INTRODUCTION

CHAPTER 1

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1.1 General Introduction: Actinobacteria

Actinobacteria are filamentous bacteria classified in Actinomycetales and belong to Actinobacteria species. Most actinobacteria are free-living saprophytes with a high percentage of G+C (greater than 55%). They can be found in almost any environment, including plants, soil, and water [1]. One of the most abundant groups of soil organisms, the actinobacterial population, has been shown to contribute to the cycle of soil components. Among the most important ways is through the breakdown of organic matter. By producing extracellular enzymes such as chitinase, lignin peroxidase, cellulase, and several others, they contribute to the cycles of mineralisation and degradation. In other words, they break down organic matter into minerals [2]. Actinobacteria are considered essential prokaryotes in the healthcare and biotechnology industries due to their ability to produce many bioactive molecules. This recognition is primarily because actinobacteria are abundant [3, 4].

Remarkably, three-quarters of all known antibiotics are produced by actinobacteria, making them significant antibiotic producers. They have approximately half the known bioactive secondary metabolites, including enzymes, anti-cancer agents, antibiotics, and immunosuppressant medications. Streptomyces, a type of actinobacteria that lives in soil, comprise 80% of antibiotics. Micromonospora and Actinopolyspora, relatively uncommon actinobacteria, come in second and third, making only around one-tenth as many antibiotics as Streptomyces [5]. The contribution of rare actinobacteria as producers of bioactive molecules became evident when approximately 25% of the antibiotics found in actinobacteria between 1975 and 1980 came from these organisms. Actinobacteria that are considered rare have traditionally been understood to refer to those actinobacterial isolates whose frequency of isolation using traditional techniques is significantly lower than that of streptomycetes. The physiology and productivity of molecules produced by uncommon actinobacteria gradually improved, which allowed for an expansion of the screening source into more unusual environments.

Since the beginning of the last half-century, terrestrial actinobacteria have been of significant interest to researchers worldwide because cutting-edge biologically active metabolites have been found in previously undiscovered genera. Antibiotics, chemotherapeutic agents, and other secondary metabolites with unique structures and functions have been discovered recently as potentially coming from marine actinobacteria. Actinobacteria, on the other hand, have almost always been found on land. The initial report of actinobacteria found in marine sediments came out just a few years ago. Actinobacteria have been found in terrestrial sources only. It has been speculated that river water and floods carry terrestrial actinobacteria into the ocean, where they would remain viable as terrestrial polluting microorganisms for many years. Despite this, the rate at which novel chemicals are extracted from terrestrial actinobacteria is declining [6, 7]. It is of the utmost importance that new families of terrestrial actinobacteria should be explored as potential sources of novel secondary metabolites with bioactive properties in habitats that have yet to be thoroughly investigated or exploited. In recent years, actinobacteria have come to the forefront of scientific discussion due to their ability to produce antibiotics such as rifamycin streptomycin, gentamicin, and erythromycin, among others; these are utilised not only in the pharmaceutical sector but additionally in agricultural practices to limit the growth of various phytopathogens [8] including Erwinia amylovora causes apple fire blight. In contrast, Agrobacterium tumefaciens causes crown gall [9].

1.2 Actinobacteria: Source of Natural agents to fight against Pathogens

Phytopathogenic fungi present significant global challenges in cultivating commercially valuable plants, particularly in tropical and subtropical areas [10]. Chemical fungicides are widely employed in present-day agriculture. Nevertheless, their overuse can adversely affect human health and cause environmental degradation and the emergence of fungicide-resistant pathogens. Due to the exacerbation of issues related to fungal disease management, there is a pressing need to thoroughly explore antimicrobial compounds to ascertain substitute approaches for safeguarding plants that are less reliant on chemicals and exhibit greater environmental sustainability. Microbial inhibitors are extensively employed for the management of fungal diseases of plants. Numerous types of actinobacteria, specifically Streptomyces, are widely recognised as biocontrol agents with antifungal properties that impede the growth of various fungi harmful to plants. The inhibitory effect of Streptomyces on pathogenic fungi is commonly attributed to the synthesis of antifungal agents and extracellular hydrolytic enzymes. Enzymes like chitinase and 1,3-glucanase aid in the fungal cell wall degradation [11]. Despite novel antimicrobial agents and the recurrent propagation of epidemic illnesses, several pathogenic microorganisms that afflict humans develop resistance to recently introduced medications that are derivatives of conventional antibiotics. The rapid emergence of many pathogens resistant to antibiotics, which can lead to severe and potentially fatal diseases, has increased the urgency with which new antibiotics must be developed [12]. Therefore, it is imperative to explore new antibiotics, specifically from microorganisms, to address the challenge posed by a growing number of antibiotic-resistant bacteria.

1.3 *Actinomycetes*: Characteristics

Actinomycetes represent a group of filamentous Gram-positive bacteria with a high G+C content (>55%) in their DNA and precise aerial hyphae, making them visually appealing. The Actinobacteria phylum is a significant taxonomic unit among the 18 main lineages within the bacterial domain, exhibiting considerable variation within the soil category [13. 14]. According to Bergey's Manual of Systematic Bacteriology, *Actinomycetes* are classified into eight distinct families based on their principle for classification [15] and comprise a total of 63 genera [16]. *Actinomycetes* are saprophytic, free-living microbes that colonise plants, are prevalent in water, and are widely dispersed in the soil. Initially, *actinomycetes* were identified based on their morphological characteristics. However, it was later discovered that the taxonomy of *actinomycetes* based solely on morphology is inadequate for distinguishing between multiple genera and closely related species. Furthermore, using phylogenetic and molecular methodologies has significantly contributed to the advancement of classification techniques [17,18]. In contrast, certain organisms erroneously assigned to ineligible taxonomic categories have been reclassified accurately due to the implementation of molecular methodologies [19]. The categorisation of species and their evolutionary relationships has recently been commonly established by utilising 16S rRNA and Polymerase Chain Reaction (PCR) for its sequencing and analysis. [20, 21].

Actinomycetes are a group of bacteria distinguished from other types by their distinctive characteristics. Some distinguishing features of *actinomycetes* are as follows:

Morphology: *Actinomycetes* are rod-shaped bacteria that form branching filaments resembling fungi. They are typically 0.5 to 1.0 micrometres in diameter and 1.0 to 10.0 micrometres in length.

Cell wall: *Actinomycetes*' cell walls contain peptidoglycan, a polymer of amino acids and sugars that provides structural support to the bacteria.

Habitat: *Actinomycetes* are found in various environments, including soil, water, and marine sediments. They are also present in the human microbiome.

Metabolism: *Actinomycetes* are aerobic bacteria, meaning they require oxygen to grow. They are also heterotrophic, meaning they obtain their energy and nutrients from organic compounds.

Secondary metabolites: *Actinomycetes* are recognised for their capacity to synthesise secondary metabolites, encompassing antibiotics, anticancer, and antifungal agents.

Growth rate: *Actinomycetes* grow relatively slowly compared to other types of bacteria, with a generation time of 12 to 24 hours.

Antibiotic resistance: Some species of *actinomycetes* have developed resistance to multiple antibiotics, making them difficult to treat.

Actinomycetes play an essential role in the environment and human health, both as a source of beneficial compounds and a potential pathogen.

1.4 *Actinomycetes*: Life Cycle and Importance of Metabolites

The life cycle of *actinomycetes* typically exhibits three distinct morphological stages under microscopic classification, namely vegetative mycelium (growth), aerial mycelium with spore-bearing cuffs, and the distinctive arrangement of spores. The latter two features are beneficial for identification and provide valuable investigative information. [22]. The categorisation of *actinomycetes* into genera, such as Streptomyces, is facilitated by researchers' combined utilisation of cultural and microscopic features. The life cycle of *Actinomycetes* involves a complex and dynamic series of events that enable these bacteria to adapt to environmental stress and reproduce efficiently. The life cycle can be divided into three stages: vegetative growth, sporulation, and germination.

Actinomycetes multiply by binary fission during the vegetative growth phase, forming a complex network of branching filaments known as hyphae. These hyphae elongate and branch, resulting in the formation of a mycelium. The mycelium is responsible for producing secondary metabolites, such as antibiotics and enzymes, which are essential for the survival of the *Actinomycetes* in their natural environment.

Under unfavourable conditions, *Actinomycetes* enter the sporulation phase. During this phase, the hyphae differentiate into specialised structures called sporangia, which produce spores. The spores of *Actinomycetes* are highly resistant to environmental stress and can remain dormant for extended periods. This survival strategy allows *Actinomycetes* to endure harsh environmental conditions and ensures the continued propagation of the species.

The germination phase involves the activation of spores to initiate the next cycle of vegetative growth and sporulation. When conditions become favourable, the spores of *Actinomycetes* germinate, resulting in the formation of new hyphae. These hyphae continue to elongate and branch, forming a new mycelium, and the cycle of vegetative growth and sporulation begins anew.

The life cycle of *Actinomycetes* is a complex and dynamic process that involves intricate cellular mechanisms that enable these bacteria to adapt to environmental stress and ensure the continued propagation of the species. Understanding the life cycle of *Actinomycetes* is essential for developing novel therapeutics and managing environmental and clinical infections caused by these bacteria [23].

Several studies have been conducted wherein the *actinomycetes* isolated strains were identified as a Streptomyces species based on their properties and characteristics [24, 25]. *Actinomycetes* are commonly recognised as synthesisers of antibiotics and other valuable secondary metabolites, as illustrated [26]. To date, a significant proportion of antibiotics and antimicrobial secondary metabolites that are commercially available have been identified and described from various species of *actinomycetes*, amounting to approximately 70-80% [27]. Concealed secondary metabolites are recognised as a great reservoir of biologically potent substances, including antibiotics, enzymes, agrochemicals, antiparasitics, immunosuppressants, and antitumor agents [28]. In the past decade, there has been a decline in the popularity of searching for antibiotics and secondary metabolites that are bioactive in *actinomycetes* species and other microorganisms. This is due to the critical success of discovering new strains.

Consequently, there has been an increase in screening for novel potential chemical compounds, but this has yet to yield significant results. This is unsurprising, given that *actinomycetes* have developed their capabilities over time. *Actinomycetes* are responsible for the production of approximately 80% of antibiotics that are rationally designed. Notably, Streptomyces and Micromonospora are among the most prolific producers of antibiotics [29]. The study conducted by sources [30, 31] elucidates that *actinomycetes* can produce bioactive compounds, which consist of a diverse range of components such as anthracyclines, aminoglycosides, macrolides, glycopeptides, polyenes, β-lactams, nucleosides, peptides, terpenes, polyethers, and tetracyclines. These compounds exhibit a broad spectrum of biological activities.

Furthermore, many antibiotics employed in clinical settings are derived exclusively from naturally occurring microbes and their secondary metabolites or are semi-synthetic derivatives [32]. The isolation and characterisation of novel *actinomycetes* from sources naturally occur in achieving an excited state of isolation. This has been noted in previous literature [33]. Streptomyces strains are widely distributed in the natural environment, particularly in soil, and are among the most prevalent isolated *actinomycetes*. These microorganisms are known for their hostile behaviour against other organisms [34]. The genus Streptomyces is widely acknowledged within the *actinomycetes* family due to their remarkable capacity for producing and secretion of a wide range of biologically active secondary metabolites. Soil *actinomycetes*-

derived compounds have diverse biological effects, including but not limited to antimicrobial, cytotoxic, plant growth hormone, immunosuppressive, antifungal, neurotoxic, antimitotic, antineoplastic, and antiviral activities supported by previous research [35].

1.5 *Actinomycetes*: Role in Pesticide Degradation

Since the late 20th century, there has been a notable increase in global grain production, rising 700 million tonnes from 500 million tonnes [36]. Cereals are responsible for 80% of the total food consumed by humans [37]. Pests pose a threat to food safety both during the natural growth process and during storage. China is predominantly an agricultural nation; however, approximately 8.8% of its total grain production, equivalent to 40 million tonnes, is lost annually due to various insect pests [38]. On an annual basis, India generates an average of 250 million tonnes of grain. However, approximately 11-15% of its production, equivalent to roughly 27.5-37.5 million tonnes per year, is lost due to pests and other related causes [39]. To mitigate potential damages, implementing pesticides is commonly employed to manage agricultural and domestic pests [40]. The utilisation of pesticides has significantly decreased food loss. However, these pesticides have been extensively disseminated throughout the environment, including soil, air, water, and agricultural commodities. The extensive utilisation of pesticides poses a significant environmental hazard [41, 42]. The contamination caused by these entities not only affects the soil and crops but also has a detrimental impact on the groundwater and marine ecosystem, posing a direct threat to human health and the environment [43].

The following two factors address the dispute between stable or high-yield farming and environmental damage; the discovery and development of pesticides with low toxicity, high effectiveness, and few pesticide residues are crucial. However, methods for getting rid of pesticide residues are just as important. Since the 1940s, people have been studying how microbes break down herbicide residues. The approach and mechanisms underlying organic pollutant degradation have been extensively explored in light of growing ecological concerns [44]. *Actinomycetes* are diverse microorganisms found in air, water, and soil. Degrading pesticides is an important step, given the widespread use of these chemicals for crop protection against diseases and pests. Using pesticides has been found to have detrimental effects on the environment, including the contamination of soil, water, and air. *Actinomycetes* are known to be able to degrade various pesticides. Their potential as a viable approach to mitigating the environmental effects of pesticides renders them a promising solution.

One of the primary mechanisms by which *actinomycetes* degrade pesticides is through the production of enzymes that can break down the chemical structures of the pesticides. These enzymes include hydrolases, oxidases, and dehalogenases, which can break down pesticides into simpler compounds that can be metabolised by other microorganisms or assimilated by plants. In the presence of different carbon sources, it has been discovered that *actinomycetes* can break down pesticides through a process known as co-metabolism. In this process, Pesticides are a vital carbon and energy source for *actinomycetes*. Several studies have shown that *actinomycetes* can effectively degrade different pesticides, including organophosphates, carbamates, and pyrethroids. For example, the actinomycete strain Streptomyces griseus was found to degrade the organophosphate pesticide chlorpyrifos, while another strain, Streptomyces sp. EMM02 was found to degrade the pyrethroid pesticide deltamethrin. *Actinomycetes* have also been found to degrade the herbicide glyphosate, widely used in agriculture.

The ability of *actinomycetes* to degrade pesticides has several advantages over other methods of pesticide removal, such as chemical and physical treatments. *Actinomycetes* are natural microorganisms found in the environment, and their use for pesticide degradation does not require adding chemicals or other external inputs. Additionally, *actinomycetes* can degrade pesticides under various environmental conditions, including high temperatures and low nutrient availability.

The proposed approach is expected to mitigate secondary contamination risks and exhibit cost-effectiveness while being eco-friendly, as it relies on the natural ability of bacteria to degrade pesticide residues. The efficacy of the pesticides' biodegradation by microbes may have been compromised due to the intricate and volatile nature of the environment, resulting in suboptimal outcomes. Scholars have meticulously examined microbes, leading to a thorough understanding of the degradation process of organic pesticides. Several bacterial strains capable of degrading and transforming pesticides have been isolated [45-47]. Furthermore, the predominant biodegradable pathways and pesticide mechanisms were elucidated lucidly [48-50]. As per the existing literature, the present investigations about biodegradable pesticides primarily focus on microorganisms inhabiting the soil, including fungi, bacteria, and *actinomycetes* [51]. Among these, key players include bacteria and fungi. Due to the bacteria's tendency to induce mutant strains with diverse biochemical capabilities that allow for adaptation to various environments, further extensive investigation can be conducted.

In conclusion, *actinomycetes* play a crucial role in pesticide degradation, a significant environmental concern. The enzymatic degradation and co-metabolism capabilities of *actinomycetes* render them a promising remedy for mitigating the environmental impact of pesticides, owing to their ability to degrade a broad spectrum of pesticides. Understanding mechanisms that lead to pesticide degradation by *actinomycetes* and discovering novel strains with improved pesticide-degrading capabilities requires more study [52-56].

1.6 Hypothesis

Despite being reported by the World Health Organization (WHO), monocrotophos has not yet been banned in India because it's cheaper than other pesticides and more effective in controlling pests. The significant population of the states of India does not have access to quality crops, even after huge investments and massive efforts, concerning higher levels of monocrotophos contamination in rural and urban areas. The higher indirect consumption of this pesticide results in deaths in India. This particular pesticide functions as a cholinesterase inhibitor with direct action and can penetrate the skin. The observed symptoms resemble those exhibited by other organophosphate compounds, albeit with a rapid onset that can manifest within minutes or up to a day. The activity of cholinesterase inhibition induces effects on the nervous system. The symptoms of severe poisoning resulting from monocrotophos exposure may result in a heart attack or respiratory failure, ultimately resulting in a fatality in the most challenging scenarios. However, till now, no effective methods are available for the bio-degradation of monocrotophos pesticides, as they persist in the soil and water for longer durations, and their contact with humans and animals causes severe diseases and deaths. Hence, an imperative requirement exists for the biodegradation of pesticides of this nature into non-toxic configurations. In this scenario, the application of actinobacteria in the bioremediation of such types of pesticides may be an effective method because the metabolic diversity of such type bacteria is huge and exploiting their metabolic diversity may prove to be an essential step in bio-degradation of monocrotophos into non-toxic forms. The Bio-formulation of the promising candidates (if found) of this study may patented.

1.7 Key Questions

- How can rare actinobacterial genera be harnessed for monocrotophos bioremediation and plant growth promotion?
- Which molecular techniques are optimal for identifying genes in actinobacteria responsible for monocrotophos degradation?
- How does gene expression in an E. coli system enhance in vivo monocrotophos bioremediation, and what are the challenges?
- How can the research findings translate into practical, field-ready bio-formulations for agriculture and improved environmental health?

1.8 Objectives

The genera of microbes, including *Streptosporangium*, *Nocardiodes*, *Actinomadura*, *Planobispora*, *Micromonospra*, *Saccharopolyspora*, etc., are rarely exploited in the bioremediation of pesticides. In this context, The current investigation has been formulated to isolate the rare genera of actinobacteria. Degrading the monocrotophos pesticides into nontoxic forms and their bio-formulation and application in agriculture with the following objectives:

1. Isolation and screening of *actinomycetes* for bioremediation of Monocrotophos pesticides (MCP) and their PGPR traits.
2. Identification of promising isolates using polyphasic taxonomy
3. Characterisation of genes and their products responsible for degrading monocrotophos pesticides
4. Characterisation of degraded effects of Monocrotophos pesticides and assessment of their toxicity
5. Expression of genes in *E. coli* system and their application in bioremediation (*In vivo*)

Organisation of thesis

Chapter I Introduction: This chapter provides an overview of the roles of Actinobacteria and Actinomycetes in bioremediation. It sets the stage by introducing the hypothesis and objectives of the study, which focus on the potential of these bacteria to degrade monocrotophos pesticides.

Chapter II Review of Literature This chapter reviews existing literature on actinomycetes, their potential applications, agricultural practices in India, pesticide usage, and studies on biodegradation of pesticides.

Chapter III Materials and Methods This chapter outlines the methodologies used in the study, including the collection and preparation of samples, screening processes, and techniques used for pesticide biodegradation and actinobacterial strain formulation.

Chapter IV Results: This chapter presents the study findings derived from the methodologies employed in Chapter 3.

Chapter V Discussion In this chapter, the results are analysed and discussed in the context of the literature reviewed in Chapter 2.

Chapter VI Conclusion and Future Prospects This chapter concludes the study and presents potential future research directions based on the findings.

Chapter VII This chapter summarises the entire study, encapsulating the critical points of the previous chapters.

Chapter VIII. Bibliography: this chapter lists all the references cited throughout the thesis.