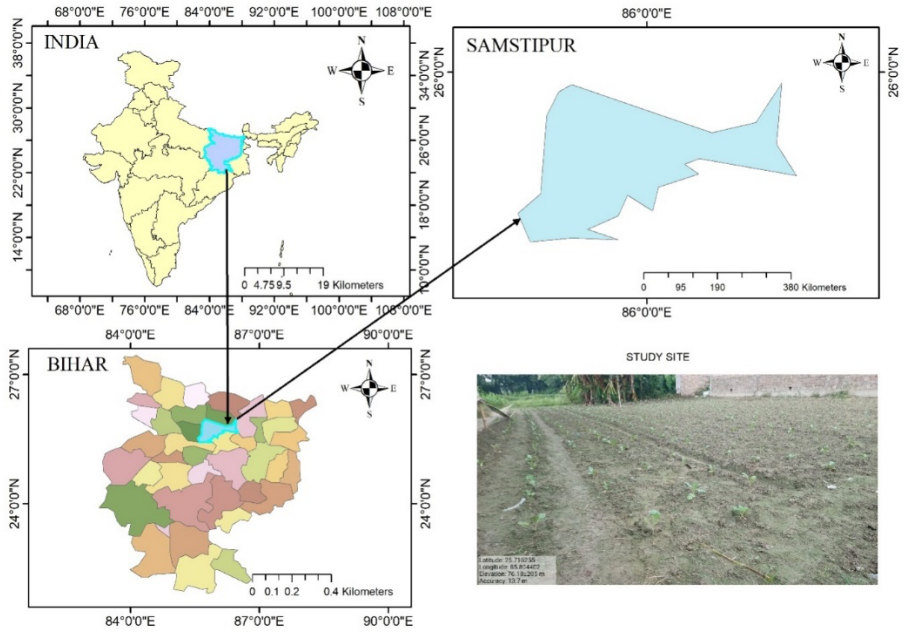

Figure 1. Map of the study site Samastipur, Bihar, India

Preparation of chemical and media

A stock solution of the pesticide Endosulfan was prepared by dissolving it in acetonitrile. This solution was meticulously filtered to remove any impurities and sterilized to ensure its purity. Subsequently, the sterilized Endosulfan stock solution was carefully stored in a refrigerator, preserving its integrity for future use. For the isolation and cultivation of bacterial strains capable of degrading Endosulfan, a specialized growth medium known as Bushnell agar was employed. This medium was adjusted to a neutral pH of 7.0, ensuring optimal conditions for bacterial growth. Using this pH-adjusted Bushnell agar, the process of isolating and nurturing Endosulfan-degrading bacterial strains was carried out with precision and care.

Analysis of physico-chemical parameter

According to chemical analysis, representative samples of the initial soil were taken, air-dried, and crushed to a one-millimetre thickness. A 10g sample of soil was dried overnight at 105 °C in order to determine the gravimetric soil moisture content (Reynolds, 1970).

A percentage (%) was used to show the moisture content. A glass electrode pH meter and electrical conductivity meter (pH HI1131) was used to measure the pH (soil: deionized water=1:2.5 w/v) of the sample. Standard procedures were used to analyse the phosphate, magnesium, and chloride (Radhika and Kannahi, 2014).

Isolation of endosulfan degrading bacteria

Minimal media containing pesticide as a carbon source was used to isolate insecticide (Endosulfan) degrading bacteria. The bacterial species were isolated from Endosulfan contaminated soil by serial dilution method (Figure 2). Different bacterial colonies were observed in Bushnell Haas agar medium. These colonies were identified by Gram's staining and biochemical tests. Endosulfan was used at different concentration of 0.1%, 0.5% and 1% in Bushnell Haas Medium as a selective enrichment agent for isolation of bacteria.

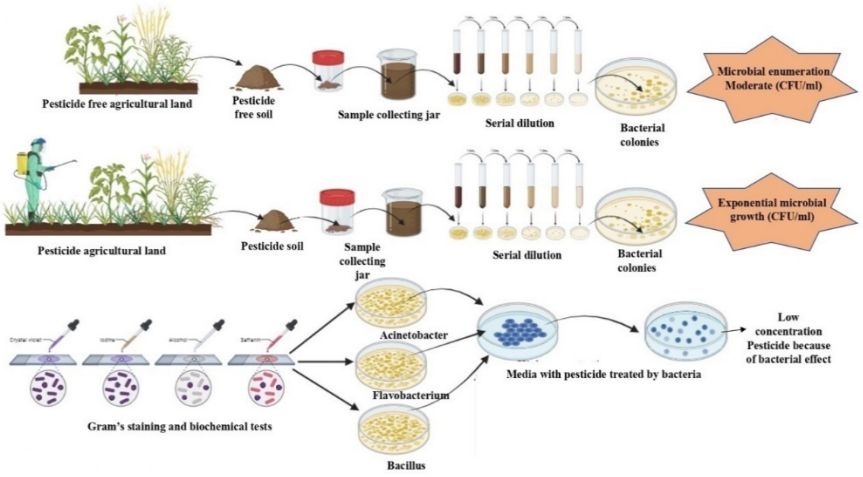

Figure 2. Experimental workflow: Isolation and degradation of Endosulfan by soil bacteria

Identification of bacteria

The phylogeny was prepared using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1403 positions in the final dataset. Evolutionary analyses were conducted in MEGA11(Tamura *et al.,* 2021).

Results

Physico-chemical characteristics of soil sample analysis

The soil sample under examination exhibited various physicochemical properties. These characteristics provide valuable insights into the composition and condition of the soil. The pH level of the soil sample is recorded at 7.6. This pH value indicates the soil's acidity or alkalinity and is a crucial factor influencing nutrient availability and microbial activity within the soil.

The temperature of the soil is recorded at 27°C. Soil temperature is shown to be a significant factor affecting biological activity, nutrient cycling,

and plant growth in the soil ecosystem. The moisture content of the soil was relatively high, at 86%. This measurement represented the proportion of water present in the soil, which is vital for sustaining plant life and various soil organisms. The soil sample contained phosphate at a concentration of 30.6 milligrams per unit of measurement. Phosphate is an essential nutrient for plant growth and is indicative of the soil's fertility. The soil is found to contain magnesium with a concentration of 13.0 milligrams per unit. Magnesium is another critical nutrient for plants and plays a role in various metabolic processes within the soil. Chloride is presented in the soil at a concentration of 10.5 milligrams per unit, with a small measurement uncertainty of ±0.6 milligrams. Chloride levels can influence plant health and relevant for assessing soil salinity. These physicochemical characteristics are summarized in Table 1, providing a comprehensive overview of the soil sample's properties. This information is essential for understanding the soil's suitability for various agricultural or environmental purposes and for making informed decisions regarding soil management and improvement strategies.

Table 1. Physicochemical characterization of soil sample

Sl. No.	Parameters	Values of soil samples
1	pH	7.6
2	Temperature ^{0}C	27^{0}C
3	Moisture content	86%
4	Phosphate(mg)	30.6±0.0mg
5	Magnesium(mg)	13.0±0.5mg
6	Chloride(mg)	10.5±0.6mg

*Values are Mean ±standard deviation

Isolation of endosulfan degrading bacteria

Bacterial species were obtained from soil samples collected from the pesticide-contaminated soil of farming land. To isolate these bacteria, a serial dilution method was employed, which involved diluting the soil sample multiple times to obtain individual bacterial colonies. Subsequently, the isolated bacterial colonies were grown on Bushnell Haas agar medium, where they developed into distinct colonies. These colonies were then subjected to a series of laboratory tests for identification purposes.

Firstly, Gram's staining was performed, a technique that categorizes bacteria into two groups based on the characteristics of their cell walls: Gram-positive and Gram-negative. This staining process helped in distinguishing between different types of bacteria. Following Gram's staining, the bacteria underwent a series of biochemical tests. These tests encompassed a range of

experiments designed to detect specific metabolic characteristics and chemical reactions exhibited by the bacteria. These reactions included the utilization of certain nutrients, the production of specific enzymes, or the fermentation of sugars. By analyzing the results of these tests, it was able to identify and classify the bacterial species presented in the pesticide-contaminated soil accurately.

Identification and growth conditions of isolates

The 16S rRNA sequencing results identified as *Acinetobacter oryzae* strain B23 based on 98-100% similarity to a GeneBank entry with accession number NR181031.1. (Figure 3). Similarly, *Flavobacterium indicum* strain GPTSA100-9=DSM17447 exhibited a similarity level of 98-100% to a GeneBank with accession number NR043269.1 (Figure 4) and *Bacillus subtilis* strain IAM 12118 exhibited a similarity level of 98-100% to a GeneBank with accession number NR112116.2 (Figure 5). All identified species matched with 100% bootstrap support in the phylogenetic tree.

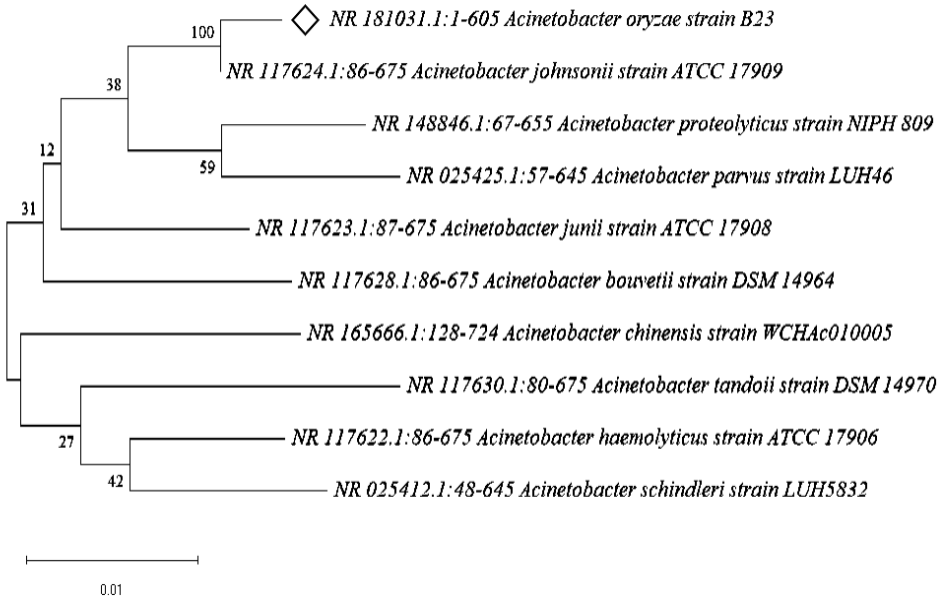

Figure 3. Phylogenetic tree of *Acinetobacter oryzae* strain B23 prepared by Neighbor joining method using MEGA11

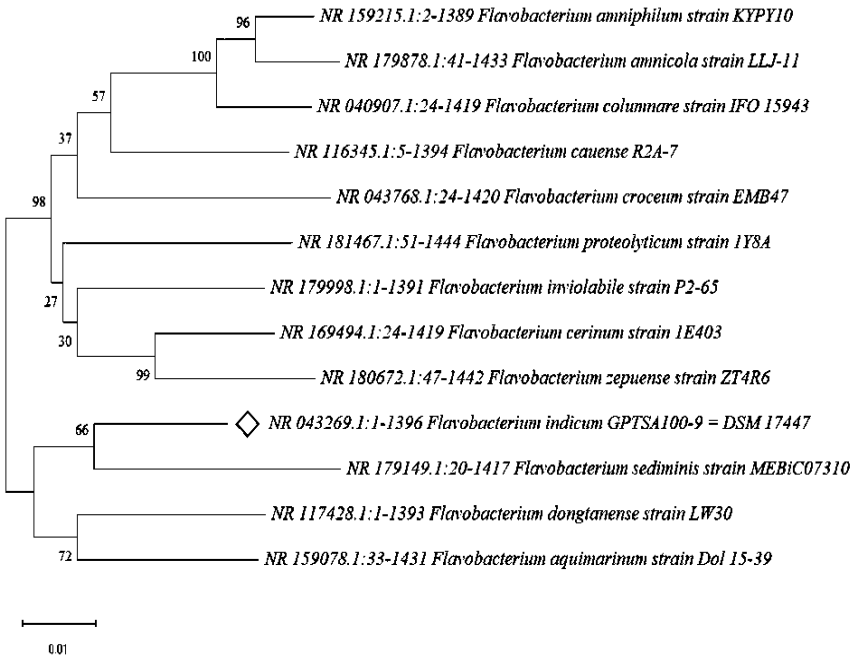

Figure 4. Phylogenetic tree of *Flavobacterium indicum GPTSA100-9=DSM17447* prepared by Neighbor joining method using MEGA11

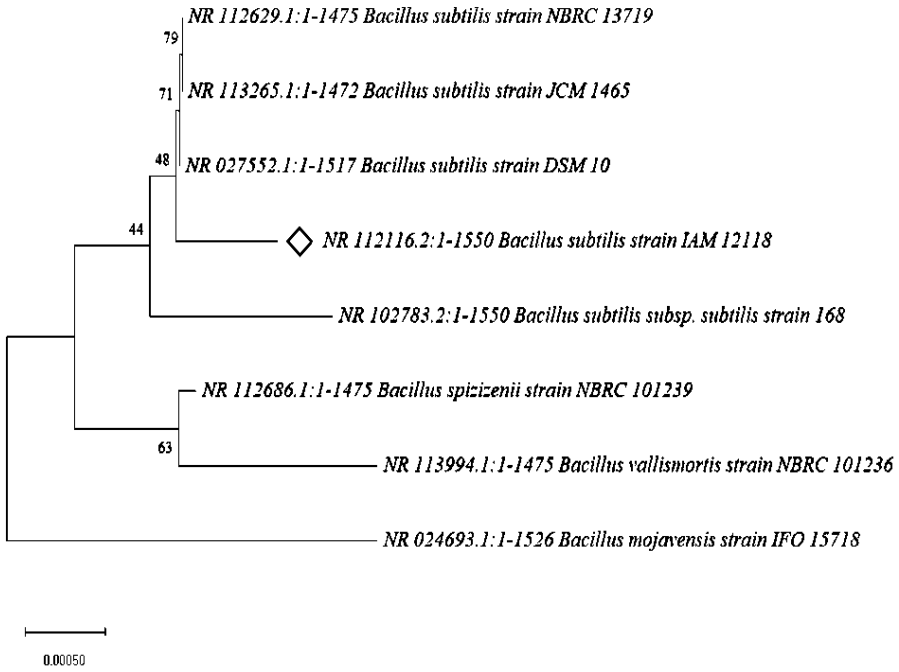

Figure 5. Phylogenetic tree of *Bacillus subtilis* strain IAM 12118 prepared by Neighbor joining method using MEGA11

In this study, the distinctive traits associated with the growth of isolated microorganisms were examined. The method was involved to measure the absorbance of light at a wavelength of 600 nanometres (nm). This particular wavelength is commonly used in microbiology to assess the growth and density of bacterial cultures. The microorganisms focused in this analysis were *Acinetobacter oryzae*, *Flavobacterium indicum*, and *Bacillus subtilis*. Their growth characteristics were assessed by quantifying the absorbance values at 600nm for each of these organisms. The outcomes unveiled distinct absorbance measurements associated with each of the microorganisms, as indicated in Table 2. For *Acinetobacter oryzae*, the absorbance value was recorded as 0.55 at 600nm, indicating the extent of its growth under the given conditions. Similarly, *Flavobacterium indicum* exhibited a slightly higher absorbance value of 0.61 at the same wavelength, suggesting its growth was slightly more robust compared to *Acinetobacter oryzae* under the experimental conditions. In contrast, *Bacillus subtilis* displayed the highest absorbance value among the three microorganisms, registering at 1.25 at 600nm. It was significantly higher absorbance value implies that *Bacillus subtilis* exhibited the most pronounced growth among the isolated organisms, demonstrating a thriving and flourishing population under the experimental conditions. The absorbance measurements at 600nm provided valuable insights into the growth characteristics of *Acinetobacter oryzae*, *Flavobacterium indicum* and *Bacillus subtilis*allowing to distinguish the varying degrees of growth exhibited by these isolated microorganisms.

Table 2. Analysis of Endosulfan degrading organisms

S. No.	Organisms	OD value at 600nm
1	*Acinetobacter oryzae*	0.55±0.35
2	*Flavobacterium indicum*	0.61±0.30
3	*Bacillus subtilis*	1.25± 0.5

*Values are Mean±standard deviation

Endosulfan utilizing bacteria

The results of the degradation of Endosulfan by *Acinetobacter oryzae*, *Flavobacterium indicum*, and *Bacillus* are presented. For *Acinetobacter oryzae*, the maximum zones of accumulation were observed at different concentrations of Endosulfan. Specifically, at concentrations of 0.1%, 0.5%, and 1.0%, the maximum zones of accumulation were measured at 2.17±0.40, 1.93±0.3, and 0.77±0.20, respectively (Table 3). These values indicated the highest points of Endosulfan degradation achieved by *Acinetobacter oryzae* at each concentration. In the case of *Flavobacterium indicum*, similar concentration-dependent trends were observed. At concentrations of 0.1%, 0.5%, and 1.0% of Endosulfan, the maximum zones of accumulation were

recorded at 2.07±0.3, 2.12±0.8, and 0.66±0.14, respectively. These values highlighted the peak levels of Endosulfan degradation attained by *Flavobacterium indicum* under the specified conditions. Similarly, for *Bacillus subtilis*, the maximum zones of accumulation were noted at varying concentrations of Endosulfan. At concentrations of 0.1%, 0.5%, and 1.0%, *Bacillus subtilis* exhibited maximum zones of accumulation measuring 2.28±0.50, 1.97±0.4, and 0.87±0.10, respectively (Table 3). These figures signify the highest levels of Endosulfan degradation achieved by *Bacillus subtilis* at the respective concentrations. A comprehensive overview of the degradation capabilities of *Acinetobacter oryzae*, *Flavobacterium indicum*, and *Bacillus subtilis* in response to different concentrations of Endosulfan are presented in Table 3. These results showcased the concentration-dependent patterns of Endosulfan degradation exhibited by these microorganisms, shedding light on their effectiveness in environmental remediation efforts.

Table 3. Total bacterial population and degradation of Endosulfan at concentrations of (0.01 and 0.1%) in contaminated soil

S. No.	Organisms	Dilution	Total viable counts (CFU/g)	Endosulfan counts (CFU/g)	Endosulfan resistance in concentration	bacterial different (%)
				0.1%	**0.5%**	**1.0%**
1	*Acinetobacter oryzae*	10^{-4} 10^{-5} 10^{-6} 10^{-7}	5.50±0.30 4.25±0.50 3.76±0.45 3.54±0.45	3.55±0.40 2.54±1.40 1.41±0.30 1.30±0.20	2.54±0.3 2.33±0.2 1.34±0.6 1.39±0.3	1.00±0.20 0.70±0.10 0.65±0.55 0.56±0.55
2	*Flavobacterium indicum*	10^{-4} 10^{-5} 10^{-6} 10^{-7}	5.50±0.40 4.60±0.30 3.95±0.50 3.67±0.45	3.32±0.30 2.54±0.40 1.30±0.30 1.21±0.20	3.52±0.30 2.41±0.40 1.20±0.20 1.00±0.01	1.07±0.10 0.50±0.07 0.50±0.05 0.40±0.54
3	*Bacillus subtilis*	10^{-4} 10^{-5} 10^{-6} 10^{-7}	5.60±0.50 4.32±0.45 3.94±0.55 3.32±0.35	3.65±0.50 2.62±0.50 1.50±0.40 1.41±0.31	2.62±0.4 2.44±0.5 1.40±0.4 1.40±0.2	1.07±0.10 0.90±0.54 0.80±0.47 0.60±0.46

*Values are Mean ±standard deviation

Discussion

The study explored the properties of pesticide-contaminated soil and isolated bacterial species that can degrade endosulfan, a harmful pesticide. By examining the soil's chemical and physical characteristics, we gained insights into the factors that influence microbial activity and the potential for certain bacteria to break down the pesticide. Three specific bacteria were identified: *Acinetobacter oryzae*, *Flavobacterium indicum*, and *Bacillus subtilis*. The soil had a slightly alkaline pH of 7.6, which is ideal for many microorganisms, including those that help degrade pesticides. This pH level supports the function of enzymes that break down complex molecules like endosulfan. The soil temperature was 27°C, which is a suitable range for bacteria to thrive, especially those involved in biodegradation. Additionally, the soil's high moisture content (86%) is essential for microbial metabolism, enabling the transport of nutrients and contaminants (Brady and Weil, 2008). Nutrients like phosphate and magnesium were present in the soil, providing a fertile environment for microbial communities to thrive (Marschner, 2012), while chloride levels were within safe limits, indicating no salinity issues that could hinder microbial activity.To isolate the bacteria, we used a serial dilution method and cultured the samples on a specific growth medium. They identified the three bacterial species through staining and biochemical tests. Each species is known for its ability to degrade pesticides, with past studies confirming their roles (Rahman *et al.,* 2016; Zhang *et al.,* 2018).

The bacteria's growth was measured by their optical density (OD) at 600 nm. Among the isolates, *Bacillus subtilis* showed the strongest growth, with an OD value of 1.25, indicating that it thrived under the experimental conditions. This is likely due to *B. subtilis's* resilience and versatility, which make it well-suited to surviving in contaminated environments. *Flavobacterium indicum* showed moderate growth, while *Acinetobacter oryzae* grew the least. These variations reflect differences in how each species adapts to the environment and their metabolic capabilities (Gir *et al.,* 2022).

The degradation tests revealed that *Bacillus subtilis* was the most effective at breaking down endosulfan, especially at lower concentrations. It performed better than the other two species across all tested concentrations (0.1%, 0.5%, and 1.0%), suggesting that it has efficient enzymes capable of metabolizing endosulfan. However, its effectiveness slightly decreased at higher concentrations, possibly due to saturation. On the other hand, Acinetobacter oryzae and Flavobacterium indicum also showed the ability to degrade endosulfan, but not as effectively as *Bacillus subtilis*. As the endosulfan concentration increased, the degradation efficiency of these two species declined, likely due to inhibition or reduced enzyme activity.

Further, the study showed that as the concentration of endosulfan increased, the overall microbial population in the soil decreased. However, *Bacillus subtilis* maintained relatively high numbers across all

concentrations, reinforcing its potential for bioremediation. In contrast, the other two bacteria saw a more significant decline in their populations, which could limit their use in highly contaminated environments.

Implications for bioremediation

We concluded that *Bacillus subtilis* is particularly promising for use in cleaning up pesticide-contaminated soils due to its robust growth and strong degradation capabilities. This bacterium could help improve soil health and reduce pesticide residues, making it useful in agricultural settings. The study also emphasizes the need to select bacterial strains with high resistance and metabolic efficiency to ensure the success of biodegradation efforts.

Limitations and future directions

While the findings are promising, the study was conducted in a lab setting, which may not fully replicate real-world conditions. Field trials are necessary to test how well these bacteria perform in natural environments, where factors like temperature fluctuations and competition with other microorganisms could affect their effectiveness. Additionally, further research into the specific enzymes and metabolic pathways involved in endosulfan degradation could provide valuable insights into improving bioremediation techniques.

Acknowledgements

We would like to express our sincere gratitude to our supervisor (Dr. Deokant), for their support, mentorship, and insightful guidance were indispensable throughout this journey. We are indebted to our co-authors, for their tireless collaboration, creative input, and unwavering dedication. This research is our collective efforts and teamwork.

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(Received: 6 October 2023, Revised: 16 November 2024, Accepted: 19 November 2024)