

**BIOPROSPECTING OF INSECT NEST ASSOCIATED ACTINOBACTERIA WITH
SPECIAL REFERENCE TO ANTIMICROBIAL PROPERTIES**

Submitted in Partial Fulfilment of the requirement for the award of
Master of Technology degree in Biotechnology

by

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DEPARTMENT OF BIOTECHNOLOGY

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SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade "A" by NAAC

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This is to certify that this Project Report is the bonafide work of **SHEEBA.J** (41810019), who carried out the project entitled "**BIOPROSPECTING OF INSECT NEST ASSOCIATED ACTINOBACTERIA WITH SPECIAL REFERENCE TO ANTIMICROBIAL PROPERTIES**" under our supervision from January 2023 to May 2023

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ABSTRACT

Symbiotic interaction between insects and microbes are vastly spread in nature, and most often a source for ecological innovation. The present study attempts to explore actinobacteria from insect nest samples (ant, termite, wasp) for antimicrobial activity. Totally 15 actinobacterial colonies were recovered from ant, termite and wasp nest samples by adopting standard spread plate method. In primary recovery only 4 out of 15 cultures were grown on ISP2 agar plates. Antimicrobial activity of 4 actinobacterial strains isolated from ant and termite nest were determined by agar plug method. Screening of antimicrobial properties of actinobacterial strains was determined by agar plug method. Two actinobacterial strains AN1 and AN5 showed promising activity (23-26 mm inhibition) against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Mycobacterium smegmatis*. The strain AA5 produced antimicrobial compound much earlier on ISP2 agar when compared to ISP2 broth. Crude extracts from the strain AA5 was produced by adopting agar surface fermentation and extracted using ethyl acetate. Based on the studied molecular analysis, actinobacterial strain AA5 isolated from ant nest was identified as *Streptomyces rochei*. In addition to antimicrobial activity, extracts also exhibited anti-TB, anti-oxidant and anti-biofilm activity. This study concluded that insect nest is a promising source for bioactive actinobacteria. The potential strain AA5 isolated from ant nest will be promising sources for antimicrobial metabolites against drug resistant bacteria and mycobacterial pathogens.

Keywords: Actinobacteria, insect nest, symbiosis, antimicrobial.

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CHAPTER 1

1. INTRODUCTION

Our comprehension of the mechanistic basis of interactions between insects and microorganisms has significantly improved over the past quarter century. Even though insects aren't the only ones who live close to microbes, it's easy to see how their success is partly due to their ability to deal with them. The nature of the receptors, signalling cascades, and effectors that make up insect immune systems has been made clear through research into how insects respond to pathogens. Insect immunity is clearly multifaceted, specific, and highly effective, despite not being "adaptive" in the traditional immunological sense. Huge headway has additionally been made on the microbial side with the distinguishing proof of an assortment of destructiveness instruments that empower organisms to dodge, stifle or oppose the bug's invulnerable framework. In addition, better ways to protect beneficial insects, such as pollinators, can be developed by better understanding the underlying mechanisms of insect host resistance to microbes and limiting their capacity to transmit diseases that harm human health or crops (Jeremy et al .,2012.) The majority of interactions between insects and microbes are not pathogenic but rather commensal or mutualistic. The mutualistic bacterium *Buchnera*, which supplies its aphid host with essential amino acids that plant saps lack, is one example of an insect that incorporates and exploits the vast metabolic potential of microbes for their own benefit. We are beginning to realize, more and more, that insect-microbe symbioses are much more numerous and diverse than initially thought. In addition to being necessary for the supply of nutrients, the microbial flora that live in insect guts have recently been linked to the insect's capacity to resist infection. While the ability of endosymbiotic *Wolbachia* to control their host's reproduction has been studied for a long time, the ability to give their hosts resistance to viral infection, which is largely responsible for their prevalence, was overlooked until just a few years ago. (Flórez et al. 2015)

Endosymbionts made up of highly integrated bacteria are found in several insect species. These endosymbionts give their hosts more metabolic capacity, allowing them to survive on a diet that lacks nutrients. The *Buchnera* symbiont of Aphids and *Wigglesworthia*, which gives tsetse flies the ability to eat only blood meals because it contains complex B-vitamins, are two examples. It has long been known that

raising insects at higher temperatures can result in the loss of endosymbionts. The aposymbiotic insects that result often have drastically reduced fitness. Wernegreen discusses the unanticipated impact of climate change on endosymbiotic interactions in light of global warming models that predict an increase in average temperature of 1.8–4.8°C. Actinobacteria are well-known antibiotic producers, particularly those that produce novel therapeutic compounds. Actinobacteria, the majority of which are isolated from soils, account for nearly two-thirds of the antibiotics on the market. However, there has been a decrease in the intensive search for newer actinobacterial strains that produce novel bioactive metabolites. Numerous researchers diverted their research on actinobacteria from less explored niches in order to address this concern. Because of their unique mutualistic mechanisms, actinobacterial symbionts of plants, animals, and insects seem to have received more attention (Jeremy et al., 2012.) Chitinases and lipases are produced by entomopathogenic fungi, which can penetrate insect cuticles. They are attractive agents for controlling crop pests and disease vectors because they are important pathogens of insects in the wild. The development of genetic and genomic tools has recently benefited the study of entomopathogenic fungi. The recent development of novel and more potent entomopathogenic fungal strains that can be engineered with a comprehensive understanding of virulence mechanisms and other aspects of fungal biology, which may enhance the effectiveness of this method of insect control. These diverse reviews show how quickly research on insect–microbe interactions is progressing and how much it affects human health and agricultural productivity. A growing need for a holistic approach is also reflected, with more complex models incorporating the simultaneous investigation of a variety of microbes and environmental conditions. The study of the interaction between insects and their pathogens in the context of mutualists and non-pathogenic commensals and vice versa is likely to advance research. Additionally, researchers are becoming increasingly aware of the complex scenarios in which microbes tend to occupy condition-dependent positions on the spectrum from mutualists to pathogens.

1.1 Actinobacteria:

Actinobacteria are a diverse group of bacteria that are characterized by their ability to produce a wide range of biologically active compounds. These bacteria are found in a variety of environments, including soil, water, and the human body, and are known for their ability to survive in extreme conditions.

One of the most important characteristics of actinobacteria is their ability to produce a wide range of biologically active compounds, including antibiotics, enzymes, and other substances that have potential medical and industrial uses. In fact, many of the antibiotics used today, including penicillin and tetracycline, were derived from actinobacteria.

Actinobacteria are also known for their ability to survive in extreme conditions, such as high or low temperatures, low oxygen levels, and high levels of radiation. This ability to survive in harsh environments makes actinobacteria of interest for bioremediation, as they can potentially be used to clean up contaminated sites.

In addition to their potential industrial and medical uses, actinobacteria also play important roles in the environment. For example, they are important decomposers, breaking down organic matter and releasing nutrients back into the soil. They are also thought to play a role in the nitrogen cycle, fixing nitrogen from the air and making it available to plants.

Overall, actinobacteria are a diverse and important group of bacteria that have potential uses in a variety of fields. Their ability to produce biologically active compounds and survive in extreme conditions make them of particular interest for bioprospecting and other applications.

In the past few decades, a large number of actinomycetes have been isolated and screened from soil. These actinomycetes are responsible for between 70% and 80% of the relevant secondary metabolites that can be purchased in stores. Numerous bioactive compounds with important applications in human medicine and diverse clinical effects can be derived from actinomycetes. Actinomycetes are thought to

have produced approximately one-third of the thousands of naturally occurring antibiotics. Hotam S. Chaudhary and coworkers, 2013).

In the fight against emerging diseases and antibiotic-resistant pathogens, it may be necessary to investigate and discover novel microorganisms that produce novel secondary metabolites (Berdy, 2012). Actinobacteria play a crucial role in the breakdown of organic matter in both natural and man-made environments. Berdy (2012) says that they are also well-known for their abundance of bioactive secondary metabolites. Organic compounds that are not specifically associated with an organism's normal growth, improvement, or propagation are referred to as secondary metabolites. This group stands out because of the variety of actinobacteria and their capacity to produce novel substances. According to Mohan & Rajamanickam (2018), they are in charge of the production of approximately fifty percent of the investigated bioactive secondary metabolites, which include enzymes, antibiotics, anticancer agents, and antiinflammatory agents, among others. In light of the outstanding reputation of actinobacteria, the successful isolation of new actinobacteria from various sources for medication screening programs has been the focus of much progress over the past 50 years.

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Overall, actinobacteria are a diverse and important group of bacteria that have potential uses in a variety of fields. Their ability to produce biologically active compounds and survive in extreme conditions make them of particular interest for bioprospecting and other applications.

1.2 ANTIBACTERIAL ACTIVITY OF ACTINOBACTERIA

Hotam S. Chaudhary et al., (2013) The main objective of the present study was isolation, purification, and characterization of actinomycetes from soil samples,

having antimicrobial activity against 12 selected pathogenic strains. Soils samples were taken from different niche habitats of Sheopur district, Madhya Pradesh, India. These samples were serially diluted and plated on actinomycete isolation agar media. Potential colonies were screened, purified, and stored in glycerol stock. Isolates were morphologically and biochemically characterized. These isolates were subjected to extraction for production of the antibacterial compound. Antibacterial activity and Minimum Inhibitory Concentration (MIC) of the purified extract of isolates were evaluated. Totally 31 actinomycete isolates were tested for antagonistic activity against 12 pathogenic microorganisms. Isolates AS14, AS27, and AS28 were highly active, while AS1 showed less activity against the pathogenic microorganisms. Isolate AS7 exhibited the highest antagonistic activity against *Bacillus cereus* (24 mm) and AS16 showed the highest activity against *Enterococcus faecalis* (21 mm). MIC was also determined for actinomycete isolates against all the tested microorganisms. MIC of actinomycete isolates was found to be 2.5 mg/ml against *Shigella dysenteriae*, Vancomycin-resistant enterococci, and *Klebsiella pneumoniae*, and was 1.25 mg/ml for *Staphylococcus*, and was 1.25 mg/ml for *Staphylococcus saprophyticus*, *Streptococcus pyogenes*, *Staphylococcus epidermidis*, Methicillin-resistance *Staphylococcus aureus*, *Enterococcus faecalis*, and *Staphylococcus aureus*. All actinomycetes isolated showed antibacterial activity against *S. aureus*, while they showed less activity against *S. dysenteriae*. These isolates had antibacterial activity and could be used in the development of new antibiotics for pharmaceutical or agricultural purposes.

1.3. INSECTS:

Insects are a diverse group of arthropods that are characterized by their small size, segmented bodies, and wings. They are found in almost every habitat on earth and are incredibly adaptable, allowing them to thrive in a wide range of environments.

Insects are an important part of many ecosystems, playing a variety of roles such as pollinators, decomposers, and predators. They are also a vital source of food for many other animals, including birds, reptiles, and mammals.

Insects are known for their incredible diversity, with over one million known species. This diversity is reflected in their wide range of sizes, shapes, and colors, as well as in their varied behaviors and lifestyles. Some insects are social, living in large

colonies, while others are solitary. Some insects are herbivores, feeding on plants, while others are carnivorous, preying on other insects or small animals.

Overall, insects are an integral part of many ecosystems and play a vital role in the balance of nature. Despite their small size, they are an incredibly diverse and important group of animals that deserve our attention and protection.

One of the most diverse and ancient forms of life on Earth, insects have the potential to wreak havoc if their numbers reach a certain threshold. According to Misof et al., their existence can be traced back to the Paleozoic period, when Orthopterans and Hemipterans first appeared on the planet. 2014). They have progressed since then and have successfully adapted to a variety of extreme climates. In contrast, despite the ecological and economic importance of insects, insect pests, most of which are likely the result of man-made habitat changes and many of which are caused by climate change, cause more than 20% of crop losses annually (Deutsch et al., 2018). In addition, insect migration patterns, the timing of their life cycles, and population dynamics are being affected by shifting climate conditions. They have been able to expand their host range, changed their behavior and biology, and helped invade and colonize various agroclimatic zones around the world as a result of overcoming these obstacles (Shrestha, 2019). They have been able to successfully combat all adverse conditions thanks to their large population size, short reproductive cycles, and high reproductive rates. They have invaded numerous regions of the planet due to their lightness, allowing them to be carried away by wind currents. At the present time, they are found in almost every ecosystem on Earth. In addition, their successful evolution and diversification have been greatly aided by their intricate relationship with beneficial microbes (Janson et al., 2008).

Throughout their evolutionary history, insects are known to have been associated with microbes such as bacteria and fungi. According to Thompson & Simpson (2009), some bacterial species live in insect specialized cells called bacteriocytes and are referred to as "endosymbionts," while others live on the body surface and are referred to as "ectosymbionts." They do, however, spend the majority of their time in the digestive tract, where they play a crucial role in influencing the diverse

diets and ecological niches of their insect hosts. It is known that an insect's gut microflora (1) make it easier for it to eat even stubborn food; 2) provide protection and immunity from a variety of parasites, pathogens, and predators; (3) repay the supplement less than stellar eating routine (e.g., on account of sap-sucking bugs); (4) Facilitate communication between and within specifics; (5) control mating and the success of reproduction; (6) aid in digestion, and 7) provide essential amino acids, nutrients, and metabolic components (Arbuthnott et al., 2016; 2015 Douglas; 2018 by Engl and Kaltenpoth; Russell and other, 2014; Wielkopolan and Palska-Stepłowska, 2016). Jing and co. 2020) have demonstrated that symbionts' primary function is to provide essential nutrients, followed by digestion and detoxification. As a result, it suggests that insects rely heavily on their gut microbiome for both normal life cycle processes and survival. In addition, their relationship can be categorized as obligate (or primary) or facultative (or secondary) depending on the degree of dependence (Baumann, 2005; Moran and others, 2008). However, facultative bacteria can change into obligate under certain conditions, so there is no clear distinction between these two groups (Ferrari & Vavre, 2011). The endosymbionts and their insect host have developed a complex and fascinating relationship that has yet to be fully understood in all of its facets. These symbionts are referred to by some researchers as "intracellular parasites" because they have taken over the insect body and developed a variety of survival mechanisms that also benefit their host. However, it is equally possible that the insect's survival necessitated the establishment of this relationship with its microbiome. Whatever the circumstance, they have now adjusted to one another. Bacterial species present inside a bug stomach can show mutualism, commensalism, or really might be pathogenic (Dillon and Dillon, 2004). Insects can selectively maintain beneficial microbes while triggering an immune response against pathogenic bacteria (Mikonranta et al., 2014). Experimentation has revealed that symbiotic antimicrobial peptides (AMPs) regulate symbiotic interactions by limiting symbiotic bacteria's reproduction, sometimes transforming them into a distinct form, and eliminating undesirable, sensitive bacteria (Mergaert, 2018). A few structural groups of AMPs are accounted for from bugs that incorporate apidaecin, hymenoptaecin, defensins, cecropins, drosocins, attacins, dipterocins, ponerocins, metchnikowins, and melittin (Kwong et al., 2017; Wu and other, 2018). Upon microbial acquisition, the AMPs apidaecin and hymenoptaecin are upregulated in bee gut tissue (Kwong et al., 2017). However, it

has been demonstrated that the endosymbiotic bacteria do not elicit antibacterial responses in insects, such as the expression of AMP-coding genes (Eleftherianos et al., 2013). For example, *Wolbachia* when present as a facultative symbiont in *Aedes albopictus* doesn't set off the union of AMPs (Bourtzis et al., 2000); However, *Anopheles gambiae*, which is not its natural host, experiences an immune response as a result (Hughes et al., 2011). Similarly, according to Burke & Moran (2011), the expression of defense-related genes is unaffected by the presence of *Serratia symbiotica* in aphids. Additionally, it has been observed that distinct symbionts interact with the insect immune system in distinct ways; Some can get around the insect's cellular immune response, but others affect the melanization response, which is an insect defense mechanism (Thomas et al., 2011).

In a similar vein, the most recent data in this context appear to indicate a number of ways in which the symbionts can circumvent insect immunity. However, it is still unknown how endosymbionts acquired this ability to evade the immune system of the insect or how the insect distinguishes between "beneficial symbionts" and potentially pathogenic ones. But what is clear is that when a symbiotic relationship starts, both the bacteria and the insect go through a lot of changes. These changes are caused by different forces in evolution, so it's possible that the bacteria's symbionts can get around the insect's immune system. Acquisition, colonization, and transmission are all components of microbial symbiosis. While some bacteria are vertically transmitted or inherited, others are readily acquired by insects during their life cycle. Post securing, successful colonization is pivotal for their endurance and perseverance, which, thusly, is profoundly affected by the physical and physiological states of the bug stomach. After that, transmission across genera becomes crucial, particularly for obligate endosymbionts, which are completely dependent on their host for survival. As a result, microbes have developed a number of fascinating mechanisms to guarantee transmission, which will be discussed further in this communication. Endosymbiont acquisition, maintenance, and transmission have been the subject of previous research. However, we only have a limited understanding of the mechanisms that drive this entire transition, which involves the transformation of a free-living microbe into an insect's obligate symbiont. Interestingly, it is known that the genome of bacteria undergoes

modifications in order to adapt to the environment in the gut. According to Yuval (2017), some microbes are even capable of manipulating their insect host to ensure their survival in addition to modifying themselves. Our comprehension of insect-microbe interactions has also improved as a result of the increased focus on the microbiome in general and the insect microbiome in particular over the past ten years. Insects and their symbionts are now shown to be tightly connected at almost every evolutionary stage, according to researchers. According to Engel & Moran (2013), the insect's host has a significant impact on the microbiome, and these endosymbiotic bacteria have now become so integrated into the insect's body that they coevolve with their host. With actinobacterial symbiotic relationships, insects are playing a significant role in the production of bioactive metabolites.

CHAPTER 2

2. RIVIEW OF LITERATURE

2.1. DIVERSITY OF INSECT NEST MICROBIOME:

Insects and bacteria have a diverse and complex interaction that plays a significant role in various ecological and biological processes.

One of the most common ways that insects interact with bacteria is through the process of symbiosis, where both organisms benefit from the relationship. For example, some insects, such as termites, have a symbiotic relationship with bacteria that live in their gut and help them digest cellulose, a component of plant cell walls. These bacteria produce enzymes that break down the cellulose, allowing the insects to extract nutrients from plants that would otherwise be indigestible. In return, the insects provide a suitable environment and nutrients for the bacteria to grow and thrive.

Insects can also serve as hosts for bacteria that cause disease in humans and other animals. Mosquitoes, for example, transmit a range of diseases, including malaria and dengue fever, through the bites they deliver to their hosts. These diseases are caused by bacteria or viruses that are transmitted from the mosquito to the host through the mosquito's saliva.

In addition to these interactions, insects can also be impacted by bacteria in more indirect ways. For example, some bacteria produce toxins that are harmful to insects, while others produce compounds that can repel or attract insects. These interactions can have significant effects on insect behavior and population dynamics.

Overall, the diversity of insect-bacteria interactions is vast and complex, and it is an area of active research in many fields, including ecology, evolutionary biology, and medicine. Understanding these interactions can provide important insights into the relationships between different species and the role that bacteria play in various ecological processes.

There are prokaryotic microorganisms in every environment on Earth, and they interact with many eukaryotic taxa, including insects, in a variety of ways. Symbiotic, pathogenic, or vectoring relationships are all possible. The adhesion of the microorganism to the host, entry and "invasion" of the host, establishment and dissemination within the host by avoiding immune responses, and transmission back to the environment or to a new host are all stages of an interaction, regardless of the type. These processes can now be dissected thanks to advances in genomics and genetics, which have revealed crucial details about the factors that shape each association's members. Moreover, numerous mechanisms engaged with the foundation of the affiliations have been examined, along with the advancement of new strategies for the administration of bug populaces. Over a million distinct insect species can be found almost everywhere, with the exception of the ocean, making them the most diverse group of animals (Vilmos et al.,1998). Insects are unavoidably associated with a vast array of microscopic life forms because of their widespread distribution, which includes viruses, bacteria, fungi, protozoa, and nematode and parasites with multiple cells. Although some of these microorganisms have a wide range of hosts, many of them form highly specialized associations that include specific insect species as well as specific life stages of the insect host (Aronson et al.,1986). This reflects the characteristics of the ecological niche that the insects and associated microbes occupy, the requirements of the insect or microorganism, and the genetic mechanisms that the microorganism employs to establish the interaction.

This review aims to provide an overview of the numerous associations between bacteria and insects, as well as their potential and current uses. In the first section, we focus on the molecular mechanisms that underlie the various interactions between these groups of organisms and their characteristics, highlighting several examples. In the second section, we go over the aspects of these interactions that have the potential to control insects, including the use of insecticidal toxins, and summarize a number of strategies and recent developments with implications for agriculture and epidemiology. Symbiotic or pathogenic interactions can occur

between insects and bacteria. In his 1879 monograph "Die Erscheinung der Symbios" (Strasbourg, 1879), Heinrich Anton de Bary first used the term "symbiosis" to describe "the living together of unlike organisms. "There is a well-established biochemical foundation to most symbioses: In some cases, one partner receives nutrients from the other partner, and in others, the waste products produced by one partner are recycled by the other partner. The diversity of insects is thought to have been shaped by the existence of such associations throughout their evolutionary history. According to Moya et al., symbiotic associations can be classified as commensalism, mutualism, or parasitism based on the fitness effects on the members of the relationship. 2008) Parasitism occurs when one species benefits from the association while the other species suffers from it. Some commensal microorganisms, like those found in the gut of insects, could be considered mutualists: By competing with other microbes for nutrients, the insect gut microbiome helps digestion, produces vital vitamins, and keeps out potentially harmful microbes. Mutualism is a less adaptable association in which an insect and a microbe benefit from one another. Mutualism and symbiosis are frequently used interchangeably. Although pathogenic interactions differ greatly from the perspective of the host, they also necessitate a high level of specialization and close contact with the host. In this way, mutualists and bacterial pathogens use many of the same molecular mechanisms. The arbitrary definition of bacterial behaviour is further complicated by the fact that the same microorganism can behave in different ways depending on the host's fitness and the environment. This can make the microorganism beneficial or harmful.

2.2 INSECT MICROBIAL SYMBIOSIS:

Insect symbionts are organisms that live in close association with insects and benefit from the relationship. These symbionts can be bacteria, fungi, protozoa, or other types of microorganisms.

One of the most common types of insect symbiosis is mutualism, where both the insect and the symbiont benefit from the relationship. For example, some insects, such as termites and certain species of ants, have a symbiotic relationship with bacteria that live in their gut and help them digest cellulose, a component of plant

cell walls. These bacteria produce enzymes that break down the cellulose, allowing the insects to extract nutrients from plants that would otherwise be indigestible. In return, the insects provide a suitable environment and nutrients for the bacteria to grow and thrive.

Other insects have symbiotic relationships with fungi that help them defend against predators or parasites. For example, some species of beetles and ants have symbiotic relationships with fungi that produce toxins that are harmful to their predators or parasites. The insects provide the fungi with a suitable environment and nutrients, and in return, the fungi provide protection for the insects.

In addition to mutualistic relationships, insect symbionts can also have other types of interactions, such as commensalism, where the symbiont benefits from the relationship but the insect is not affected, or parasitism, where the symbiont benefits at the expense of the insect.

Overall, insect symbiosis is a diverse and complex phenomenon that plays a significant role in many ecological and biological processes. Understanding these relationships can provide important insights into the interactions between different species and the role that symbionts play in various ecological processes.

2.3 Ant actinobacteria symbiosis relation

Ants are insects that are found in a wide range of habitats around the world. They are known for their highly organized social structures and their ability to work together to achieve common goals. Many species of ants have a symbiotic relationship with actinobacteria, which are a group of bacteria that are known for their ability to produce a wide range of antibiotics and other biologically active compounds.

The actinobacteria in ants are often found in the ants' mandibular glands, which are located on the head of the ants. The actinobacteria in ants produce a range of antibiotics that help protect the ants from infections and parasites. These antibiotics

can also help protect the ants from mites and other parasites that can harm the ants or reduce their fitness.

In addition to producing antibiotics, the actinobacteria in ants can also provide other benefits to the ants. For example, some actinobacteria produce compounds that can attract or repel other insects, which can affect the behavior and population dynamics of the ants. Actinobacteria can also produce compounds that can help protect the ants from environmental stressors, such as UV radiation or extreme temperatures.

Overall, the actinobacterial symbiosis in ants is an important and complex phenomenon that plays a significant role in many ecological and biological processes. Understanding this relationship can provide important insights into the interactions between different species and the role that actinobacteria play in various ecological processes.

Beneficial metabolites provided by microbial symbionts are utilized by hosts in numerous host-microbe mutualisms. It is believed that fungi-growing (attine) ants collaborate with *Pseudonocardia* bacteria to produce antibiotics that specifically suppress the coevolving pathogen *Escovopsis*, which reduces growth in the ants' fungal gardens. The *Pseudonocardia* and *Escovopsis* coevolution model's four key assumptions are put to the test here. Multiple *Pseudonocardia* species and occasionally *Amycolatopsis* (a close relative of *Pseudonocardia*) cooccur on workers from a single nest, contradicting the assumption of a single *pseudonocardiaceus* strain per nest, according to culture-dependent and culture-independent (tag-encoded 454-pyrosequencing) surveys. *Pseudonocardia* may also be transmitted horizontally during mating, given that it can affect males. The previous finding that *Pseudonocardia* had a growth-enhancing effect on the cultivars was contradicted by the fact that *Amycolatopsis* and *Pseudonocardia* secretions killed or significantly suppressed ant-cultivated fungi. Therefore, if attine ants apply *pseudonocardiaceus* secretions to gardens that are actively growing, they run the risk of harming their own cultivar. Contrary to the initial report of specific antibiosis against *Escovopsis* alone, *Pseudonocardia* and *Amycolatopsis* isolates also exhibit nonspecific antifungal activities against saprophytic, endophytic, entomopathogenic, and *gardenpathogenic* fungi. We conclude that *pseudonocardia-ceous* bacteria associated with attine do not possess derived

antibiotic properties that specifically inhibit *Escovopsis*. In order to replace the current *Pseudonocardia-Escovopsis* coevolution model, we examine hypotheses regarding the nonadaptive and adaptive functions of attine integumental bacteria and develop a new conceptual framework. On the off chance that relationship with *Pseudonocardia* is versatile to attine subterranean insects, substitute jobs of such microorganisms could incorporate the security of subterranean insects or sterilization of the home (Ruchira et al., 2009).

The "New World," which spans North America and South America, is home to approximately 250 species of fungi-farming ants. Attine ants cultivate basidiomycete fungi for food by collecting plant and other material from their environment. The foraged material has changed over the course of these interactions, resulting in the so-called "basal" and "highly evolved" agricultural systems that are based on the substrate that the fungal crop is fed (Schultz & Brady, 2008). Specialized pathogenic fungi from the genus *Escovopsis* can suppress the crop fungal cultivar and destroy the ant colony in this multipartite symbiosis (Currie et al., 1999), and a symbiotic actinobacterium typically belonging to the genus *Pseudonocardia* that produces small molecules that inhibit crop fungi more effectively than pathogenic fungi (Cafaro & Currie, 2005).

Actinobacteria cover some attine ants' exoskeletons, while others don't show the same obvious association. Subterranean insects of *Atta* sort, for example, don't show particular cryptsd morphological designs that harbor actinobacteriadin their bodies. According to Currie et al., the ant's internalization of the bacterial symbionts (2003) or even in other colony locations. As a result, the hypothesis that the colony's microorganisms prevented opportunistic pathogens was tested. 99 yeast strains were isolated from various *Atta sexdens* colonies, including the fungal garden, waste deposit, and surface of ants' collected leaves. The inhibitory properties of these strains were evaluated not only against each other but also against six reporter yeast strains. The findings revealed that 77 strains, or 78 percent, prevented the rival strain from growing. The presence of a role in sustaining the nest microbial community was suggested by the high number of active strains.

Atta sexdens ants collected from eucalyptus plantations in the Brazilian state of Rio de Janeiro were also the subject of research into the function that bacterial strains

associated with the fungal gardens played. *Burkholderia* sp. was found to be associated with many colonies. strains that prevented the growth of a variety of entomopathogenic fungi, including *Metarhizium anisopliae*, *Beauveria bassiana*, the saprophytic fungi *Verticillium lecanii*, and the specific ant pathogen *Escovopsis weberi*. Even though these strains were effective against a variety of fungi, they were ineffective against the fungal cultivar. sp. *burkholderia* strains were disengaged from 32 out of 57 insect homes (56%), additionally recommending a significant environmental job. Proteobacteria can also carry out a variety of other ecological tasks in addition to producing small molecules with defensive functions. Volatile pyrazines, such as 2,5-dimethylpyrazine (1) and 3-ethyl-2,5- dimethylpyrazine (2), are produced by *Serratia marcescens* isolated from *Atta sexdens rubropilosa* colonies and are components of ant trail pheromone. However, the ant's dependence on trail pheromone biosynthesis by microorganisms remains unclear. Some insects have been shown to have microbial involvement in the production of pheromones. However, more research is needed to test the connection between the presence of particular symbionts, changes in the chemistry of the host, and behavioral effects (Engl & Kaltenpoth, 2018). With queen ants fertilized during the nuptial flight, leaf-cutter ants establish new colonies; However, mortality is extremely high during and immediately following the nuptial flight (Holldobler & Wilson, 1990, p. 596). After their nuptial flight, it was discovered that queen ants of *A. sexdens rubropilosa* were infected with the entomopathogenic fungus *Aspergillus nomius*. The parasites created aflatoxin B1 (3) and aflatoxin G1 (4) both in situ and in vitro. According to Pupo et al., these compounds may play a significant role in the fungal pathogenicity of the *Atta* queens. 2017).

Leaf-cutter ants of the genus *Acromyrmex*, in contrast to *Atta* ants, typically carry the symbiotic bacteria in crypts in their exoskeletons. At the USP campus in Piracicaba-SP, Brazil, worker ants of the species *Acromyrmex subterraneus brunneus* were sampled for the presence of actinobacteria, and twenty different actinobacteria strains were identified. *Streptomyces* is home to 17 strains, with *Pseudonocardia*, *Kitasatospora*, and *Propionimonas* making up the remaining three genera. According to Zucchi et al., the majority of *Streptomyces* isolates prevented the nest pathogen *Escovopsis weberi* from growing. 2011). These studies suggested that, in addition to *Pseudonocardia*, other ant-associated microbes from

Brazil-collected samples produced biologically active secondary metabolites; However, the chemical compounds that have antimicrobial properties remain elusive. Actinobacteria have evolved protective symbiotic interactions with various organisms, and they are able to produce compounds with a great deal of chemical diversity and a wide range of biological activities. Indeed, it has been demonstrated that *Streptomyces* is an excellent source of insect antimicrobial defenses (Currie, Scott, et al., 2019). The antimycins urauchimycin A (5) and urauchimycin B (6), which have broad and potent antifungal activity against medically important *Candida* strains, were the first compounds from *Streptomyces* strains associated with fungi-farming ants in Brazil. Recently, Brazil-based interdisciplinary research groups have been mainly focused on the characterization of biologically active natural products from microbial symbionts of social insects

2.4 Actinobacteria symbiosis in stingless bees

Stingless bees, also known as meliponines, are a group of small bees that are found in tropical and subtropical regions around the world. They are known for their ability to produce honey and wax, and they play a vital role in the pollination of a wide range of plants.

One of the most notable features of stingless bees is their symbiotic relationship with actinobacteria, which are a group of bacteria that are known for their ability to produce a wide range of antibiotics and other biologically active compounds. The actinobacteria in stingless bees are found in the bees' mandibular glands, which are located on the head of the bees.

The actinobacteria in stingless bees produce a range of antibiotics that help protect the bees from infections and parasites. These antibiotics can also help protect the bees from mites and other parasites that can harm the bees or reduce their fitness. In addition to producing antibiotics, the actinobacteria in stingless bees can also produce compounds that can attract or repel other insects, which can affect the behavior and population dynamics of the bees.

Overall, the actinobacterial symbiosis in stingless bees is an important and complex phenomenon that plays a significant role in many ecological and biological

processes. Understanding this relationship can provide important insights into the interactions between different species and the role that actinobacteria play in various ecological processes.

Bees have been linked to a wide range of microorganisms, including yeasts, fungi, and bacteria; Their function as beneficial symbionts is poorly understood (Morais et al., 2013). Indeed, a diverse bacterial community can be found worldwide in honey bees (Kwong & Moran, 2016; Moran, 2015). It is believed that this particular microbiota coevolved with their hosts and played roles in nutrition and defense against pathogens. Lactic acid bacteria (LAB) are frequently isolated from the guts of honey bees and bumble bees. As a result, the microbial pathogens of honey bees and bumble bees can serve as a foundation for an ecological approach to the study of the bacterial symbionts of stingless bees that are involved in defensive responses. The role of the associated microbiota in protecting bees from pathogens has been the subject of research. The bacterial pathogens that are responsible for both European and American foulbrood diseases could be controlled through LAB. According to Leonhardt & Kaltenpoth (2014), stingless bees from various regions also carry LAB, which may perform similar functions. Additionally, stingless bees from other tropical and subtropical regions have been found to contain actinobacteria. *Streptomyces* species that produce antibiotics were found to have antimicrobial properties against a wide range of human pathogens when they were isolated from the Costa Rican stingless bee *Tetragonisca angustula*. In Thailand, colonies of honey bees (*A. mellifera*, *A. cerana*, and *A. florae*) and stingless bees (*Trigona laeviceps*, and *T. fuscobalteata*) harbored actinobacteria strains that were effective against *P. larvae* and *M. plutonius* (Promnuan et al., 2009). However, little research has been done on the small molecules that act as mediators between these defensive symbioses.

The antimicrobial properties of actinomycetes isolated from the combs of the giant honey bee *Apis dorsata* were the subject of a study. The antimicrobial activity of 25 isolates against four plant pathogenic bacteria (*Ralstonia solanacearum*, *Xanthomonas campestris* pv.) was tested on three distinct media. *Xanthomonas oryzae* pv. *campestris oryzae*, and *Pectobacterium vehicle otovorum*). Antimicrobial activity was observed in three of these strains: DSC3-6, DGA3-20, and DGA8- 3. All of the isolates belonged to the genus *Streptomyces* based on the sequences of their

16S rRNA genes. Isolates DSC3-6, DGA3-20, and DGA8-3 were found to be closely related to *Streptomyces ramulosus* (99.42 percent), *Streptomyces axinellae* (99.70 percent), and *Streptomyces drozdowiczii* (99.71 percent), respectively, in the maximum likelihood phylogenetic tree. This was the primary report on antibacterial action against phytopathogenic microscopic organisms from actinomycetes secluded from the goliath bumble bee (Promnuan et al., 2020).

2.5 Actinobacteria symbiosis in wasps

Actinobacteria are a group of bacteria that are known for their ability to produce a wide range of antibiotics and other biologically active compounds. Many insects, including wasps, have a symbiotic relationship with actinobacteria, which can provide various benefits to the insects.

One of the most common ways that wasps benefit from actinobacterial symbiosis is through the production of antibiotics that help protect the wasps from infections and parasites. For example, some species of wasps have a symbiotic relationship with actinobacteria that produce antibiotics that help protect the wasps from diseases. These antibiotics can also help protect the wasps from parasites, such as mites and worms, that can harm the wasps or reduce their fitness.

In addition to producing antibiotics, actinobacteria can also provide other benefits to wasps. For example, some actinobacteria produce compounds that can attract or repel other insects, which can affect the behavior and population dynamics of the wasps. Actinobacteria can also produce compounds that can help protect the wasps from environmental stressors, such as UV radiation or extreme temperatures.

Overall, actinobacterial symbiosis is an important and diverse phenomenon that plays a significant role in many ecological and biological processes. Understanding these relationships can provide important insights into the interactions between different species and the role that actinobacteria play in various ecological processes.

Past exploration endeavors on digger wasps of the family Philanthus (beewolves; The symbiotic actinobacterium *Candidatus Streptomyces philanthi* (Hymenoptera, Crab-ronidae), which includes more than 100 species found in Europe, Africa, Asia,

and North America (Bohart et al., 1976). The symbionts are raised in specialized reservoirs for the antennal glands before being moved to the brood cells. There, they make antibiotics like streptochlorin and piericidin derivatives, which protect the wasps' larvae from pathogens. The reservoirs of the antennal glands of the Brazilian digger wasp *Trachypus boharti* also contain bacteria. According to gene sequences, the Brazilian wasps cultivate the actinobacteria that are most distantly related to all of the antennal symbionts that have been described. However, the underlying chemistry of this protective symbiosis has not yet been discovered.

Actinomycetes from the genus *Streptomyces*, *Micromonospora*, and *Actinoplanes* were easily cultured from nest material across multiple invasive *P. dominulus* populations, according to a preliminary assessment of the actinomycetes found in the nests of *Polistes dominulus* (Christ). Antimicrobial activity against the challenge bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens*, and *Bacillus subtilis* was tested on thirty of these isolates. A little over half of segregates hindered the development of no less than one test strain. This study examines the antimicrobial-producing actinomycetes isolated from social wasps and the bacteria associated with *P. dominulus* nests. This provides a novel method for studying nest-associated actinomycetes, an ubiquitous and global insect group (Madden et al., 2013).

New antibiotic-producing actinomycetes from wasp mud nests were the subject of a study. Nine distinct kinds of wasp active mud nests from Rajshahi, Bangladesh, were gathered for this purpose. For each home, home material was aseptically homogenized with a 1X saline arrangement and afterward weakened homogenate was plated in Actinomycetes Separation Agar medium to segregate actinomycetes. Nine wasp mud nests collected contained a total of 27 purified bacteria cultures. The homogenate was filtered and centrifuged to obtain the mud nest extract. The disc diffusion method was used to test the extracts' ability to stop bacterial growth. Notwithstanding, just concentrate of home number 9 (N9) showed antimicrobial adequacy against tried microbes, *E. coli*. The antimicrobial efficacy of the 27 isolates was then evaluated using the disc diffusion and agar cross-streak methods. Only the isolate N9C2 was found to be capable of inhibiting the growth of the studied bacteria, *E. coli*, out of the 27 isolates. The isolate N9C2 was then identified by isolating, amplifying, and sequencing its 16S rDNA. As indicated by NCBI impact,

the most noteworthy comparability of grouping (the vast majority) of 16S rDNA of the disengage N9C2 was demonstrated to be that of *Streptomyces coelestis* strain AS 4.1594.

2.6 Actinobacteria symbiosis in termites

Termites are insects that are found in a wide range of habitats around the world. They are known for their ability to consume and digest cellulose, a component of plant cell walls, which is indigestible to most animals. One of the key factors that enables termites to digest cellulose is their symbiotic relationship with actinobacteria, which are a group of bacteria that are known for their ability to produce a wide range of antibiotics and other biologically active compounds.

The actinobacteria in termites are found in the termites' gut, where they help the termites digest cellulose by producing enzymes that break down the cellulose into simpler sugars that the termites can then absorb and use as a source of energy. In return, the termites provide the actinobacteria with a suitable environment and nutrients for growth and reproduction.

In addition to helping termites digest cellulose, the actinobacteria in termites can also provide other benefits. For example, some actinobacteria produce antibiotics that help protect the termites from infections and parasites. These antibiotics can also help protect the termites from mites and other parasites that can harm the termites or reduce their fitness.

Overall, the actinobacterial symbiosis in termites is an important and complex phenomenon that plays a significant role in many ecological and biological processes. Understanding this relationship can provide important insights into the interactions between different species and the role that actinobacteria play in various ecological processes.

Despite the fact that social insects that nest in soil environments are constantly in contact with entomopathogens, they have developed a variety of defense mechanisms, resulting in both individual and social immunity that reduces the likelihood of epizootics in the colony, such as subterranean termites. Here, we report that the fecal nest supports the growth of actinobacteria, which provide an additional

level of protection to the social group against entomopathogens. *Coptotermes formosanus* uses its feces as building material for its nest structure, which results in a "carton material." From multiple termite colonies' nest materials, a *Streptomyces* species with in vivo antimicrobial activity against fungal entomopathogens was discovered. Termite groups were exposed to the fungal entomopathogen *Metarhizium anisopliae* while foraging, and the presence of *Streptomyces* within the nest structure helped the termites survive. As a result, the termite exosymbiosis described in this article takes the form of a defensive mutualism resulting from the termite's use of feces in its nesting structure. In addition to their individual and social immunity, the termite group's association with an actinobacteria community in the termite fecal material confers an extended disease resistance as an additional level of defense (Chouvenc et al., 2013).

In order to evaluate their potential as a source of bioactive actinobacteria with antimicrobial activity, a total of 118 actinobacterial isolates were collected from the three different kinds of termite nests—mound, carton, and subterranean nests. Carton nests yielded the greatest number of actinobacterial isolates (67) and generic abundance (7 known genera). In each type of termite nest, *Streptomyces* dominated the genus. In the non-*Streptomyces* group, mound and carton nests were dominated by *Nocardia*, while subterranean nests were dominated by *Pseudonocardia*. 12 isolates from the genera *Streptomyces*, *Amycolatopsis*, *Pseudonocardia*, *Micromonospora*, and *Nocardia* exhibited distinct antimicrobial activities, representing a trend of novel species discovery (20 percent of bioactive actinobacteria that could inhibit the growth of at least one test organism). *Streptomyces* sp. The most distinct bioactive isolate was CMU-NKS-3. It and *S. padanus* MITKK-103T shared 99 percent of their 16S rRNA gene sequences, indicating that they were closely related. The isolate CMU-NKS-3, which was grown in potato dextrose broth and exhibited a wide range of minimum inhibitory concentrations (6.10 104e1.25 mg mL⁻¹) against various pathogens, produced the highest amount of extracellular antimicrobial substances. According to Sujada et al., we concluded that termite nests are a rich source of bioactive strains of cultivable actinobacteria for upcoming biotechnological requirements. 2014).

From *Nasutitermes* sp.'s nest, ten actinomycetes isolates were collected. within the Pananjung Pangandaran National Park. The dual culture method was used to test

these isolates for antimicrobial activity against challenge bacteria (*E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Serratia marcescens*) and fungi (*Fomitopsis palustris*, *Fusarium oxysporum*, and *Trichoderma viride*). The outcome uncovered that few detaches were dynamic against parasites and microbes. With an inhibition rate of more than 80%, isolate Pn-TN2 exhibited the highest levels of antibacterial and antifungal inhibition. Pn-TN2, according to morphological and 16S rRNA gene sequence analysis, belonged to *Streptomyces prasinopilosus*. According to Krishna et al.'s findings, the termite nest could provide future biotechnological needs with bioactive strains of cultivable Actinomycetes (Krishanti et al., 2018).

2.7 BIOPROSPECTING

Bioprospecting is the process of discovering and studying new biological resources and substances that can be used for a variety of purposes, including medicine, agriculture, and industry. It involves the exploration of diverse environments, such as forests, oceans, and soils, to find new species and evaluate their potential uses.

One of the main benefits of bioprospecting is that it allows for the discovery of new and potentially valuable substances that can be used in the development of new drugs, pesticides, and other products. For example, the anti-cancer drug Taxol was developed from the Pacific yew tree, which was discovered through bioprospecting. Similarly, bioprospecting has led to the discovery of many other important drugs, such as penicillin and aspirin.

Another benefit of bioprospecting is that it can help to preserve biodiversity. By studying and protecting the environments in which new species are discovered, bioprospecting can help to ensure that these ecosystems remain healthy and diverse. Additionally, bioprospecting can provide economic benefits to local communities by providing employment and other opportunities.

However, bioprospecting can also raise ethical and legal issues. There is often debate over who owns the rights to the biological resources that are discovered and how the profits from their use should be shared. In order to address these issues, many countries have adopted legal frameworks that establishes guidelines for the regulation of bio prospecting

2.8 Bioprospecting of insect nest microbiome:

Bioprospecting of the insect nest microbiome refers to the process of studying and exploring the diverse microbial communities found within the nests of insects in order to identify new and potentially valuable biological resources. This type of bioprospecting has gained increasing attention in recent years due to the growing awareness of the important role that the microbiome plays in the health and well-being of insects and the potential applications of these microorganisms in various fields.

One of the main benefits of bioprospecting the insect nest microbiome is the potential to discover new antimicrobial agents. Many of the microorganisms found in insect nests have evolved to survive in a highly diverse and often harsh environment, making them well-suited for the production of new and novel antimicrobial compounds. In fact, numerous studies have already identified a range of antimicrobial compounds from the microbiomes of insect nests, including bacteria, fungi, and viruses. These compounds have the potential to be used in the development of new antibiotics, as well as for other applications such as the control of plant diseases.

In addition to the potential for discovering new antimicrobial agents, bioprospecting the insect nest microbiome also has the potential to provide insights into the evolution and adaptation of microorganisms. By studying the microbial communities found within insect nests, scientists can learn more about how these microorganisms have evolved to survive and thrive in these environments, and how they interact with the insects themselves. This knowledge could potentially be used

to develop new approaches to managing the health and well-being of insects, as well as to better understand the role of the microbiome in human health and disease.

However, bioprospecting the insect nest microbiome also raises a number of ethical and legal issues. There is often debate over who owns the rights to the biological resources that are discovered and how the profits from their use should be shared. In addition, there are concerns about the potential impacts of bioprospecting on the insects and their habitats, as well as the potential for the introduction of non-native species or pathogens into these ecosystems.

To address these issues, it is important for bioprospecting of the insect nest microbiome to be conducted in an ethical and responsible manner, with appropriate safeguards in place to protect the rights of local communities and to ensure the fair distribution of benefits. This may include the development of clear legal frameworks and guidelines for the regulation of bioprospecting activities, as well as the implementation of conservation measures to protect the insects and their habitats.

Overall, bioprospecting of the insect nest microbiome has the potential to lead to the discovery of new and valuable biological resources, as well as to provide insights into the evolution and adaptation of microorganisms. However, it is important to ensure that these activities are conducted in a way that is both ethical and sustainable, in order to maximize the benefits while minimizing any negative impacts.

2.9 SECONDARY METABOLITES OF ACTINOBACTERIA

Jolanta Solecka et al., (2012) reviewed secondary metabolites obtained from Actinomycetes provide a potential source of many novel compounds with antibacterial, antitumor, antifungal, antiviral, antiparasitic and other properties. The majority of these compounds are widely used as medicines for combating multidrug-resistant Gram-positive and Gram-negative bacterial strains. Members of the genus *Streptomyces* are profile producers of previously known secondary metabolites. Actinomycetes have been isolated from terrestrial soils, from the rhizospheres of

plant roots, and recently from marine sediments. This review demonstrates the diversity of secondary metabolites produced by actinomycete strains with respect to their chemical structure, biological activity and origin. On the basis of this diversity, this review concludes that the discovery of new bioactive compounds will continue to pose great challenge for scientist. Aleksandra Rajnisz Mateusiak (2018) reviewed the growing resistance of microorganisms towards antibiotics has become a serious global problem. Therapeutics with novel chemical scaffolds and/or mechanisms of action are urgently needed to combat infections caused by multidrug resistant pathogens, including bacteria, fungi and viruses. Development of novel antimicrobial agents is still highly dependent on the discovery of new natural products. At present, most antimicrobial drugs used in medicine are of natural origin. Among the natural producers of bioactive substances, Actinobacteria continue to be an important source of novel secondary metabolites for drug application. In this review, the authors report on the bioactive antimicrobial secondary metabolites of Actinobacteria that were described between 2011 and April 2018. Special attention is paid to the chemical scaffolds, biological activities, and origin of these novel antibacterial, antifungal and 17 antiviral compounds. *Arenimycin C*, *chromopeptide lactone RSP 01*, *kocurin*, *macrolactins A1* and *B1*, *chaxamycin D* as well as anthracimycin are regarded as the most effective compounds with antibacterial activity. In turn, the highest potency among selected antifungal compounds is exhibited by *enduspeptide B*, *neomaclafungins A-I* and *kribelloside D*, while *ahmpatinin iBu*, *antimycin A1a*, and *pentapeptide 4862F* are recognized as the strongest antiviral agents.

2.10 DRUG DISCOVERY

Rapid globalization and intensive use of antibiotics, an increasing threat of multidrug-resistant pathogens. The urge to discover novel lead antibiotic compounds and to refill the industrial antibiotic pipeline to meet current and future challenges. Nowadays the major drawback of natural products research and drug discovery represents the re-isolation of known compounds and the 5 random nature in terms of organisms explored by which this research is performed. Most compounds are still isolated from random sources and tested against random

targets to find more or less useful bioactivities. More rational approaches are necessary to enhance the efficacy, efficiency, and speed of drug discovery in general and antibiotic discovery in particular. In recent years, the exploration of the chemical basis of specific and well described bacteria host or fungal host interactions in combination with analytical dereplication processes has emerged as a powerful strategy to identify novel chemical entities (Tawifke et al., 2013). Since the initial appearance, natural products and the respective complex biosynthetic machineries have been in a constant state of evolutionary based refinement for at least a billion year (Fischbach et al., 2008). They function as chemical modulators and signaling molecules for intra and inter kingdom interaction such as defense, protection, behavior, virulence, and central physiological functions; thereby generating evolutionary benefits for the producer in natural habitants (Kaltenpoth, 2009; Oliver et al., 2014). Recent developments in analytical chemistry, genome sequencing and molecular biology facilitate the analyse of minute amounts of biological material and enable a more efficient interaction to molecule discovery approach (Johnston et al., 2015; Kuhlisch et al., 2015). These studies also place the natural products into a genomic, regulatory, functional, and ecological context, and might allow drawing more general conclusions about the biosynthetic origins, the ecology and evolution of symbiotic associations. However, even in this ecological context natural product chemistry is highly capricious, because so far, we are not able to calculate or predict which molecular structures are responsible for certain biological function. Despite this aspect, natural products originating from insect microbial symbioses have a vast biochemical diversity which is powerful resource for drug discovery 6 (Challinor, 2015). Surprisingly, despite the success of insects in terms of numbers and diversity, the most successful drugs derived from natural products, including artemisinin, quinine, aspirin, cocodamol, simvastatin, and cyclosporine, have been isolated from plants, marine organisms, and microbes. Altogether, 939 nature-derived approved drugs were developed between 1961 and 2010 but none of these were from insects and only a few originated from invertebrates such as leeches, sponges, and cone snails (Fuqing Huang et al., 2014).

CHAPTER 3

3.1 AIM

To isolate and and characterize actinobacteria from insect nests.

3.2 OBJECTIVE

1. Isolation and characterization of actinobacteria from insect nest.
2. Invitro screening for antimicrobial activity by agar plug method and selection of potential strain
 - Activity against *Mycobacterium Smegmatis*, *Pseudomonas Aeruginosa*, *Klebsiella Pneumonia*
3. Production and extraction of antimicrobial metabolites from potential actinobacterial strain.
4. Characterization, Taxonomy and Molecular analysis of the potential strain

CHAPTER 4

MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 SAMPLE COLLECTION

To isolate Actinobacteria, insect nest samples were collected. Aseptically obtained at the Sathyabama Institute of Science and Technology, the samples were brought as soon as possible to the lab. The sample that was collected was dry and had a powdery texture (Figure 3.1). To get rid of any moisture, the sample was dried at room temperature for 24 hours. The pretreatment sample was then heated dry for 10 minutes in a hot air oven using roughly 5 grammes (Radhakrishnan et al., 2007)



FIG 4.1- Collection of Samples

4.2.1 MEDIUM USED

- Starch casein agar (SCA)
- Actinomycete isolation agar (AIA)
- International *Streptomyces* project-2 (ISP2)
- Nutrient agar medium
- Nutrient broth
- Muller-Hinton agar (MHA)
- Muller-Hinton broth (MHB)

4.2 METHODS

4.2.1 ISOLATION AND CHARACTERIZATION OF ACTINOBACTERIA FROM INSECT NEST

4.2.1.1 ISOLATION OF ACTINOBACTERIA FROM INSECT NEST SAMPLE

Using Starch Casein Agar (SCA) and Actinomycete Isolation Agar (AIA) media, actinobacteria were isolated using the usual spread plate method.

The obtained sample is pre-treated by placing the dry sample in a petri plate and heating it over a hot plate at 60°C for 15 minutes. This is done to prevent the growth of any unwelcome bacteria. To 50 ml of sterile distilled water, 5 g of the pre-treated insect nest sample was introduced. For 30 minutes, the flask was shaken in a shaker to thoroughly mix the sample. 100 microliters of each of the 10⁻⁴ and 10⁻⁵ dilutions were obtained and distributed over the agar medium using a sterile L-rod after the particles settled and the suspension was serially diluted up to 10⁻³. A sterile L-rod was used to disseminate 100 microliters of each of the 10⁻⁴ and 10⁻⁵ dilutions onto the agar medium. (Radhakrishnan et al., 2014) At 28 °C, all plates were incubated.

4.2.1.2 RECOVERY AND PURIFICATION OF ACTINOBACTERIA

To inoculate colonies onto International Streptomyces Project-2 (ISP2 medium) plates, colonies that displayed suspicious actinobacterial morphology, such as powdery growth, pigmented, and filamentous colonies, were collected from isolation plates using a sterile L-shaped needle. Seven days were spent incubating the plates at 28 °C. After visually checking purity, individual actinobacterial colonies from morphologically distinct actinobacterial cultures were chosen and kept. They were then subcultured on ISP2 agar plates in duplicate, and incubated at 28 °C for 7–14 days. Before further analysis, all culture medium were kept in the refrigerator.

4.2.1.3 CHARACTERIZATION OF ACTINOBACTERIA

All actinobacterial cultures were inoculated in ISP2 agar medium to perform cultural characterization. All plates were incubated for 7–14 days at 28 °C. According to Shirling and Gottlieb (1966), cultural traits that were observed were growth, colony uniformity, Ariel mass colour, the presence of lateral pigments, and the generation

of soluble pigments. The substrate and aerial mycelium of each isolate were also examined under a microscope

4.2.2 IN VITRO SCREENING FOR ANTIMICROBIAL ACTIVITY AND SELECTION OF POTENTIAL STRAIN

4.2.2.1 TEST PATHOGENS

Test pathogens used in this study include bacterial pathogens such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Mycobacterium smegmatis* and *Bacillus cereus*. All the bacterial cultures were maintained in nutrient agar slants as well as stab culture using nutrient agar medium.

4.2.2.2 TESTING FOR ANTIMICROBIAL ACTIVITY

The agar plug method was used to assess the antibacterial activity of actinobacterial cultures (Radhakrishnan et al., 2014). A sterile spatula was used to scrape mycelial growth off of actinobacteria cultures that had been cultivated on nutrient agar plates. Using a sterile cotton swab, the test microorganisms were injected into Mueller-Hinton agar plates with 0.5 McFarland standards. From ISP2 agar seeded with actinobacterial cultures, 5 mm-diameter agar plugs were cut out and layered over nutritional agar containing the test pathogens. All plates underwent a 24-hour incubation period at 37 °C. The actinobacterial strain AA5, which was discovered in an ant colony, displayed the greatest activity against the test pathogens in the initial screening. In triplicate, the action was verified. The actinobacterial strain AA5 was therefore chosen as a suitable strain for additional research.

4.2.3 PRODUCTION AND EXTRACTION OF ANTIMICROBIAL METABOLITES FROM POTENTIAL ACTINOBACTERIAL STRAIN

4.2.3.1 EFFECT OF FERMENTATION METHOD ON COMPOUND PRODUCTION

Effect of solid-state and submerged fermentation on strain AA5's ability to produce antibacterial chemicals. ISP2 agar plates and 100 ml of ISP2 broth were infected with actinobacterial strain spores. A 28°C incubator was used for the agar plates. ISP2 broth in centrifuge tubes was incubated in a rotating shaker at 100 RPM. A plug of agar was removed from the ISP2 agar plate every 24 hours and analysed for the presence of *Staphylococcus aureus* (Radhakrishnan et al., 2016). For 10

minutes, 2 ml of each ISP2 broth sample was centrifuged at 10,000 rpm. The well diffusion method was used to examine the cell-free supernatant's ability to inhibit *S. aureus* growth (Bavya et al., 2011). Until the eighth day of incubation, activity was monitored every 24 hours.

4.2.3.2 PRODUCTION AND EXTRACTION OF BIOACTIVE COMPOUNDS FROM POTENTIAL ACTINOBACTERIAL STRAINS

A substantial number of antibacterial compounds were produced by agar surface fermentation with MHA medium. After being plated onto agar, the potential actinobacterial strain was allowed to develop for 10 days at 28 °C. A sterile spatula was used to scrape away the mycelial growth following incubation. The released antimicrobial metabolites were extracted from the agar medium for 24 hours using a 1:2 ratio of ethanol, a medium polar solvent. The solvent component was then collected and concentrated using a rotary evaporator to produce the crude extract..

4.3 OPTIMIZATION OF BIOACTIVE COMPOUNDS PRODUCTION (MEDIA OPTIMIZATION)

It is a method used to determine the parameter that needs to be altered in order to obtain a higher yield during production, since ISP2 is being used regularly, the strain tends to grow accustomed to the media and its composition, therefore the yield obtained for the strain in standard ISP2 is a saturated yield, whereas when the components of the media are slightly tweaked by increasing or decreasing the concentration, the yield produced may vary a reduction or increase in yield can be observed.

Classical method:

One factor at a time

Effect of critical medium components and culture conditions on bioactive compounds production from the strain AA 5 was determined by adopting classical one factor at a time method. To screen for suitable substrates and environmental conditions the strain AA5 was inoculated into basal medium containing different carbon source, nitrogen source, minerals, pH, seawater and temperature. After incubation, agar plug from all the media was tested against the *S.Aureus*.

4.4 Anti-Biofilm:

TSB broth with 1% glucose was prepared and autoclaved. Pathogens were inoculated and kept in the incubator for 24 hours.

DAY -1:

Antibiofilm activity was performed against 4 biofilm-forming pathogens. The four pathogens are *E.coli*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*. Four 96 well plates were taken and the pathogens' names were written on it. 100 µl of TSB broth is added to all wells. An extra 100 µl of TSB broth was added to column 2 alone. Water was added to all four sides of the 96-well plate. The extract was added to the first well. 100 µl was taken in a pipette from the first well and transferred to the second well for serial dilution. Again, 100 µl was taken from the second well and transferred to the third well. The same is repeated for well three too. Now, the final concentration is reached upto 25 µl/mL. 50 µL of pathogen inoculum was added to all wells. Then the plates were incubated at 37°C for 24 hours.

DAY-2:

The next day the plates were washed with 1X PBS 2 times and let to air dry completely.

DAY-3:

150-180 µL of 0.1% crystal violet was added to all wells and left for 30 minutes. Then it was rinsed with 1X PBS 2 times. The wells were resolubilized with 150-180 µL of 95% ethanol and the OD readings were taken at 570 nm. Biofilm inhibition was calculated using the formula.

% Biofilm inhibition = $\frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$

4.5 ANTI – OXIDANT:

In DPPH assay 0.1 mM solution of DPPH was prepared in methanol and 500 µl of DPPH solution was added to 2500 µl of the BS in different concentrations i.e. 10, 30, 100, 500 and 1000 Mg/ml respectively. Then, absorbance was measured at 517 nm using spectrophotometer. The mixture was shaken vigorously and allowed to

stand at room temperature for 30 min (Zou et al., 2011). The percent DPPH scavenging effect was calculated by using following equation:

- inhibition = $(A_0 - A_1 / A_0) * 100$. Where A_0 was the absorbance of control reaction and A_1 was the absorbance in presence of test or standard sample

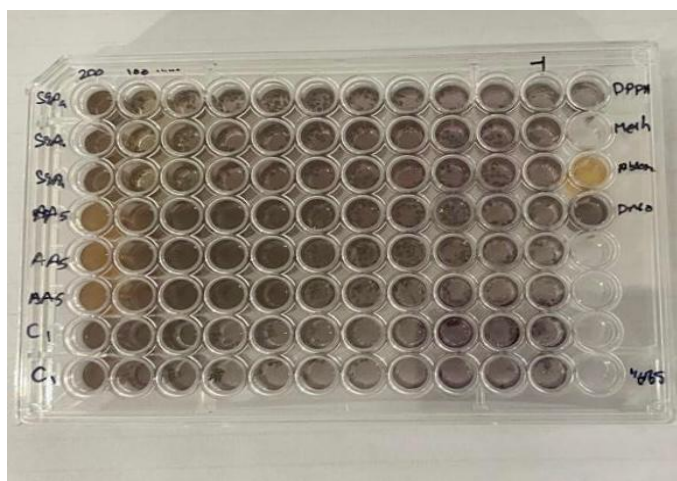


FIG 4.2 ANTI-OXIDANT ASSAY

4.6 ANTI – TB ACTIVITY:

Microplate Alamar Blue Assay

MABA is used for measuring cell proliferation and viability by monitoring the oxidation/reduction state of the environment of cellular growth. As with the tetrazolium salts, Alamar blue is a soluble redox dye that is stable in culture medium and nontoxic (Ansar Ahmed et al., 1994). The oxidized dye is blue and nonfluorescent; upon reduction it turns pink and fluorescent, therefore, growth can be determined by a visual colour change or by using a fluorometer. Alamar blue has been used to assess the susceptibility of *M. tuberculosis* antimicrobials in several laboratories (Collins & Franzblau, 1997; Franzblau et al., 1998; Yajko et al., 1995)

MABA is used to check activity against *M. tuberculosis* and *M. smegmatis* for crude extract of AA5.

4.7 CHARACTERIZATION AND TAXONOMY OF POTENTIAL ACTINOBACTERIAL STRAIN

4.7.1 MICROMORPHOLOGY

Transplantation Embedding Technique was used to examine the micromorphology of a possible actinobacterial strain (Xuet al., 2007). In a nutshell, a sterile knife was used to cut a rectangular hole in an ISP2 agar plate. The actinobacterial strain's spore was then inoculated on the hole's borders under aseptic conditions. The inoculated actinobacterial culture on the ISP2 agar plate was touched, and a sterile coverslip was placed on top of the agar. The plate was incubated for 7–14 days at 280°C. The coverslip was then aseptically removed with sterile forceps, put over a clean microscopic slide, and taped with cellophane. Micromorphology of actinobacterial strains were observed under bright field microscope under 10x and 40x magnifications. Multiple coverslips were placed on the actinobacterial strain inoculated area on ISP2 agar plates to study the different stages of actinobacterial growth.

4.7.2 CULTURAL CHARACTERISTICS

Cultural characteristics were studied by inoculating the growth of actinobacterial strains into different ISP (International Streptomyces Project) media such as yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol-asparagine 54 agar (ISP5), peptone-yeast extract-iron agar (ISP6) and tyrosine agar (ISP7). Following the procedures outlined by Shirling and Gottlieb (1966), all the media were created. All the plates underwent triple plating and a 10-day incubation period at 280°C. The type of growth, uniformity, aerial mass colour, the existence of reverse side pigment, and the specifics of soluble pigment formation, if any, are among the cultural traits that are noted.

4.7.3 PHYSIOLOGICAL CHARACTERISTICS

Using ISP2 media supplemented with various sugar sources, the research of the actinobacterial strains, i.e., potential strain, utilisation of carbon sources was conducted. Glucose, lactose, mannitol, inositol, rhamnase, and raffinose were among the sugars employed in this investigation. By adding the actinobacterial strain AA5 to ISP2 media that also contained peptone, yeast, malt, potassium nitrate, and soybean extract, the effect of nitrogen supply on growth was examined. After 10 days of 28oC incubation, the plates were checked for growth. By inoculating

the actinobacterial strain AA 5 onto ISP2 agar medium adjusted to different pH values, namely 7 and 9, the effect of pH on growth was examined. After 10 days of 28°C incubation, all of the plates were checked for growth.

4.7.4 MOLECULAR CHARACTERIZATION

Genomic DNA of the potential actinobacterial strain AA5 extracted using solute ready genomic DNA kit. The 16SrRNA gene was amplified by PCR using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') (Patel Rashmita *et al.*, 2014). The reaction mixture (50µl) contained 100 ng of 2µl genomic DNA, 5µl of 10x buffer, 4µl of BSA (1mg/ml), 1µl of dNTP mixture (2.5mM), 1µl of each primer (10µM) and 3.5U of Taq DNA polymerase. PCR condition followed as 6min at 94°C followed by 35 cycles of 45s at 94°C, 45s at 55°C and 1.5min at 72°C, followed by a 8 min extension at 72°C. PCR amplification was performed in an Eppendorf Master cycler Gradient. Purified PCR product was sequenced bidirectional to obtain complete coverage at Bioserve, Hydrabed. Sequences were edited and contig was assembled in DNA baserv.3 and compared with GenBank sequences by BLAST analysis. The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank using CLC sequence viewer 6.0 programs. The alignment was constructed to phylogenetic tree using neighbor joining in MEGA7 software (**Lane 1991**). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resampling of the neighbor joining data set (**Saitou. 1987.**). The partial 16S rRNA nucleotide sequence of the potential actinobacteria strain ANA 5 was deposited to GenBank database.

4.8 IDENTIFICATION OF ANTINO BACTERIAL STRAIN

Based on the microscopic and cultural characteristics examined, the potential actinobacterial strain AA5 was identified as a genus-level streptomycete.

4.9 LARGE SCALE PRODUCTION OF BIOACTIVE POTENTIAL STREPTOMYCES:

A bioreactor is a distinctively designed vessel that provides controlled physicochemical parameters to support the growth of microorganisms. The design of the fermenter should be such that it is able to provide optimal parameters for the growth of microorganisms. A Fermentors, also known as bioreactors, are sterilised and enclosed vessels that are used for the growth of microorganisms under optimal conditions. The microorganisms can be grown in large quantities to produce metabolites for commercial uses.



FIG 4.3 FERMENTOR

4.9.1 WATER STERILIZATION:

The bioreactor or the vessel is cleaned by using water sterilization. In this step water is passed into the fermentor it is then heated by the steam which passes through the jacket. The steam in the jacket heats the water till the temperature rises to 121.5. After the required temperature is obtained a holding period of about 30mins is maintained for the process of sterilization. After which the water is cooled and drained out.

4.9.2 MEDIA STERILIZATION:

ISP2 broth is prepared for a volume of 6 liters. The broth is transferred from the conical flask to the fermentor vessel using a funnel. The media is to be autoclaved or sterilized which is also performed by the fermentor as well. The media sterilization is similar to the procedure of water sterilization where the temperature is maintained at 121.5 and holding period of 30 mins as well is maintained. The media is the slowly allowed to cool down on its own, once the temperature drops to 90 the cooling system is turned on where a supply of cool water around the jacket is supplied this cools the media further down.

4.9.3 INNOCULUM PERPARATION:

Tow 1000ml conical flask were taken and sterilized 300ml of ISP2 broth were prepared and taken in each flask separately and autoclaved. 4-6 plugs from the culture plate was cut using well cutter. It was then added to both the flasks and sealed close with a sterile cotton plug. The conical flasks containing the inoculum is then placed in a shaker incubator for a period of 7 days. Once the inoculum is prepared and the media in the fermentor cools down, the inoculum is transferred to the media this is called inoculation.

Inoculum must be in a healthy, active state (lag phase minimized) and should be available in large volume. It is checked for any contamination. 600 mL of pure culture is inoculated in the fermenter under sterile conditions. Growth of organisms in the fermenter under sterile conditions for product formation: The organism grows in the production media for 4-5 days where it undergoes changes in dissolved oxygen and pH. Parameters like temperature, agitation, pH, and dO₂ are observed after every 2 hours. The gradual decrease in dissolved oxygen marks the growth of bacteria which shows the log phase of growth kinetics.

4.9.4 Extraction of the product

The product was harvested in extraction bottles and it was centrifuged to remove the cells. Cell-free supernatant was taken in a separate beaker and 40g of resin was autoclaved and added to it and kept in a rotary shaker for 24hours. The resin was removed and dried and methanol was added to it and left for 24 hours. The next day

the resin was removed and the methanol mixture was lyophilized. The weight of the compound was 2.21 grams. The extracts are then used for activity testing against *S.Aureus*.

4.10 HPLC-HRMS/MS Analysis and Metabolites Identification Using GNPS

Dereplication:

Crude extract was analyzed by LC-HRMS and LC-HRMS/MS using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific Spa, Rodano, Italy) coupled to an Agilent model 1100 LC system (Agilent Technologies, Cernusco sul Naviglio, Italy) equipped with a solvent reservoir, an in-line degasser, a binary pump, and a refrigerated autosampler [13]. The spectrum was recorded by infusion into the ESI source using MeOH as the solvent. A 5 μ m Kinetex C18 column (50 mm $\times$ 2.1 mm), maintained at 25 °C, was operated using a gradient elution of H₂O and MeOH both with 0.1% formic acid, running at 200 μ L/min. The gradient program was as follows: 10% MeOH for 3 min, 10–90% MeOH over 30 min, and 90% MeOH for 3 min. Data dependent acquisition mode MS spectra was recorded in positive ion mode with a spray voltage of 5 kV, a capillary temperature of 230 °C, a sheath gas rate of 12 units N₂ (ca. 120 mL/min), and an auxiliary gas rate of 5 units N₂ (ca. 50 mL/min). HRMS/MS scans were obtained for selected ions with collision-induced dissociation (CID) fragmentation, isolation width of 2.0, normalized collision energy of 35, activation Q of 0.250, and activation time of 30 ms.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 ISOLATION AND CHARACTERIZATION OF ACTINOBACTERIA FROM ANT NEST

5.1.1 ISOLATION OF ACTINOBACTERIA FROM ANT NEST SAMPLE

Actinobacterial colonies with various morphologies and typically slow growth rates were seen on starch casein agar and Actinomycete isolation agar during the isolation of actinobacteria. Nine different strains from ISP2 agar plates were taken out and stored at 4 °C. Figuring 4.1 On ISP2 agar medium during recovery and preservation, all of the actinobacterial cultures displayed good development and the presence of both aerial and substrate mycelium, with *Streptomyces* making up the majority of them.

Researchers Kumar et al.⁵ and Zucchi et al.¹⁵ studied the diversity of actinobacteria in ant nests and talked about how crucial this diversity is to preserving the asepsis of the nest. According to the literature, such stories of Indian ecology have not been thoroughly investigated.



FIG-5.1 SCA and AIA showing actinobacterial strains

5.1.2 RECOVERY AND PURIFICATION OF ACTINOBACTERIA

An examination of a termite and ant nest produced nine actinobacterial colonies when the standard spread plate method was used. Colonies with putative actinobacterial morphology, including pigmented, filamentous, and powdery growth, were taken from the isolation plate and injected into plates of yeast extract-malt

extract agar (ISP2 medium), using a sterile L-shaped needle. 7 days at 28 °C.

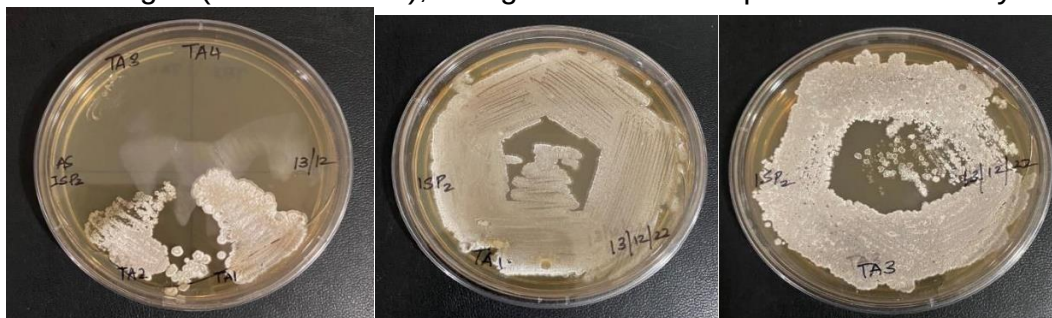


FIG-5.2 Morphological characterization

5.1.3 CHARACTERIZATION OF ACTINOBACTERIA

Growth, colony uniformity, aerial mass colour, the presence of side pigment, and the production of soluble pigment are among the cultural traits that have been observed (Shirling and Gottlieb, 1996). For the purpose of identifying the substrate and aerial mycelium, every culture was examined under a microscope. In table 4.1, the culture and five morphological traits were noted and listed.

Table 5.1 CULTURE AND MORPHOLOGICAL CHARACTERISTICS OF ANT AND TERMITE NEST ACTINOBACTERIAL STRAINS

S.no	Strain no	Cultural Characteristics				Aerial Mycelium	Substrate mycelium
		Growth	Consistency	RSP	SP		
1	AA1	Good	Powdery	+	+	+	+
2	AA2	Good	Slimy	+	+	+	+
3	AA3	Good	Slimy	+	+	+	+
4	AA4	Good	Slimy	+	+	+	+
5	AA5	Good	Powdery	+	+	+	+
6	TA1	Good	Powdery	+	+	+	+
7	TA2	Good	Slimy	+	+	+	+
8	TA3	Good	Powdery	+	+	+	+
9	TA4	Good	Slimy	+	+	+	+

5.2 INVITRO SCREENING FOR ANTIMICROBIAL ACTIVITY AND SELECTION OF POTENTIAL STRAIN

In this study, only 9 actinobacterial strains recovered from the ant nest sample were screened for antimicrobial activity against Gram-positive and Gram-negative bacterial pathogens (figure 5.3). Table 5.2 lists the actinobacterial strains' antimicrobial activities. Actinobacterial strain AA5 out of the five cultures examined had encouraging activity against all of the bacterial pathogens examined.

Antimicrobial activity was independently verified in triplicate. AA5 showed positive action in this as well against all the test pathogens (Figure 5.4). As a result, the actinobacterial strain AA5 was chosen as one of the candidates for further research. It was recently discovered that a *Streptomyces* species connected to the higher attine *Acromyrmex octospinosus* produces the well-known antifungal *candidicin* (Hutchings et al., 2010). *Streptomyces* sp. TD025 was chosen for the isolation of bioactive compounds since it demonstrated robust and broad-spectrum *Candida* inhibition (Pagnocca et al., 2013). Less research has been done on actinobacteria connected with ant nests' antibacterial properties.

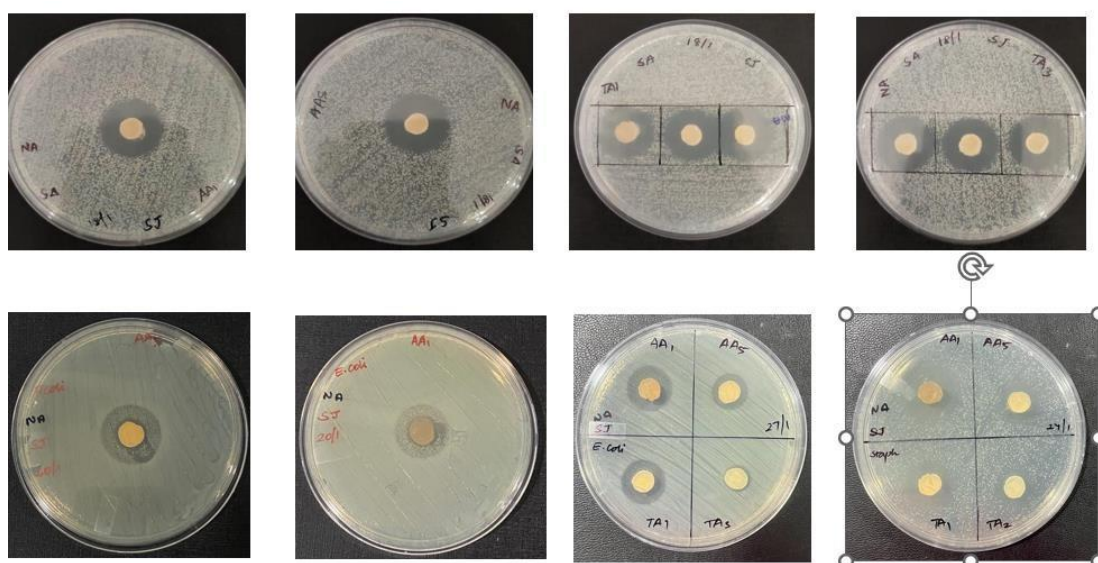


FIG-5.3Antimicobial activity of isolated colonies by agar plug method

Table 5.2 Antimicrobial activity of actinobacteria isolated from ant and termite nest

Test pathogens	Actinobacterial strains (Zone of inhibition in millimeter in diameter)									
	AA1	AA2	AA3	AA4	AA5	TA1	TA2	TA3	TA4	TA5
<i>Staphylococcus Aureus</i>	20	-	-	-	24	26	-	25	-	-
<i>Mycobacterium smegmatis</i>	-	-	-	-	24	-	-	-	-	-
<i>Pseudomonas aeruginosa</i>	11	-	-	-	15	-	-	-	-	-
<i>Klebsiella pneumonia</i>	-	-	-	-	18	-	-	-	-	-
<i>Vibrio Cholera</i>	13	-	-	-	22	-	-	18	-	-
<i>Escherichia Coli</i>	16	-	-	-	22	20	-	18	-	-

(-)- No growth

5.2.1 CONFIRMATORY TEST FOR SELECTION OF POTENTIAL STRAIN

From the above table we can infer that the stain AA5 has a broad spectrum against both gram positive and gram negative pathogens thus it is selected as the potential strain, and further more it is being processed and utilized in the various testing and screening processes.

5.3 PRODUCTION AND EXTRACTION OF ANTIMICROBIAL METABOLITES FROM POTENTIAL ACTINOBACTERIAL STRAIN

5.3.1 EFFECT OF SUBMERGED AND SOLID-STATE FERMENTATION

The influence of fermentation on the production of antibacterial chemicals by strain AA5. The results showed that actinobacterial strain AA5 was found to produce the antimicrobial compound on the sixth day of submerged fermentation, whereas strain AA5 was found to produce the antimicrobial compound starting on the second day of agar surface fermentation. The bacterium AA5 was subjected to agar surface fermentation using ISP2 agar medium in an effort to develop additional antibacterial chemicals.



FIG-5.5 Pure forms of potential strain in ISP2 agar



FIG-5.6 production and extraction of antimicrobial metabolites from potential actinobacterial strain

Table 5.3 Effect of fermentation conditions on antimicrobial compound production

Incubation period (Days)	Zone of inhibition in millimeter in diameter	
	Strain AA5	
	Nutrient agar	Nutrient Broth
1	-	-
2	-	-
3	12	-
4	14	-
5	16	-

6	16	4
7	16	6
8	16	8

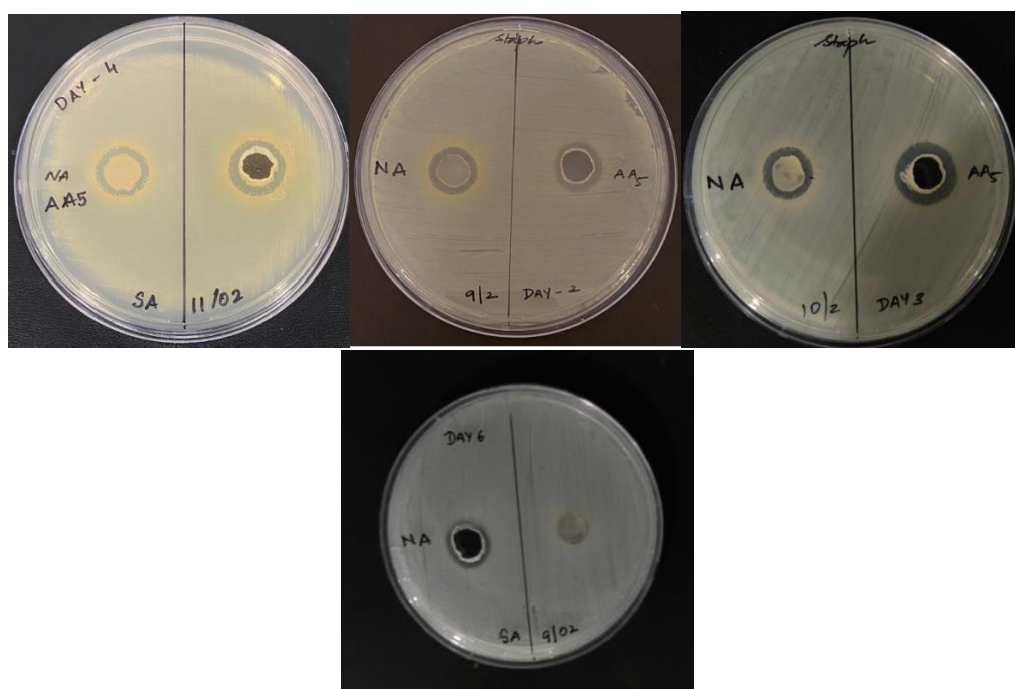


FIG-5.7 Effect of fermentation condition on antimicrobial compound production

5.3.2 PRODUCTION AND EXTRACTION OF ANTIMICROBIAL COMPOUNDS

Actinobacterial strain AA5 showed good growth on ISP2 agar medium. Strain AA5 yielded 158mg mg of crude obtained after 4 batches of extraction using ethyl acetate as the solvent.

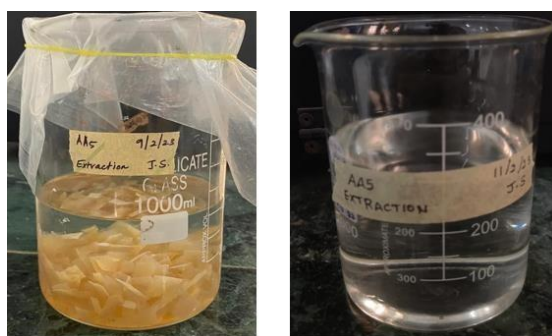


FIG-5.8 Production and extraction of antimicrobial metabolites from potential actinobacterial strains

5.4 OPTIMIZATION OF BIOACTIVE COMPOUNDS PRODUCTION

Among the various factors tested most of the carbon, nitrogen and minerals sources were found to influence the antimicrobial activity of strain AA 5.

Table 5.4 Zone of inhibition in mm antimicrobial activity

Variables	Media	Zone of inhibition in mm Antimicrobial activity
Utilization of carbon sources	d-Glucose	14
	Lactose	15
	Raffinose	12
	Cellulose	13
	manitol	9
Utilization of nitrogen sources	Peptone -	15
	KNO ₃ -	9
	Soybean	14
	ASP	12
Different pH	pH 0	6
	pH 1	-
	pH 7	15
	pH 9	16
	pH 11	-

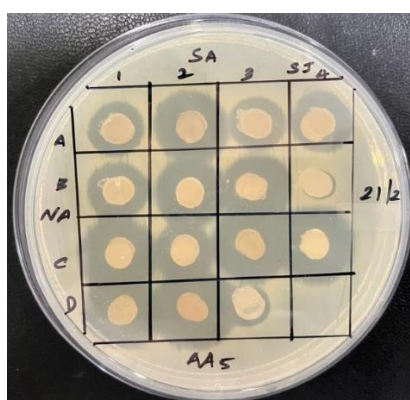


FIG-5.9 Optimization of strain AA5 on different media composition

5.4 CHARACTERIZATION AND TAXONOMY OF POTENTIAL ACTINOBACTERIAL STRAIN

5.4.1 MICROMORPHOLOGY

The transplanting embedding technique was used to examine the micro morphology of the AA5 strain. Using a sterile knife, a rectangular hole was briefly pierced into an ISP2 agar plate. Next, while maintaining an aseptic environment, the spore of the AA5 strain was implanted on the hole's edge (Xie et al., 2007). A sterile cover slip was placed over the inoculated actinobacterial culture agar. The plate was incubated at 280°C for 7–14 days. Using sterile forceps, the cover slip was then aseptically removed, placed over a clean microscope slide, and taped down. At 10x and 40x magnifications, bright field microscope photographs of the micro morphology of the potential strain were acquired. The strain AA5 demonstrated the existence of aerial and substrate mycelium without fragmentation when examined using brilliant field microscopy

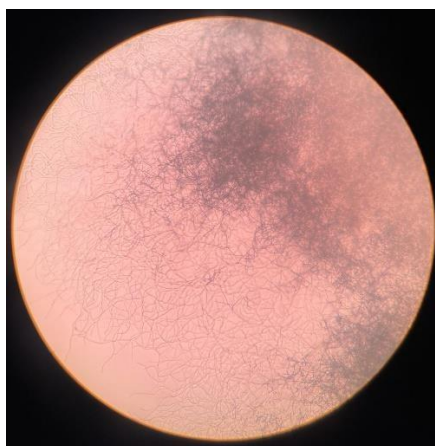


FIG-5.10 Microscopic observation of potential strain

5.4.2 CULTURAL CHARACTERISTICS

Cultural qualities were investigated by including the AA5 strain's development into various ISP (International Streptomyces Protocol) compositions. This includes ISP2, ISP3, and other media. All the media were produced using the methods described by Shirling and Gottlieb (1966). All of the plates were incubated at 350C for 10 days. Culture features include growth type, uniformity, aerial mass colour, mycelium growth, the presence of reverse side pigments, and any available data on soluble pigment production. Table 5.5: Culture characteristics of actinobacterial strains AA5

Medium	Growth of actinobacterial strains
Yeast extract malt extract agar (ISP 2 medium)	Good
Oat Meal agar (ISP 3 medium)	Good
Inorganic salts - starch agar (ISP4 medium)	Good
Glycerol asparagine agar (ISP 5 medium)	Good
Peptone yeast extract iron agar (ISP6 medium)	Moderate
Peptone yeast extract iron agar (ISP6 medium)	No growth

5.4.3 PHYSIOLOGICAL CHARACTERISTICS

For the study of carbon uptake by the AA5 strains, ISP2 media supplemented with a variety of sugar sources—including glucose, lactose, sucrose, and raffinose—was utilised. The effects of nitrogen sources on growth were investigated by introducing the AA5 strains to ISP2 media enriched with peptone, yeast, malt, peptone, casein, and soybean extract. The growth of the plates was examined after 10 days of incubation (Gayathri et al., 2010). The impact of minerals on growth was investigated by inoculating the AA5 strains into ISP2 media enhanced with calcium chloride and manganese chloride. The effects of pH on growth were studied by putting the AA5 strains into ISP2 media that had been altered to various pH values between 6 and 9. After 7–10 days of incubation, the development of each plate was evaluated (table 5.6).

Table 5.6 Physiological characteristics of AA5

CHARACTERISTICS	VARIABLES	ANA5
Utilization of nitrogen source	Yeast	Growth
	Malt	Moderate
	Peptone	No growth
	Soy meal	Good
	casein	No Growth
Utilization of carbon source	Sucrose	Good
	Raffinose	Moderate
	Starch	Good
	Glycerol	Good
Utilization of mineral source	Mncl ₂	Moderate
	Cacl ₂	Good

5.4.5 MOLECULAR CHARACTERIZATION

5.5.4.1 Contig formation of potential strain sequence

>Streptomyces rochei|AA5|

GAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAGTAACACA
GGCATCTGCGAGGTTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCT
ATCAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGG
CCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTA
CGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGG
GAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCT
TAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGA
TCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
GTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGG
CACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAA
GTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC

GGGGGCCCCGCACAAGCGGCGGAGCATGTGGCTTAATTTCGACGCAACGCGAA
 GAACCTTACCAAGGCTTGACATACACCGGAAAACCCTGGAGACAGGGTCCCC
 CTTGTGGTCGGTGTACAGGTGGTGC

5.6 IDENTIFICATION OF ACTINOBACTERIAL STRAIN

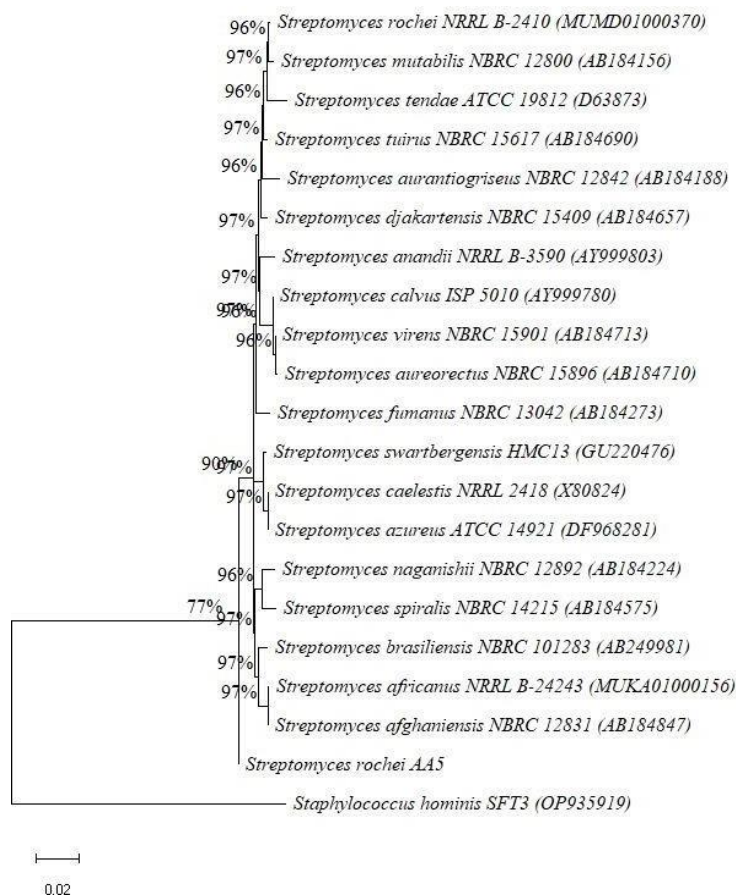


Fig 5.11 Phylogenetic tree

The PCR amplification of 16S r RNA gene of actinobacterial strain AA 5 yielded about 1670 base pair sequence. The BLAST analysis showed 99% similarity to *Streptomyces rochei*(Fig5.11).

Study on *Streptomyces rochei* from ant nest is scanty. The strain AA5 may represent a unique species of *Streptomyces*, according to the findings of the current investigation, which also showed that it produces metabolites with a broad spectrum of activity (against both Gram-positive and Gram-negative bacteria).

To clarify the nature of the active metabolite generated by the isolate AA5, however, more research is necessary. We offer a brand-new approach to investigate

actinomycetes connected with nests from a widespread and global group of insects. Understanding insect–actinobacteria systems at the molecular level requires complex, multidisciplinary approaches. Proper chemical characterization of active metabolites and appropriate bioactivity assays should become the base of ecological research in this area. Insect ecology remains unclear without works at the intersection of microbiology, chemistry and animal studies.

We have found that the potential of this area is much greater than just an exotic source of compounds. The creation of new methods and agents for the prevention of infectious diseases and other related areas may be stimulated by the study of insect-associated actinomycetes and their secondary metabolites.

5.7 ANTI - BIOFILM ASSAY

TABLE 5.7 INHIBITION OF BOFILM FORMATION OF TEST PATHOGENS

Sam ple ID	<i>A. baumannii</i> µg/mL			<i>E. coli</i> µg/mL			<i>K. pneumonia</i> µg/mL			<i>P. aeruginosa</i>		
	200 µ/mL	100 µ/mL	50 µ/mL	200 µ/mL	100 µ/mL	50 µ/mL	200 µ/mL	100 µ/mL	50 µ/mL	200 µ/mL	100 µ/mL	50 µ/mL
S1	30.32	27.09	28.28	27.24	29.64	24.85	21.06	38.95	32.86	23.55	24.77	23.31

Result:

- The crude extract did not show any significant anti – biofilm activity.

5.8 ANTI – OXIDANT ASSAY

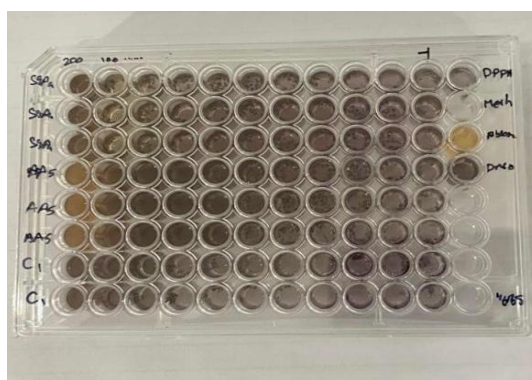


FIG 5.13 ANTI-OXIDANT ACTIVITY OF THE STRAIN AA5

CHAPTER 6

SUMMARY AND CONCLUSION

The ecosystems that have not yet been fully investigated or examined hold great promise for the discovery of bioactive actinobacteria. The goal of the current study was to investigate the antibacterial potential of actinobacteria from ant nest samples. On an ISP2 agar plate, primary 9 cultures were recovered and grown. The agar plug method was used to assess the antimicrobial activity of nine actinobacterial strains that were isolated from ant and termite nests. When used against the pathogens *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholera*, and *Aeromonas caviae*, Actinobacterial strain AA5 demonstrated promising activity (18–27 mm inhibition).

The strain AA5 also demonstrated various significant properties such as, Anti-oxidant activity yielding a significant result, the strain also exhibited properties of anti-biofilm against *E. coli*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*. This proves that the strain AA5 has great potential.

Bioactive metabolites from the strain AA5 were produced by adopting agar surface fermentation and submerged fermentation using ISP2 agar and ISP2 broth, respectively. Antimicrobial activity of agar plug from ISP2 agar and cell supernatant from ISP2 broth was tested against *S.aureus* by agar plug and well diffusion method, respectively for every 24 hours up to 8 days. The strain produced antimicrobial compound earlier on ISP2 agar when compared to ISP2 broth.

Crude antimicrobial from the strain AA5 was produced by adopting agar surface fermentation and extracted by ethyl acetate. Strain AA5 produced 32.2 mg of crude extract per 100 ml of ISP2 agar medium.

Microscopic, cultural and physiological and molecular characteristics the AA5 strain was studied by adopting standard procedures. Based on the studied phenotypic characteristics, actinobacterial strains AA5 was identified as *Streptomyces*. BLAST and phylogenetic tree analysis of AA 5 shows 97% similarity to sequence of *Streptomyces rochei*.

Effect of various medium components on antimicrobial compound production by the strains AA5 was studied by adopting agar plug method. Various sugars and nitrogen

compounds and minerals were found to influence the antimicrobial compound production.

Through HRMS (high resolution mass spectrometry) the atomic mass of the potential compound was determined. Our results concur with the predictions that the ant-actinobacterial symbiosis may be a source of bioactive metabolites for biotechnology and medical applications.

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