PROBIOTIC MICROBIAL SPRAY FOR DEGRADATION OF PESTICIDE RESIDUES ON FRESH PRODUCE

Submitted by

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UNDER THE GUIDANCE OF

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VIVEKANAND COLLEGE, KOLHAPUR

(AN EMPOWERED AUTONOMOUS INSTITUTE)

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"Dissemination of education for Knowledge, Science and culture"
- Shikshanmaharshi Dr. Bapuji Salunkhe

Shri Swami Vivekanand Shikshan Sanstha's

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CERTIFICATE
OF
RESEARCH PROJECT COMPLETION

This is to certify that Ms. **RUSHITA DINKAR POWAR** studying in M. Sc. part II, Sem-IV at Vivekanand College, Kolhapur (An Empowered Autonomous Institute) has sincerely completed research project work entitled **"PROBIOTIC MICROBIAL SPRAY FOR DEGRADATION OF PESTICIDE RESIDUES ON FRESH PRODUCE"** during academic year 2025-26.

24/4/26.

24.04.26

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Place: Kolhapur

Date:

Ms. Rushita D. Powar
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INDEX

1.0

INTRODUCTION

The extensive use of chemical pesticides in modern agriculture has greatly enhanced global food productivity and pest control efficiency. However, their excessive and indiscriminate application has resulted in the accumulation of toxic residues in soil, water, and food chains, posing severe environmental and health concerns (Singh et al., 2020). Many pesticides are chemically stable and resistant to natural degradation, leading to their persistence in the ecosystem and potential bioaccumulation in living organisms (Kumar et. al., 2021). This persistence disrupts soil microbial diversity, affects nutrient cycling, and may lead to long-term ecological imbalance (Dragun et. al., 1984). Biotechnol Bioeng Pesticides play a crucial role in modern agriculture by protecting crops from pests and increasing food production; however, their extensive and indiscriminate use has resulted in severe environmental contamination and ecological imbalance (Aktar et. al., 2009). Residual pesticides often persist in soil, water, and air entering the food chain and causing adverse effects on non-target organisms including humans (Carvalho, 2017). Due to their stability and resistance to degradation, many synthetic pesticides such as organochlorines, organophosphates, and carbamates accumulate in the environment and pose long-term risks (Zhang et. al., 2018). Traditional physicochemical methods for pesticide removal, such as incineration, adsorption and chemical oxidation, are often costly, inefficient, and may generate secondary pollutants (Singh and Walker, 2006). Conventional methods for pesticide removal, including chemical oxidation, adsorption, and incineration, are often costly and may produce harmful by-products (Yadav et. al., 2019).

In contrast, various bacteria (e.g., *Pseudomonas, Bacillus, Flavobacterium*), fungi (e.g., *Aspergillus, Trichoderma, Penicillium)*, and actinomycetes have demonstrated the ability to degrade a wide range of pesticide compounds through enzymatic processes such as hydrolysis, oxidation, and dechlorination (Agarwal et. al., 2020; Hussain et. al., 2009). The identification and characterization of these pesticide-degrading microorganisms are therefore essential for understanding their metabolic pathways, optimizing degradation conditions, and applying them in large-scale bioremediation systems aimed at restoring pesticide-polluted ecosystems (Cycoń et. al., 2017).

Microorganisms such as bacteria, fungi, and actinomycetes possess enzymatic systems that enable them to metabolize, transform, or completely mineralize pesticides into non- toxic compounds (Cycoń et. al., 2017). Several bacterial genera, including Pseudomonas, Bacillus, Burkholderia, and Flavobacterium, have demonstrated significant potential in degrading diverse classes of pesticides such as organophosphates, carbamates, and organochlorines (Abraham & Silambarasan, 2022). These microorganisms utilize pesticides as sources of carbon, nitrogen, or phosphorus, or degrade them co-metabolically, depending on environmental conditions and the structural complexity of the compound (Zhang et. al., 2020). Similarly, certain fungi such as Aspergillus and Trichoderma secrete extracellular enzymes like hydrolases and oxidases that play a vital role in pesticide degradation and detoxification (Ahemad & Khan, 2012).

The process of microbial degradation not only reduces pesticide toxicity but also contributes to improving soil fertility and restoring ecological balance (Gupta et. al., 2018). Furthermore, understanding the genetic and biochemical mechanisms involved in microbial degradation can facilitate the development of bioaugmentation and biostimulation strategies for large-scale environmental remediation (Sharma et. al., 2021). Thus, the study of pesticide-degrading organisms forms a cornerstone in the development of sustainable agricultural practices and in mitigating the adverse effects of chemical pollution on ecosystems.

Microbial degradation of pesticides is primarily governed by the metabolic versatility of microorganisms and the presence of specific catabolic enzymes that can transform complex pesticide molecules into simpler, non-toxic compounds (Singh et al., 2016). Bacteria are often the most effective degraders due to their rapid adaptability and diverse enzymatic systems capable of utilizing pesticides as carbon or energy sources (Aislabie and Lloyd-Jones, 1995). For instance, species such as *Pseudomonas putida, Bacillus subtilis,* and *Flavobacterium sp.* have been reported to degrade organophosphates and chlorinated hydrocarbons effectively under aerobic conditions (Cycoń and Piotrowska-Seget, 2016).

Fungi also contribute significantly to pesticide degradation through the secretion of extracellular oxidative enzymes such as laccases, peroxidases, and hydrolases, which can break down complex aromatic structures of persistent pesticides (Bollag and Liu, 1990). Moreover, (Chaudhry et. al., 2002).

Actinomycetes such as *Streptomyces* possess strong catabolic potential for the degradation of herbicides and insecticides due to their ability to produce a wide array of degradative enzymes Environmental factors such as pH, temperature, nutrient availability, oxygen concentration, and soil type significantly influence the efficiency of microbial degradation processes (Arora et. al., 2014). In addition, the presence of co-metabolites or easily degradable organic substrates can enhance the rate of biodegradation by inducing enzyme production or promoting microbial growth (Kumar et. al., 2018). The degradation mechanism typically involves several steps, including adsorption of the pesticide onto microbial cell surfaces, enzymatic cleavage of chemical bonds, and mineralization into harmless end products such as CO_2, H_2O, and inorganic ions (Singh, 2008).

The genes responsible for these degradative enzymes are often located on plasmids or mobile genetic elements, allowing horizontal gene transfer among microbial populations and thus enhancing biodegradation potential in contaminated environments (Rani et. al., 2019).

The study of pesticide-degrading microorganisms not only provides insight into natural detoxification processes but also supports the development of engineered bioremediation systems and bioaugmentation strategies for large-scale environmental cleanup. By isolating efficient strains, optimizing degradation conditions, and applying advanced molecular techniques such as metagenomics and transcriptomics, researchers can better understand the microbial pathways involved and enhance their practical applications in sustainable agriculture and environmental protection (Rani and Kumar, 2020).Despite extensive research on pesticide degradation, significant knowledge gaps remain regarding the diversity, functional mechanisms, and ecological interactions of pesticide- degrading microorganisms in different environmental matrices. Most studies have focused on laboratory-scale degradation under controlled conditions, which do not accurately reflect the complex physicochemical and biological dynamics present in natural ecosystems such asagricultural soils and aquatic environments (Singh et. al., 2011). only under specific conditions or for a narrow range of pesticide compounds, limiting their practical applicability for large-scale bioremediation (Kumar et. al.,2018).

The persistence and bioaccumulation of certain pesticides such as chlorpyrifos, endosulfan, and atrazine highlight the urgent need for identifying novel and robust microbial species capable of tolerating environmental stress while maintaining degradation potential (Cycoń et. al., 2017).

Additionally, the genetic basis of pesticide degradation, including the regulatory networks, enzyme-coding genes, and pathways responsible for detoxification, remains inadequately explored for many microbial taxa (Rani et. al., 2019). Advanced molecular techniques such as metagenomics, next-generation sequencing (NGS), and bioinformatics tools have opened new avenues for discovering unculturable microorganisms and their associated degradative genes (Rani & Kumar, 2020). However, the integration of these molecular insights with practical bioremediation approaches is still limited. There is also a lack of comprehensive studies correlating microbial diversity, enzyme activity, and environmental parameters affecting degradation efficiency (Arora et. al., 2014).

In recent years, probiotic microorganisms have attracted considerable attention for their potential in bioremediation of pesticide residues. Traditionally defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host‖ (FAO/WHO, 2002), probiotics are well known for improving gut health, enhancing immunity, and restoring microbial balance. However, several probiotic species, including *Lactobacillus, Bacillus, Pseudomonas*, and *Saccharomyces,* have demonstrated remarkable metabolic adaptability that enables them to survive in contaminated environments and degrade xenobiotic compounds, including organophosphates, carbamates, and chlorinated hydrocarbons (Patel et. al., 2022).The ability of probiotics to degrade pesticides is primarily attributed to their enzymatic systems—such as hydrolases, esterases, dehalogenases, and phosphatases—that catalyze the breakdown of toxic pesticide molecules into less harmful or non-toxic metabolites (Abdel-Rahman et. al., 2021). These microbes can also adsorb or sequester pesticide residues on their cell surfaces, further reducing environmental contamination. Moreover, the use of probiotic strains offers additional ecological and agricultural benefits: they enhance soil fertility, promote plant growth, suppress pathogens, and restore microbial biodiversity (Choudhary & Sharma, 2019).

Integrating probiotic organisms into agricultural practices provides a dual advantage— improving the microbiological quality of soil and crops while simultaneously degrading hazardous chemicals. Their Generally Recognized As Safe (GRAS) status makes them particularly attractive for use in food and environmental systems without risk of pathogenicity or toxicity (Hill et. al., 2014). Hence, exploring the potential of probiotic pesticide-degrading organisms represents an innovative, sustainable, and biologically safe approach for mitigating pesticide pollution and promoting eco-friendly agricultural systems.

Therefore, the present study aims to isolate, identify, and characterize efficient pesticide- degrading microorganisms from various sources and to evaluate their degradation potential. The research also seeks and analyses the effect of pesticide degrading bioformulation on the food products.

2.0
REVIEW OF LITERATURE

2.1. Degradation of pesticide

The microbial degradation studies of pesticides had been greatly developed, and most of the pesticide degrading microbial strains have also been identified, but the actual application of microbial bioremediation was limited, which was often due to its low degradable efficiency and the environment condition. Mineralization and co-metabolism were the main mechanisms for the further degradation of pesticides and their intermediate products, while the group and molecular structure of pesticide determined its degradation behavior in the microbial environment, chemical structure determined its solubility, in which molecular orientation, spatial structure, chemical functional groups, intermolecular attraction, and repulsion characteristics affecting the ingestion of pesticides by microorganism. The main research directions of microbial degradation of pesticides were the development of high efficiency pesticide degradation engineering bacteria, the cultivation of mixed bacteria, the immobilization of degrading bacteria, and the quantitative study of pesticide biodegradation models. In recent years, with the development of genetic engineering and molecular biology, on the one hand, researchers began to shift to the construction of efficient engineering bacteria, and used the gene recombination technique. On the other hand, they transformed enzyme genes to construct the vector that could express efficiently the characteristics of degrading pesticides. After that, engineering bacteria could be received. The purpose of that was to improve the expression level of specific proteins or enzymes, so as to improve the efficiency of degradation, which could overcome the problem that some enzymes in the environment could not be stabilized and maintain a high enzyme activity. In short, there was an effective method to eliminate pesticide pollution, which was using microbial agents or fertilizer preparation applied in polluted environments. The difference of pyrethroid degradation in the human body was still not very clear. Although the isolation and screening of degrading bacteria and their degradable effect were better, the research of synergistic degradation targets of various degrading bacteria was rarely seen. A large number of experimental studies could not be applied in practical production. A large number of 3-PBA residues not only caused two pollutions of agricultural products, but also led to pesticides being blocked in biomineralization, which indirectly caused the pesticide residue problems to become more specific methods serious and had a threat to food safety, the environment, and human health. The isolation and screening degradable strains of 3-PBA and the degradable characteristics of strains have been studied currently, but there were no related literature and reports that combined to study the degradation mechanism of 3PBA being degraded to phenolic compounds. For example, chromatography and mass spectrometry, degradable pathway, catalytic mechanism, enzymatic characteristics, and so on should be emphasized. Although heterologous compounds may be partially or completely decomposed by some microorganisms, they may be resistant to degradation in the environment due to their greater structure, insolubility, and highthermal stability. Therefore, we also need to pay attention to this problem to ensure strain and xenobiotic compounds degradation products harmless in pollution remediation (Yichen Huang et. al., 2018). Soil microflora includes a variety of bacteria, fungi, protozoa, algae, and viruses involved in various essential functions in agroecosystems. These microorganisms are involved in numerous fundamental functions in soil, such as nutrient cycling, soil fertility, promoting plant growth, and the decomposition of both organic and inorganic matter. Furthermore, soil microbiota impacts soil physical properties such as structure, porosity, aeration, and water infiltration by stabilizing soil aggregates (Robinson et.al., 2024). Microbial populations interact with the target compound in both physical and chemical manner and ultimately degrade it either absolutely or render it into less toxic compounds by incorporating some structural changes (laura et. al., 2013). Pesticide residues in agricultural soils pose a major environmental concern. This study highlights the critical role of the phytomicrobiome the community of microorganisms associated with plants in the biodegradation of pesticides. Plants create unique ecological niches that support diverse microbes, including endophytic bacteria and fungi, which adapt to stressful, pesticide-contaminated environments and act as ecological engineers. However, there is a pressing need for comprehensive molecular studies to better understand plant– microbe interactions, microbial survival mechanisms, and gene expression in pesticide-affected systems (M.d. Tareq Bin Salam et. al., 2024). The bioremediation process using microbes or soil microflora to degrade pesticides is a cost-effective technique till date. *Actinomycetes* and *cyanobacteria* are the most efficient degraders among the microorganisms.

Microbes possess different enzymes such as Glutathione S-transferases (GSTs), esterases and cytochrome P450 which are involved in the degradation process. (Kauret et. al., 2021). Actinomycetes have considerable potential for the biotransformation and biodegradation of pesticides. A limited number of these xenobiotic pesticides can be mineralized by single isolates, but often consortia of bacteria are required for complete degradation metabolism of pesticides is frequently observed within this group of bacteria. When compared with pesticide degradation by Gram-negative bacteria, much less information about molecular mechanisms involved in biotransformations of pesticides by actinomycetes is available. Progress in this area has been seriously hampered by a lack of suitable molecular genetic tools for most representatives of this major group of soil bacteria. Overcoming this constraint would enable a better exploitation of the biodegradation and biotransformation abilities of actinomycetes for applications such as bioremediation and construction of transgenic herbicide-resistant crops (Schrijver et. al., 2008

2.2 Degradation of pesticide by bacteria

Bacteria play a crucial role due to their high adaptability, rapid growth, and diverse metabolic capabilities. Many bacterial species possess specific enzymes that can degrade complex pesticide molecules into simpler, non-toxic compounds like carbon dioxide, water, and inorganic ions (Singh & Walker et. al., 2006; Kumar et. al., 2018). Genera such as *Pseudomonas, Bacillus, Alcaligenes*, and *Flavobacterium* have been reported to efficiently degrade organophosphates, carbamates, chlorinated hydrocarbons, and other classes of pesticides (Das et. al., 2015). Pesticide residues in agricultural soils pose a major environmental concern. Bacteria have been isolated and characterized which can degrade organophosphorus compounds as a source of phosphorus or carbon or both in the fields. Common pesticide degrading bacteria include *Pseudomonas,Bacillus, Alcaligens, Flavobacterium.*

Bacteria play important role in the biodegradation of pesticides by breaking down toxic compounds into simpler non-toxic substances. They use pesticides as source of carbon, nitrogen or energy through enzymatic reactions. The efficiency of bacterial biodegradation depends on several factors, including pesticide concentration, environmental conditions (pH, temperature, oxygen levels), and the presence of suitable co-substrates (Fenlon et. al., 2011; Cycoń et. al., 2017). Understanding the biochemical pathways and genetic mechanisms involved in bacterial degradation can help in designing bioremediation strategies for restoring contaminated ecosystems (Kumar et. al., 2018). Pesticide fate in the environment is affected by microbial activity. *Pseudomonas* species degrade Aroclor 1242 and triazine herbicides efficiently (Irene et. al., 2020).

Table 1: Main bacterial genera involved in pesticide degradation (Singh et. al., 2006)

Pesticide	Key Bacteria	Notes
Parathion	*Flavobacterium, Pseudomonas, Bacillus, Arthrobacter*	Hydrolysis to p-nitrophenol followed by aromatic ring cleavage.
Chlorpyrifos	*Enterobacter, Pseudomonas, Flavobacterium*	Converted to TCP; TCP degradation slower due to chlorine toxicity.
Glyphosate	*Arthrobacter, Pseudomonas, Rhizobium, Bacillus*	Degraded via C–P bond cleavagethrough C–P lyase pathway.
Fenitrothion, Diazinon, Monocrotophos	*Burkholderia* *Bacillus,* *Pseudomonas*	Hydrolysis and oxidation

A soil bacterium named as *Bacillus subtilis* KPA-1 was isolated from agricultural soil polluted with monocrotophos degrading efficacy using RT-qPCR approach (K.P. Acharya et. al., 2014). An endosulfan-degrading bacterium (strain ESD) was isolated from soil inoculum after repeated culture with the insecticide as the sole source of sulfur. Analysis of its 16S rRNA gene sequence, and morphological and physiological characteristics revealed it to be a new fast-growing Mycobacterium, closely related to other Mycobacterium species with xenobiotic-degrading capabilities. Degradation of endosulfan by strain ESD involved both oxidative and sulfur- separation reactions. Strain ESD did not degrade endosulfan when sulfite, sulphate or methionine were present in the medium along with the insecticide. Partial degradation occurred when the culture was grown, with endosulfan, in the presence of MOPS (3- (N- morpholino) Propane sulphonic acid), DMSO (dimethyl sulfoxide), cysteine or sulphonane and complete degradation occurred in the presence of glutathione. When both beta-endosulfan and low levels of sulphate were provided as the only sources of sulfur, biphasic exponential growth was observed with endosulfan metabolism being restricted to the latter phase of exponential growth. Conclusions: This study isolated a Mycobacterium strain (strain ESD) capable of metabolizing endosulfan by both oxidative and sulfur-separation reactions. The endosulfan-degrading reactions are a result of the sulfur-starvation response of this bacterium. (Sutherland et. al., 2002). Table 1 describes various bacterial genera involved in pesticide degradation.

2.3 Degradation of pesticide by probiotic bacteria

According to FAO/WHO, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014; Barros et al. ,2020; Cuevas-González et al., 2020). Recently, (Zendeboodi et. al., 2020) suggested a different term true probiotic to describe the viable and active microorganisms which indicate in vivo and in vitro effects. Various species in the genera *of Lactobacillus, Bifidobacterium, Streptococcus, Lactococcus, Bacillus, Streptococcus, Pediococcus, Enterococcus, Bacteroides, Akkermansia, Propionibacterium* and *Saccharomyces* have been recognised as probiotics and the most widely species include *Bifidobacterium* animalis, *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus salivarius, Lactobacillus lactis, Lactobacillus plantarum and Lactobacillus reuteri* (Khaneghah et. al., 2020). There is mounting evidence which supports the efficacy of probiotics in management of several diseases and disorders including gastrointestinal disorders, obesity, diabetes, lactose intolerance, hypertension, different types of cancers and psychiatric disorders (Barros et. al., 2019; Tenorio-Jiménez et. al., 2020) through balancing the intestinal microflora, increasing nutrients bioavailability and generation of various bioactive compounds such as bacteriocins, short-chain fatty acids, exopolysaccharides, enzymes and vitamins (Hossain et. al., 2017; Aguilar-Toalá et al., 2018; Indira et al., 2019).

Additionally, it has been highlighted that probiotics can detoxify xenobiotics and hinder their toxicity through binding to these toxicants and employing enzymatic reactions (Khorshidian et. al., 2016; Hossain et al., 2017; Cuevas-González et al., 2017). Detoxification properties of probiotics against mycotoxins, heavy metals, polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, nitrosamine and pesticides have been investigated widely in recent years, and it has been elucidated that this protective activity is dependent on several factors such as strain type, medium pH, growth phase, toxicant structure and concentration (Yousefi et al., 2019; Khorshidian et al., 2020)

An organophosphorus (OP) insecticide-mineralizing strain, *Lactobacillus plantarum* WCP931, harboring a new OP hydrolase (opdC) gene, was isolated during kimchi (Korean traditional food) fermentation. Strain WCP931 exhibited a significant survival rate of 51 to 96% under artificial gastric acid conditions at pH 2 to 3 after 3 h. The opdC gene, consisting of 831 bp encoding 276 amino acids, was cloned from strain WCP907. Recombinant *Escherichia coli* harboring the opdC gene depleted 77% chlorpyrifos (CP) in M9 minimal medium after 6 days of incubation. The OpdC enzyme represents a novel member of the GHSQG family of esterolytic enzymes or a new Opd group. The OpdC molecular mass was estimated to be approximately 31 kDa by SDS-PAGE and showed maximum activity at pH 6 and 35 °C. The mutated OpdC (Ser116 → Ala116) enzyme had no activity towards OP insecticides and ρ-nitrophenol- β-butyrate. Importantly, the relative activity of OpdC protein against chlorpyrifos, coumafos, diazinon, fenamifos, methyl parathion, and parathion was higher than that against cadosafos, dyfonate, and ethoprofos insecticides. These results suggested the involvement of OpdC in the biodegradation of OP insecticide-contaminated cabbage during fermentation. The new OpdC enzyme expands the heterogeneity of the lactic acid bacterial Opd enzyme group in nature. (Jin Lee, et. al., 2021). pH and temperature effects on OpdC enzymatic activity. The pH and temperature effects of the OpdC enzyme were examined by considering esterase activity. The effect of pH (range 3.0–11.0) on the esterase activity of OpdC protein was determined according to the abovementioned protocol at 30 ± 0.5 °C. While, the temperature effect of the OpdC enzyme was determined at 10 to 70 °C for 1 hr. The degree of OP hydrolysis was measured using HPLC. Fifty microliters of enzyme solution were poured into a solution containing 250 μL insecticide (200 mg/L) and 700 μL phosphate buffer (200 mM, pH 6.5). To calculate the experimental error, assays were performed three times. The classical spectrophotometric method was used to measure the activity of esterase provided by the native and mutant OpdC enzymes.

Lactic acid bacteria are gaining global attention, especially due to their role as a probiotic. They are increasingly being used as a flavoring agent and food preservative. Besides their role in food processing, lactic acid bacteria also have a significant role in degrading insecticide residues in the environment. The importance of lactic acid bacteria in degrading insecticide residues of various types, such as organochlorines, organophosphorus, synthetic pyrethroids, neonicotinoids, and diamides are significant. The mechanisms employed by lactic acid bacteria to degrade these insecticides, as well as their potential applications in bioremediation. The key enzymes produced by lactic acid bacteria, such as phosphatase and esterase, play a vital role in breaking down insecticide molecules. The challenges and future directions in this field. However, more research is needed to optimize the utilization of lactic acid bacteria in insecticide residue degradation and to develop practical strategies for their implementation in real-world scenarios. (Kiruthika et. al., 2024).

A convenient and cost-effective method to reduce unavoidable pesticide absorption in humans and wildlife could be the use of probiotic *lactobacilli*. *Lactobacillus* is a genus of Gram- positive gut commensal bacteria used in the production of functional foods, such as yoghurt, cheese, sauerkraut and pickles, as well as silage for animal feed. Preliminary in vitro experiments suggested that *lactobacilli* are able to degrade some pesticides. Probiotic *Lactobacillus rhamnosus* GR-1-supplemented yoghurt reduced the bioaccumulation of mercury and arsenic in pregnant women and children.

Table 2: Analytical determination of pesticide from food product

Food product	Pesticide	Determination method	Analytical range	References
Pasteurised and UHT milk	Organochlorine	GC-MS	65.42 µgkg−1 (heptachlor) 2.62 µgkg−1 heptachlor epoxide 3.86 µgkg−1 (aldrin) 4.28µg kg−1 (4,41 - DDE) 4.85µg kg−1 (dieldrin) 7.32 (2,41 - DDT) 4.74µg kg−1(β-Endosulphan)	Özdemir et al. (2019)
Raw and UHT milk samples	Chloripyrifos, Endosulphan (α andβ), profenofos and bifenthrin	GC-µ ECD	0.1–30 ng L−1	Jawaid et (2016)

	Carbaryl		0.015–0.031 mg kg−1 0.033–0.121 mg kg−1 0.005–0.006 mg kg−1	
Eggplant	Diazinon, Dimethoate, Chlorpyrifos, Quinalphos	GC-MS	0.018–1.617 mg kg−1	Aktaret al. (2017)
Grape	Azoxystrobin, boscalid, bupirimate, cyprodinil, famoxadone, fenhexamid, fludioxonil, imidacloprid, iprodione, pyrimethanil, spinosad, tebuconazole, triadimenol	GC-MS	0.011–2.737 mg kg−1	Zengin and Karaca (2018)

Carrot Melon Tomato Maskmelon	Endosulphan IEndosulphan II Endosulphan-sulphate Chlorpyrifos Iprodione Trifluralin Phosalone	GC-MS	0.02–0.04 Mgkg−1 kg−1	Hadianet al. (2019)

OBJECTIVES

- Isolate, screen , identify most potent pesticide degrading bacterial strain from suitable environmental sources
- To evaluate the probiotic potential of the selected bacterial strain
- To assess the pesticide degradation efficiency of the potent strain
- To formulate a microbial spray capable of degrading pesticide residues
- To evaluate the efficacy of formulated microbial spray

3.0
MATERIAL AND METHODS

3.1 ISOLATION OF PESTICIDE DEGRADING MICRO- ORGANISMS

3.1.1 COLLECTION OF SAMPLES

Soil samples applied with 'Hamla550' pesticide were collected from the agricultural area of Shirol (West region) using sterile containers. Soil from approximately 10–15 cm depth was collected using a spatula, transferred into properly labeled sterile containers, and transported to the laboratory for further analysis. The presence of pesticide-exposed soil in this region makes it a suitable site for isolating potential pesticide-degrading microorganisms The sample was properly labelled with the following details,

1. Date of collection: 19 July 2025
2. Place of collection: Shirol

Finally, the collected sample was transported safely to the laboratory for further analysis and study of pesticide degrading organisms.

3.1.2 COLLECTION OF PESTICIDE

The pesticide sample, Hamla550, was collected from a local agricultural pesticide store. This pesticide contains Chlorpyrifos 50% + Cypermethrin 5% EC, which is commonly used as a systemic and contact insecticide.

3.1.3 PRIMARY SCREENING OF PESTICIDE DEGRADING ORGANISMS

3.1.3.1 SERIAL DILUTION OF SAMPLE

Serial dilution of sample was carried out to reduce microbial load and obtain isolated colonies. One gm of soil sample was added to 9 ml of sterile distilled water (10^{-1} dilution). The mixture was shaken thoroughly using a vortex mixer. Further serial dilutions were prepared up to 10^{-6} or 10^{-7} by transferring 1 ml into 9 ml sterile diluent. Each dilution was mixed properly before transferring to the next tube.

3.1.3.2 PLATING OF SAMPLE

Plating was performed using spread plate technique. 0.1 ml of diluted sample was spread evenly on nutrient agar containing pesticide as selective agent. The plates were incubated at 37°C for 48 hours. Colonies appearing on plates showing clear zone indicates pesticide degradation done by organism. All cultures showing clear zone around growth were Gram stained to study morphological characteristics. Impure cultures were purified by serially subculturing and then pure cultures were labeled as Isolate 1, Isolate 2, Isolate 3 and so on.

Table 3: Composition of nutrient agar with hamla550 pesticide

Components of media	Weight
Hamla550 pesticide	1.0 ml
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Agar-agar	2.5 gm
Distilled water	100 ml
pH	4.0

3.2 SECONDARY SCREENING OF PESTICIDE DEGRADING MICROORGANISMS

All pure labelled isolates were spot inoculated on nutrient media with hamla550 pesticide. The plates were incubated at 37°C for 48 hours to check potency of each isolate for degradation of pesticide. After incubation, colony diameter and clear zone diameter of each isolate were measured and degradation index of each isolate (DI) was calculated using equation 1

DI= Diameter of clear zone / Diameter of colony … .. (Equation 1)

The isolate showing higher degradation index indicates most potent pesticide degrading culture.

3.3 IDENTIFICATION OF POTENT STRAIN

Identification of potent strain was carried out by studying colony characteristics, morphological characteristics and biochemical characteristics.

3.3.1 COLONY CHARACTERISTICS

Potent isolate was studied for colony characteristics such a shape, size, color, margin, elevation surface, opacity, consistency. For this potent isolate was streaked on nutrient agar and plate was incubated at 37°C for 24 hrs. After incubation, a well isolated colony was studied for colony characteristics.

3.3.2 MORPHOLOGICAL CHARACTERIZATION

Morphological characters of potent isolate was studied by performing Gram staining. Capsule staining was done by Chance's method to detect the presence of capsule, and endospore staining was performed by Dorner's method to identify spore formation ability. To observe motility of organism, hanging drop technology was used.

3.3.3 BIOCHEMICAL CHARACTERIZATION

Standard biochemical tests such as catalase test, sugar fermentation tests like Glucose, Sucrose, Lactose, Mannitol were performed for identification.

3.4 STUDY OF POTENT STRAIN FOR PROBIOTIC CHARACTERISTICS

Probiotics testing is essential to evaluate the functional properties and safety of potent strain. This involves a series of *in vitro* tests such as acid tolerance, bile salt tolerance, amylase test, protease test, lipase test and antibiotic sensitivity test.

3.4.1 ACID TOLERANCE TEST

The acid tolerance test is used to determine the ability of microorganisms to survive in acidic conditions, such as those found in the stomach (pH 1.5–3.5). Acid tolerance ability of isolate ensures maximum viability of probiotics after oral consumption, prevents destruction of beneficial bacteria before reaching the intestine, indicates the adaptability of microbes to harsh gastric conditions. For acid tolerance test, nutrient broth was prepared and its pH was adjusted to 3.0 using HCl. The potent strain was inoculated into the nutrient broth and incubated at 37°C. The turbidity in the tube was observed and measured at different time intervals (0, 1, 2, and 3 hours) using a colorimeter. Development of turbidity indicates that the organism is acid tolerant.

Table 4: Composition of Nutrient Broth of pH 3

Components of media	Weight
Peptone	1 ml
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Distilled water	100 ml
pH	3.0

3.4.2 BILE TOLERANCE TEST

The bile tolerance test is used to determine the ability of microorganisms to survive in the presence of bile, similar to conditions found in the small intestine. It confirms the ability of isolate to withstand bile salts in the small intestine. It helps bacteria to survive, grow, and multiply in intestinal conditions.

Nutrient broth was prepared and supplemented with sodium taurocholate (usually 0.3%–1.0%) and pH was adjusted to 9. The potent strain was inoculated into the broth and incubated at 37°C. During incubation, turbidity was observed and measured at 0, 1, 2, and 3 hours using a colorimeter. Development of turbidity indicates that the organism is bile salt tolerant.

Table 5: Composition of nutrient broth of pH 9

Components of media	Weight
Peptone	1 ml
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Distilled water	100 ml
sodium taurocholate	0.2 gm
pH	9.0

3.4.3 ANTIBIOTIC SENSITIVITY TEST

The antibiotic sensitivity test is used to determine the susceptibility of microorganisms to different antibiotics. It ensures safety of probiotic strains for human consumption. It prevents the use of bacteria carrying harmful transferable resistance genes. It maintains gut microbiota balance during infections and treatments.

For antibiotic sensitivity testing, nutrient agar plates were prepared, and the potent strain was uniformly spread over the surface using a sterile swab. Antibiotic discs were placed on the agar surface. The plates were incubated at 3 for 24 hours. After incubation, the zones of inhibition around the discs were observed and measured. A zone of inhibition indicates that the organism is sensitive to the antibiotic, while no zone indicates that the organism is resistant.

Table 6: Composition of nutrient agar

Components of media	Weight
Peptone	1 ml
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Agar -agar	2.5 gm
Distilled water	100 ml
pH	7.0

3.4.4 AMYLASE PRODUCTION TEST

The amylase test is used to determine the ability of microorganisms to produce amylase enzyme, which hydrolyzes starch. It helps in identifying bacteria that can hydrolyze starch into simple sugars. That supports digestion of complex carbohydrates in the gut. It improves nutrient absorption and energy availability.

For amylase test, starch agar plates were prepared, and the potent isolate was inoculated onto the surface of the medium. The plates were incubated at 37°C for 24 – 48 hours. After incubation, the plates were flooded with iodine solution. A clear zone around the growth indicates starch hydrolysis, showing that the organism produces amylase, while no clear zone indicates that the organism is unable to produce the enzyme.

Table 7: Composition of starch agar medium

Components of media	**Weight**
Peptone	1 gm
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Agar -agar	2.5 gm
Distilled water	100ml
Starch	1gm
pH	7

3.4.5 PROTEASE PRODUCTION TEST

The protease test is used to determine the ability of microorganisms to produce protease enzymes that hydrolyze proteins. It shows the ability of bacteria to produce proteolytic enzymes. It helps in digestion of proteins into peptides and amino acids Improves nutrient absorption and bioavailability. It contributes to gut health and metabolism. It may help in reducing protein-related digestive disorders Enhances the functional value of probiotic strains.

For protease production , Gelatine agar plates were prepared, and the potent strain was inoculated onto the surface of the medium. The plate was incubated at 37°C for 24 hours. After incubation plate was flooded with Frazier's reagent. A clear zone around growth indicates protein hydrolysis, showing that the organism produced protease, while no clear zone indicates that the organism does not produce the enzyme.

Table 8: Composition of Gelatine agar

Components of media	Weight
Peptone	1 gm
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Agar -agar	2.5 gm
Distilled water	100ml
Gelatin	1gm
pH	7

3.4.6 LIPASE PRODUCTION TEST

The lipase test was used to determine the ability of microorganisms to produce lipase enzymes that hydrolyze lipids. It indicates the ability to break down fats into fatty acids and the glycerol. It supports efficient lipid digestion. Helps in absorption of a fat-soluble vitamins (A, D, E, K). It improves overall digestive efficiency. It may be help in managing lipid metabolism and cholesterol levels. Adds to the nutritional and therapeutic value of probiotics.

For lipase production test, Tween 80 agar plates were prepared, and the potent strain was streaked onto the surface of the medium. The plates were incubated at 37°C for 24 hours. After incubation, the plates were observed for precipitation zone around the growth. A precipitation zone indicates lipid hydrolysis, showing that the organism produced lipase, while no precipitation zone indicates that the organism did not produce the enzyme.

Table 9: Composition of tween 80 agar medium

Components of media	Weight
Peptone	1 gm
Yeast extract	0.5 gm
Sodium chloride	0.5 gm
Agar-agar	2.5 gm
Distilled water	100ml
Tween 80	1ml
pH	7

3.5 ANALYSIS OF PESTICIDE DEGRADATION

To confirm pesticide degradation by the selected isolate, both qualitative and quantitative analyses were performed.

3.5.1 QUALITATIVE TEST

3.5.1.1 PHOSPHATE RELEASE TEST

The phosphate release test was performed to determine the ability of the microorganism to solubilize and release phosphate from Hamla550 pesticide. In this test, the extent of phosphate release indicates the degradation potential of the organism. The test organism was inoculated into the Nutrient broth with Hamla550 and incubated at 37°C for 24–48 hours. After incubation, 1 mL of broth was withdrawn and mixed with 1 mL of sulfanilic acid and 1ml α-naphthylamine. The development of a blue color indicates a positive result for phosphate release.

3.5.1.2 CHLORIDE RELEASE TEST

The chloride release test was performed to determine the ability of the microorganism to release chlorine or degraded chlorinated compounds from Hamla pesticide. This test helps in assessing the biodegradation potential of the organism toward pesticide residues containing chlorine.

The potent strain was inoculated into a nutreient broth containing the Hamla and incubated at 37°C for 24–48 hours under appropriate conditions. During incubation, the microorganism utilizes the compound, leading to the release of chloride ions. After incubation, a measured volume of the culture broth was collected, and a few drops of silver nitrate ($AgNO_3$) solution were added to the sample to detect the presence of released chloride ions. The formation of a white precipitate of silver chloride (AgCl) indicates a positive result for chlorine release.

3.5.1.3 THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography was performed to analyze the degradation of pesticide by the potent isolate. Thin Layer Chromatography (TLC) is a simple and effective analytical technique used to separate and identify compounds in a mixture, and in this experiment, it is applied to analyze the degradation of a pesticide by bacterial isolates. A silica gel–coated TLC plate is first prepared, and a baseline is drawn about 1–2 cm from the bottom edge using a pencil. Small, concentrated spots of the standard pesticide (control) and the degraded sample are applied on this line using a fine capillary tube, and the plate is allowed to dry. A developing chamber containing a suitable solvent system, typically hexane and ethyl acetate in a 7:3 ratio, is prepared and saturated with solvent vapors. The TLC plate is then placed carefully inside the chamber with the baseline above the solvent level, allowing the solvent to rise up the plate by capillary action and carry the compounds along at different rates. Once the solvent front reaches about three-fourths of the plate, the plate is removed, the solvent front is marked, and the plate is dried. The separated spots are visualized under ultraviolet light or in an iodine chamber. Finally, the distance traveled by each spot and the solvent front is measured, and the retention factor (Rf value) is calculated, which helps in identifying the compounds and confirming the degradation of the pesticide.

3.6 FORMULATION OF MICROBIAL SPRAY

The formulation of a microbial spray was carried out to develop a stable and efficient liquid preparation containing viable cells of the selected microbial isolate. Following the selection of a potent strain through screening procedures, the potent isolate was subjected to mass multiplication and subsequently formulated into a sprayable product. The formulation comprised an actively growing microbial culture as the core component, suspended in sterile distilled water serving as the carrier. To enhance cell viability and stability during storage and application, suitable stabilizing agents such as glycerol were incorporated in low concentrations. In addition, a mild surfactant or sticking agent was included to improve adhesion and uniform distribution on plant or soil surfaces. The final formulation was adjusted to a neutral pH (6.5–7.5) to maintain optimal microbial activity. The cell density of the formulation was standardized to approximately 10^7– 10^9 CFU/ml to ensure biological effectiveness. The prepared microbial spray was stored under sterile and cool conditions until further use.

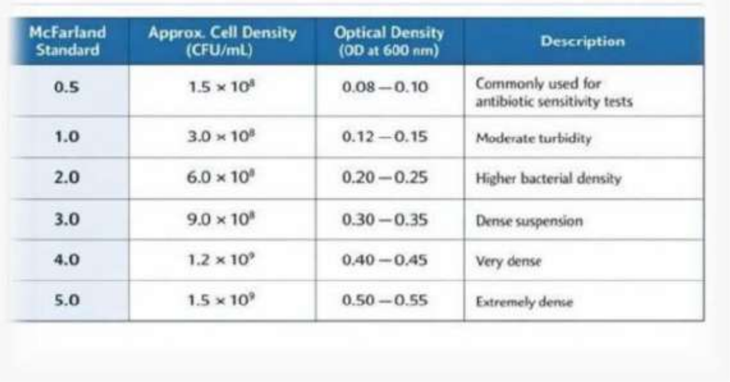
3.6.1 CULTIVATION OF ISOLATES

The selected microbial isolate was cultivated under controlled laboratory conditions to obtain sufficient biomass for formulation. A loopful of pure culture from a nutrient agar slant was aseptically inoculated into sterile nutrient broth and incubated at an optimal temperature of 37°C. During incubation, the culture was maintained under continuous agitation at 300 rpm to ensure proper aeration and uniform growth of the microorganisms. The growth of the culture was monitored at regular intervals, and turbidity was measured every 15 minutes using a spectrophotometer at 600 nm wavelength. This measurement of optical density helped in tracking the growth pattern of the organism.

3.6.2 ADJUSTMENT OF CELL DENSITY

Adjustment of cell density is an essential step in microbiological preparations to ensure that the number of microbial cells present in a suspension is uniform, reproducible, and suitable for experimental or industrial applications. Proper standardization of cell concentration is necessary because variations in microbial density can directly affect the effectiveness, reliability, and consistency of the final product, especially in formulations such as microbial sprays, probiotics, or fermentation systems. In this process, the microbial suspension is standardized using the McFarland standards, which provide a reference for estimating bacterial concentration based on turbidity. These standards are prepared using mixtures of barium chloride and sulfuric acid, producing a visible turbidity that corresponds to a specific number of bacterial cells per milliliter. By visually comparing the turbidity of the microbial culture with the McFarland standard, one can adjust the cell density accurately. Typically, a log phase (exponential phase) culture was used for this purpose because cells in this phase are metabolically active and uniform in size and physiology. The culture was diluted with sterile saline or buffer solution until its turbidity matches the desired McFarland standard. This ensures that the microbial population is neither too dense nor too dilute, thereby maintaining consistency across different batches.

Table10: McFarland standards



McFarland Standard	Approx. Cell Density (CFU/mL)	Optical Density (OD at 600 nm)	Description
0.5	1.5×10^8	0.08 – 0.10	Commonly used for antibiotic sensitivity tests
1.0	3.0×10^8	0.12 – 0.15	Moderate turbidity
2.0	6.0×10^8	0.20 – 0.25	Higher bacterial density
3.0	9.0×10^8	0.30 – 0.35	Dense suspension
4.0	1.2×10^9	0.40 – 0.45	Very dense
5.0	1.5×10^9	0.50 – 0.55	Extremely dense

After achieving the desired cell density, the formulation was often supplemented with stabilizing agents such as glycerol. Glycerol, usually added at around 1%, acts as a protective agent by preventing damage to microbial cells during environmental stress, including temperature fluctuations and dehydration. It helps maintain cell viability during storage and application.

Finally, the prepared microbial formulation was transferred aseptically into sterile containers to prevent contamination. It was stored at low temperatures to maintain stability and prolong shelf life. Before use, the formulation was gently agitated to resuspend any settled cells and ensure even application.

Table 11: Composition of microbial spray

COMPONENTS	CONCENTRATION
Microbial culture	10^8 CFU/mL
Glycerol	2%
Phosphate buffer	100 ml

3.7 APPLICATION AND EVALUATION OF MICROBIAL SPRAY

The microbial spray was applied to the target system to evaluate its efficacy. The spray was applied on cabbage leaves surfaces ensuring uniform coverage. The treated samples were kept at RT. Control setups without microbial spray were also maintained for comparative analysis. Observations were recorded at regular intervals of 30 mins, 60 mins ,and 90 mins to evaluate efficiency. The effectiveness of the microbial spray was determined by comparing treated and control samples using quantitative phosphate release methods.

3.8 ESTIMATION OF PHOSPHATE RELEASE TEST AFTER APLLICATION OF MICROBIAL SPRAY

The quantitative analysis of organophosphate pesticide degradation was performed using the phosphate release test, which estimates the amount of inorganic phosphate released during microbial activity. When organophosphate pesticides are degraded by bacterial isolates, phosphate ions are liberated as by-product In this method, the test sample was treated with ammonium molybdate and ascorbic acid reagents, which react with the released phosphate ions to form a blue-colored complex known as molybdenum blue. The intensity of the blue color is directly proportional to the concentration of phosphate present in the sample. This intensity is measured using a spectrophotometer at a wavelength of 420 nm. A standard phosphate calibration curve was prepared using known concentrations, and the absorbance values of the samples were compared with this curve to determine the phosphate concentration. A higher phosphate concentration indicates greater degradation of the organophosphate pesticide by the microorganism, making this method a reliable quantitative approach for assessing pesticide degradation.

For the estimation, a series of standard phosphate solutions is prepared using a known concentration of potassium dihydrogen phosphate (KH_2PO_4), typically ranging from 0 to 1 mg/L. To each standard and test sample, ammonium molybdate reagent was added, followed by the reducing agent. The reaction mixture is then incubated for about 10–15 minutes to allow complete development of the blue color. A blank was also prepared using distilled water and all reagents except phosphate to set the baseline for measurement The absorbance of the developed color was measured using a filter colorimeter set at 660 nm, which corresponds to the visible absorption range of the molybdenum blue complex in instruments where higher wavelengths such as 880 nm are not available. The instrument was first calibrated using the blank, and then the absorbance values of standards and test samples are recorded. A standard calibration curve was plotted by taking absorbance on the y-axis and phosphate concentration on the X-axis. The concentration of phosphate in the unknown sample is determined by locating its absorbance on the calibration curve and extrapolating the corresponding concentration value.

In this method, inorganic phosphate present in the sample reacts with ammonium molybdate under acidic conditions to form phosphomolybdic acid. This complex is then reduced by a suitable reducing agent such as ascorbic acid or stannous chloride to produce a characteristic deep blue color known as molybdenum blue.

The intensity of this blue color is directly proportional to the concentration of phosphate present in the sample and follows the principle of Beer–Lambert Law.

Table 12: Standard concentration table of KH_2PO_4(µg/ml)

Stock (ml)	Distilled Water (ml)	Concentration of KH_2PO_4 (µg/ml)	Optical density (420nm)
1	9	10	0.20
2	8	20	0.21
3	7	30	0.25
4	6	40	0.36
5	5	50	0.70
6	4	60	0.75
7	3	70	0.81
8	2	80	0.86
9	1	90	0.90
10	-	100	1.42

4.0
RESULTS AND DISCUSSION

4.1 PRIMARY SCREENING OF PESTICIDE DEGRADING MICROORGANISMS

The isolation procedure was successfully carried out using nutrient agar medium containing Hamla550 pesticide. After incubation, plates showed microbial colonies with clear zones. Impure cultures were identified by Gram staining. Pure cultures were obtained by sub- culturing isolated colonies onto fresh nutrient agar plates.

Fig.1 Isolation of pesticide degrading micro-organism

4.2 SECONDARY SCREENING OF PESTICIDE DEGRADING MICROORGANISM

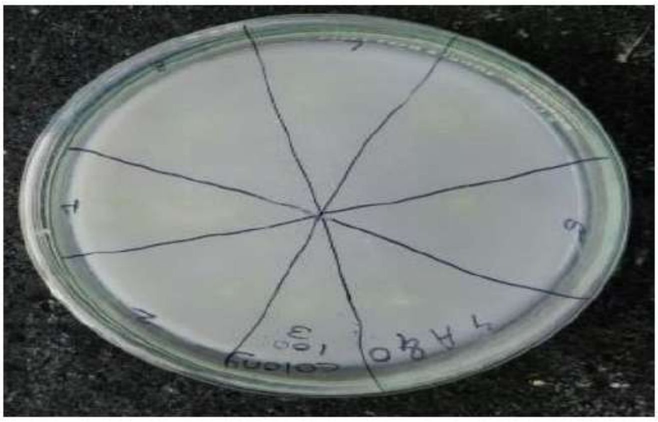


Fig.2 Spot inoculation of all strains

All pure cultures after spot inoculation and incubation at 37°C for 48 hours, showed clear zones around growth (Fig 3) The colony diameter and clear zone diameter of each isolate were measured, and the degradation indexes (DI) were calculated which is depicted in Table 11.

Table 13: Degradation indexes of pesticide degrading microorganisms

Isolates	Colony diameter (mm)	Clear zone diameter (mm)	Degradation index (DI)
Isolate 1	5	9	1.8
Isolate 2	6	10	1.6
Isolate 3	4	14	3.5
Isolate 4	5	8	1.6
Isolate 5	7	11	1.5
Isolate 6	3	8	2.6
Isolate 7	6	7	1.1
Isolate 8	5	8	1.6

Thus, the degradation index 3.5 that it is the most potent strain having pesticide degradation ability.

4.3 IDENTIFICATION OF POTENT ISOLATE

4.3.1 COLONY CHARACTERISTICS OF POTENT STRAIN

The colony characteristics of potent isolate 3 grown on nutrient agar plate after incubation at 37°C for 48 hours are presented in the Table 13.

Table 14: Colony characteristics of potent strain

Size	**Shape**	**Color**	**Margin**
D = 2 mm	Circular	White	Entire
Surface	**Elevation**	**Consistency**	**Opacity**
Smooth	Low convex	Sticky	opaque

4.3.2 MORPHOLOGICAL CHARACTERISTICS

Gram staining of isolate 3 showed that it is Gram-positive long rod-shaped bacterium arranged singly. Motility tests indicated that the organism was motile.

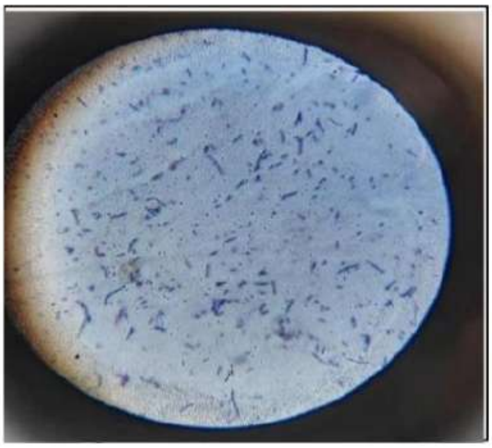


Fig 3: Gram staining

Table 15 : Spore and capsule presence

Spore formation	Spore forming
Capsule presence	Absent

4.3.3 BIOCHEMICAL CHARACTERISTICS

Sugar fermentation tests were performed to determine metabolic capabilities (Table 16)

Fig 4: Sugar fermentation test

Table 16: Sugar fermentation test

SUGARS	RESULT
Glucose	Positive
Lactose	Negative
Sucrose	Positive
Mannitol	Positive

Positive - Fermentation of sugar
Negative - No fermentation of sugar

Table 17: Catalase test

Catalase	Positive

Fig 5: Catalase test

After performing morphological and biochemical tests, the test organism was identified as a *Bacillus species*.

4.4 PROBIOTIC PROPERTIES

The isolate showed positive results for various probiotic tests including acid tolerance, bile salt tolerance, amylase production, gelatinase activity, and lipase activity. The antibiotic sensitivity test revealed that the organism was sensitive to several antibiotics, indicating that it does not carry strong antibiotic resistance.

4.4.1 ACID TOLERANCE TEST

The potent isolate showed visible turbidity after incubation in acidic nutrient broth medium (pH 2–3). The probiotic strain showed positive acid tolerance, as it survived and maintained good viability at low pH (around pH 2–3). This indicates that the organism can withstand acidic conditions

Fig 6: Acid tolerance test

4.4.2 BILE SALT TOLERANCE TEST

The potent isolate showed visible turbidity in medium containing bile salts. There is no significant decrease in cell density compared to control was observed. The bacterial cells remained viable after exposure to bile salts for the 48 hrs of incubation period.

Fig 7: Bile salt tolerance test

4.4.3 ANTIBIOTIC SENSITIVITY TEST

The antibiotic sensitivity test was carried out using the disc diffusion method, and the zones of inhibition were measured in centimeters. The test organism exhibited variable sensitivity patterns against the antibiotics tested. Among all antibiotics, the highest zone of inhibition was observed for Ofloxacin (2.5 cm), followed by Gentamicin and Streptomycin (2 cm each), indicating that the organism is highly sensitive to these antibiotics. Roxithromycin showed a zone of 1.7 cm, suggesting good sensitivity. Moderate zones of inhibition were recorded for Moxifloxacin and Chloramphenicol (1.5 cm each), indicating intermediate sensitivity. Lower zones were observed for Ceftazidime, Azithromycin, and Ceftriaxone (1 cm each), suggesting low sensitivity or resistance. No zone of inhibition was observed for Vancomycin, Lincomycin, and Clindamycin, indicating that the organism is resistant to these antibiotics (Table 15).

Fig 8: Antibiotic sensitivity test

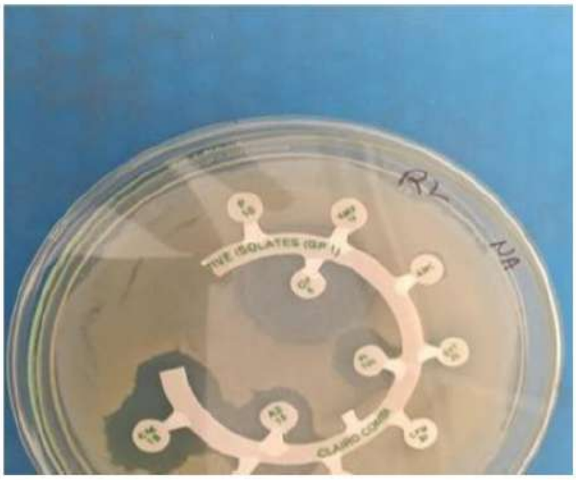


Fig 9: Antibiotic sensitivity test

Table 18: Antibiotic sensitivity test result

Name of antibiotics	Result (Diameter of clear zone in cm)
Ceftazidime	1
Moxifloxacin	1.5
Chloramphenicol	1.5
Roxithromycin	1.7
Gentamicin	2
Streptomycin	2
Ofloxacin	2
Ceftriaxone	1
Vancomycin	-
Lincomycin	-
Clindamycin	-

4.4.4 AMYLASE TEST

After incubation on starch agar, the plate was flooded with iodine solution. A clear zone (halo) was observed around the bacterial colonies. The surrounding medium turned blue-black, while the area around colonies remained colorless. The potent isolate showed a positive amylase test, indicating the production of the enzyme amylase, which hydrolyzes starch into simpler sugars.

Fig 10: Amylase test

4.4.5 PROTEASE TEST

After incubation on gelatin containing medium, a clear zone (halo) was observed around the bacterial colonies. The surrounding medium remained opaque, while the area around colonies became transparent due to protein degradation. This indicates hydrolysis of gelatin by the isolate.

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Fig 11: Protease test

4.4.6 LIPASE ACTIVITY

The result of lipase activity on Tween 80 agar was determined by observing the formation of a visible zone around the microbial growth. A positive result was indicated by the appearance of an opaque white precipitate or halo surrounding the colonies or along the streak line, which occurs due to the hydrolysis of Tween 80 by the enzyme lipase and the subsequent formation of insoluble calcium salts.

Fig 12: Lipase test

4.5. QUALITATIVE ANALYSIS

4.5.1 PHOSPHATE RELEASE TEST

The phosphate release test using ammonium molybdate and ascorbic acid showed a positive result, indicated by the development of a blue color (molybdenum blue complex). The test sample exhibited measurable absorbance, confirming the presence of soluble phosphate released by the microorganism. The intensity of the blue color was directly proportional to the amount of phosphate released. Higher absorbance indicated greater phosphate solubilization, while lower absorbance indicated lesser phosphate release by the organism.

Fig.13: Control of phosphate

Fig.14: Test of phosphate test

4.5.2 CHLORIDE RELEASE TEST

The chloride release test using silver nitrate ($AgNO_3$) showed a positive result, indicating the ability of the microorganism to release chloride ions. After incubation, the culture was centrifuged to obtain a clear supernatant. To this supernatant, silver nitrate solution was added. Immediately, a dense white precipitate of silver chloride (AgCl) was formed, confirming the presence of chloride ions in the sample. The formation of the white precipitate is due to the reaction between silver ions (Ag^+) and chloride ions (Cl^-), resulting in insoluble silver chloride.

Fig. 15: Control of chloride release test

Fig. 16: Test of chloride release test

4.5.3 THIN LAYER CHROMATOGRAPHY(TLC)

TLC analysis was performed using silica gel plates with hexane: ethyl acetate (7:3) as the solvent system. The control sample showed migration of pesticide compounds, whereas the test sample showed lack of migration, indicating degradation of pesticide compounds by the bacterial strain.

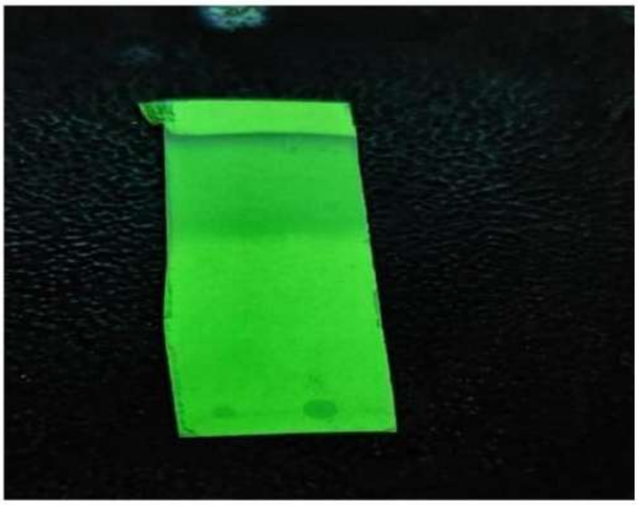

Fig 17: TLC analysis using silica gel plates hexane: ethyl acetate (7:3)

4.6 FORMULATION OF MICROBIAL SPRAY

The formulated microbial spray was successfully prepared and is presented in Figure 18. The formulation appeared as a uniform and homogeneous suspension, indicating proper mixing of microbial cultures with the carrier medium. No visible contamination, clumping, or phase separation was observed, suggesting stability of the formulation.

Fig 18 Pesticide degrading microbial spray

4.7 Quantitative Analysis

The quantitative estimation of organophosphate pesticide degradation was carried out using the phosphate release method. The development of a blue-colored complex (molybdenum blue) confirmed the presence of inorganic phosphate released due to microbial degradation of the pesticide.

A standard calibration curve was prepared using known concentrations of potassium dihydrogen phosphate (KH_2PO_4), which showed a linear relationship between absorbance and phosphate concentration, following Beer–Lambert law.

The absorbance of the test sample was recorded at 420 nm, and the corresponding phosphate concentration was determined using the standard calibration curve.

The test sample showed an absorbance of 0.30, corresponding to a phosphate concentration of 25 mg/L after 30 mins. The test sample showed an absorbance of 0.52, corresponding to a phosphate concentration of 45 mg/L after 60 mins. The test sample showed an absorbance of 1.52, corresponding to a phosphate concentration of 130 mg/L after 90 mins.

This indicates that the microbial isolate was capable of degrading organophosphate pesticide, releasing measurable inorganic phosphate. A higher phosphate concentration reflects greater degradation efficiency of the microorganism.

Fig. 18: Standard of phosphate test

Table 19 : Observation table of phosphate release

Concentration of KH_2PO_4 (μg/ml)	Optical density (420nm)
10	0.20
20	0.21
30	0.25
40	0.36
50	0.70
60	0.75
70	0.81
80	0.86
90	0.90
100	1.42
After 30 mins 25	0.30
After 60 mins 45	0.52
After 90 mins 130	1.62

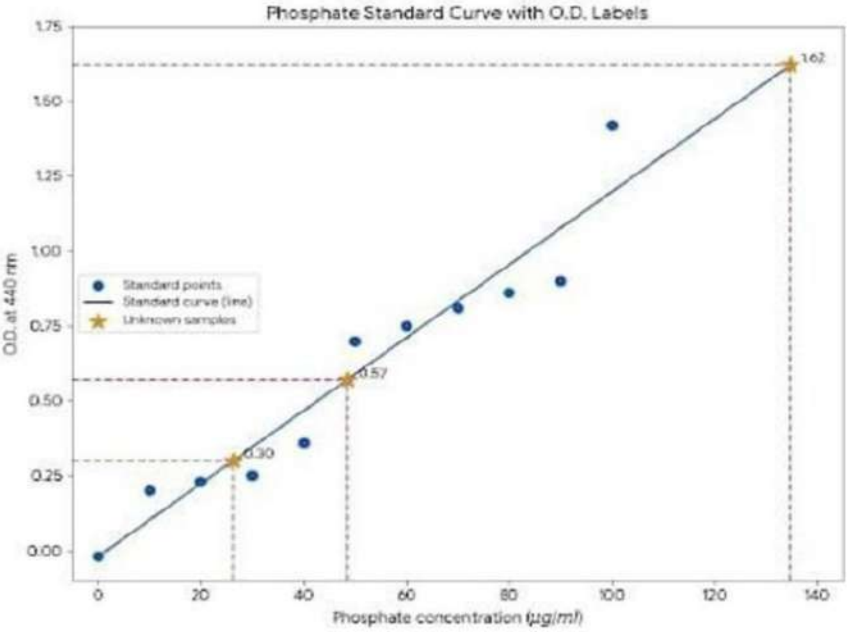

Fig.19: Standard graphof phosphate test

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SUMMARY AND CONCLUSION

In the present study, a pesticide-degrading bacterial strain was successfully isolated from contaminated soil collected from pesticide- treated agricultural fields. Based on morphological and biochemical characterization, the isolate was identified as *Bacillus* species. The organism showed typical characteristics such as Gram-positive rod- shaped cells and motile.

The isolated strain was further evaluated for various probiotic properties. The results of acid tolerance and bile salt tolerance tests indicated that the organism is capable of surviving under harsh gastrointestinal conditions. Enzyme activity tests such as amylase, lipase, and gelatinase confirmed the metabolic potential of the isolate. The antibiotic sensitivity test showed that the strain was sensitive to several antibiotics, indicating that it is safe and does not possess significant antibiotic resistance.

Qualitative analysis using Thin Layer Chromatography (TLC) suggested the degradation of pesticide compounds by the isolated microorganism. Further confirmation of pesticide degradation was obtained through chloride release and phosphate release tests, which indicated the breakdown of organophosphate pesticides.

Furthermore, the bacterial culture was formulated into a microbial spray and applied on pesticide-treated cabbage leaves. The quantitative analysis using the phosphate release assay demonstrated that the bacterial strain effectively degraded pesticide residues by releasing phosphate ions. These results confirmed the biodegradation capability of the isolated *Bacillus species*. The results suggested that the microbial spray has potential in reducing pesticide residues on vegetables.

Overall, this study demonstrates that the isolated *Bacillus* species possesses both probiotic characteristics and pesticide-degrading ability. The application of such beneficial microorganisms may provide a sustainable and eco-friendly approach for reducing pesticide contamination in agricultural products. This strategy can contribute to improve food safety, environmental protection, and sustainable microbial formulations and evaluation of their efficiency under field conditions.

The present study on isolation and characterization of pesticide-degrading probiotic microorganisms opens several promising avenues for future research and application. Although potent strains have been identified through primary and secondary screening, further advanced studies are required to enhance their efficiency, stability, and commercial viability.

One important future direction is molecular identification and characterization of the selected microbial strains using techniques such as 16S rRNA sequencing. This will help in accurate identification and understanding of genetic pathways involved in pesticide degradation. Additionally, genetic engineering approaches can be explored to improve degradation efficiency and adaptability under different environmental conditions.

Further research should focus on optimization of formulation parameters of the microbial spray, including carrier materials, shelf-life stability, and suitable additives to maintain viability during storage and application. Field-level trials are also necessary to evaluate the effectiveness of the microbial spray under real agricultural conditions, including different soil types, crops, and climatic variations.

Another important aspect is the study of synergistic interactions between multiple microbial strains to develop a consortium-based formulation, which may enhance degradation efficiency compared to single strains. The long-term impact of such formulations on soil health, plant growth promotion, and native microbial communities should also be investigated.

Toxicity and safety assessments are essential to ensure that the developed probiotic spray is non-pathogenic and environmentally safe. This includes evaluating its effects on non-target organisms, including beneficial soil microbes, plants, animals, and humans.In the future, integration of this technology with sustainable agricultural practices such as organic farming and integrated pest management (IPM) can significantly reduce chemical pesticide load. Moreover, scaling up production using bioreactors and developing cost-effective manufacturing processes will be crucial for commercialization.

Overall, the development of pesticide-degrading probiotic microbial sprays has great potential in promoting eco-friendly, sustainable, and safe agricultural practices, contributing to environmental protection and improved soil fertility.

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