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INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF NANOSCIENCE AND NANOTECHNOLOGY

ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF SILVER NANOPARTICLES SYNTHESIZED BY ENDOPHYTIC ACTINOBACTERIUM *MICROMONOSPORA* SP. CPM1

Master's Thesis

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Supervisor

Assoc. Prof. Dr. Hilal AY

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ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **"ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF SILVER NANOPARTICLES SYNTHESIZED BY ENDOPHYTIC ACTINOBACTERIUM *MICROMONOSPORA* SP. CPM1"** prepared by **Mohamed Fouad Mohamed KHALIL**, and supervised by **Assoc. Prof. Dr. Hilal AY**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date 5.9.2022.

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ÖZET

ENDOFİTİK AKTİNOBAKTERİ *MICROMONOSPORA* SP. TARAFINDAN SENTEZLENEN GÜMÜŞ NANOPARTİKÜLLERİNİN KARAKTERİZASYONU VE ANTİMİKROBİYAL AKTİVİTESİ

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Gümüş nanopartikülleri nanobilim, nanoteknoloji ve özellikle nanotıptaki önemli işlevlerinden dolayı ilgi çekmektedir. Gümüş nanopartiküllerini kullanan modern yaklaşımlar, antibakteriyel ve antifungal aktiviteye sahip yeni ilaçların keşfi ve formulasyonu için umut vadetmektedir. Gümüş nanopartikülü sentezi için çeşitli fiziksel, kimyasal ve biyolojik yöntemler kullanılmaktadır. Kimyasal ve fiziksel yöntemlere kıyasla biyolojik yöntemlerle gümüş nanopartikülü sentezi basit, hızlı, ucuz, zararsız ve çevre dostudur. Bu tez çalışmasında, gümüş nanopartikülleri *Polygonum maritimum* L. endofitik dokularından izole edilen ve karotenoid pigment üreten bir aktinobakteri izolatı olan CPM1 tarafından sentezlendi. 16S rRNA gen analizi ile CPM1 suşunun *Micromonospora* cinsinin üyesi olduğu ve *Micromonospora tulbaghiae* DSM 45142^T türü ile yakın ilişkili olduğu tespit edilmiştir. Sentezlenen gümüş nanopartikülleri UV-vis, FTIR, SEM ve SEM-EDX analizleri ile karakterize edilmiştir. UV-vis analizi, gümüş nanopartiküllerinin 439 nm'de maksimum absorpsiyona sahip olduğunu göstermiştir. SEM analizi, *Micromonospora* sp. CPM1 tarafından sentezlenen gümüş nanopartiküllerinin düzenli küresel biçimde ve ortalama 15-30 nm boyutta olduğunu göstermiştir. Gümüş nanopartiküllerinin antimikrobiyal aktivitesi *Bacillus cereus, Staphylococcus aureus, Pseudomonas aeruginosa, Enterobacter faecalis* ve *Candida albicans* patojenlerine karşı gerçekleştirilmiştir. Ayrıca, *Micromonospora* sp. CPM1'in tüm genom dizisi NCBI GenBank'tan indirilerek RAST sunucusunda (https://rast.nmpdr.org/) annote edilmiştir. *Micromonospora* sp. CPM1'in genom boyutu yaklaşık 6.4 Mb ve GC içeriği %73.2 olarak tespit edilmiştir. Protein kodlayan dizilerin sayısı 6101, RNA kodlayan dizilerin sayısı 59 olarak tespit edilmiştir. TYGS sunucusunda (https://tygs.dsmz.de/) gerçekleştirilen tüm genoma dayalı filogenetik analiz, *Micromonospora* sp. CPM1'in *Micromonospora tulbaghiae* DSM 45142^T ile yakın ilişkili olduğunu doğrulamıştır. *Micromonospora* sp. CPM1'in antiSMASH sunucusunda (https://antismash.secondarymetabolites.org/#!/start) sekonder metabolit kodlayan genlerin tespiti için gerçekleştirilen kapsamlı analizi, *Micromonospora* sp. CPM1'in terpen, lantipeptid, poliketid, ribozomal olmayan peptid ve siderofor kodlayan 14 biyosentetik gen kümesine sahip olduğunu göstermiştir. Sonuç olarak, *Micromonospora* sp. CPM1 tarafından sentezlenen gümüş nanopartiküllerinin farmasötik endüstride uygulama alanı bulabilecek gümüş nanopartiküllerinin sentezi için verimli bir kaynak olduğu değerlendirilmektedir.

Anahtar Sözcükler: *Micromonospora*, Gümüş nanopartikülleri, Genom analizi, Antimikrobiyal aktivite

ABSTRACT

ANTIMICROBIAL ACTIVITY AND CHARACTERIZATION OF SILVER NANOPARTICLES SYNTHESIZED BY ENDOPHYTIC ACTINOBACTERIUM *MICROMONOSPORA* SP. CPM1

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Silver nanoparticles (AgNPs) have attracted attention due to their important role in nanoscience and nanotechnology, especially in nanomedicine. Modern approaches using AgNPs show promise for the discovery and formulation of a variety of new drugs with antibacterial and antifungal activities. Various physical, chemical, and biological methods are used to synthesize AgNPs. The synthesis of AgNPs by biological methods is simple, fast, inexpensive, nontoxic, and environmentally friendly comparing to chemical and physical methods. In this thesis study, silver nanoparticles were synthesized by a carotenoid-producing actinobacterium, CPM1, isolated from endophytic tissues of *Polygonum maritimum* L. Strain CPM1 was identified as a member of the genus *Micromonospora*, being most closely related to *Micromonospora tulbaghiae* DSM 45142^T, by the 16S rRNA gene analysis. The synthesized AgNPs were characterized by UV-vis, FTIR, SEM and SEM-EDX analyses. The UV-vis analysis showed that the AgNPs have maximum absorbance at 439 nm. The SEM analysis revealed that the nanoparticles synthesized by *Micromonospora* sp. CPM1 are regularly spherical and 15-30 nm in average size. The antimicrobial activity analysis of the silver nanoparticles was performed against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter faecalis* and *Candida albicans* pathogens. The silver nanoparticles synthesized by *Micromonospora* sp. CPM1 inhibited the growth of *Bacillus cereus* and *Candida albicans*. In addition, the whole-genome sequence of *Micromonospora* sp. CPM1 was downloaded from NCBI GenBank and annotated on the RAST server (https://rast.nmpdr.org/). The genome size of strain *Micromonospora* sp. CPM1 was about 6.4 Mb and GC% was 73.2%. The total number of protein-coding and RNA-coding sequences were determined as 6101 and 59, respectively. A whole-genome-based phylogenomic analysis conducted on the TYGS server (https://tygs.dsmz.de/) confirmed that *Micromonospora* sp. CPM1 was closely related to *Micromonospora tulbaghiae* DSM 45142^T. A comprehensive annotation for secondary metabolite-coding gene clusters on the antiSMASH server (https://antismash.secondarymetabolites.org/#!/start) revealed that the genome of *Micromonospora* sp. CPM1 encodes for 14 biosynthetic gene clusters coding for terpenes, lantipeptides, polyketides, nonribosomal peptides and a siderophore. In conclusion, it is considered that *Micromonospora* sp. CPM1 is a promising source to synthesize silver nanoparticles with potential applications in pharmaceutical industry.

Keywords: *Micromonospora*, Silver nanoparticles, Genome analysis, Antimicrobial activity

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Mohamed Fouad Mohamed KHALIL

CONTENTS

SYMBOLS AND ABBREVIATIONS

AgNPs	: Silver nanoparticles
NPs	: Nanoparticles
NNI	: National Nanotechnology Initiative
CNTs	: Carbon Nanotubes
QDs	: Quantum dots
nm	: Nanometer
EDS	: Energy-Dispersive Spectroscopy
SEM	: Scanning Electron Microscopy
XRD	: X-Ray Diffraction
AFM	: Atomic Force Microscopy
UV	: Ultraviolet-Visible Spectroscopy
FTIR	: Fourier Transform Infra-Red
G+C	: Guanine and Cytosine

FIGURES LEGENDS

1. INTRODUCTION

The design, development, and implementation of structures, devices, and systems at the nanoscale by manipulating form and size is referred to as nanotechnology (1 nm to 100 nm). It is an exciting new field of study with potential applications in science and technology, notably in the production of new materials. Nanoparticles with distinguishing characteristics that make them helpful in materials science and biology are being created. Nanotechnology is considerably improving, if not revolutionizing, several technological and business areas, including information systems, homeland security, medical, transportation, energy, food security, and environmental research (Ghernaout et al., 2018).

Nanoscience is the study of the unique properties of materials with a diameter of 1 to 100 nanometers, and nanotechnology is the use of such research to change or build innovative items. Nanomaterials can be made thanks to the capacity to modify structures at the atomic level. Nanotechnology, in a nutshell, is the creation of intelligent things and functional systems at the atomic or molecular level. Unlike other large-scaled engineered objects and systems, which are governed by classical physics and chemistry, nanomaterials are governed by quantum mechanics. Nanostructured materials have distinct optical, electrical and/or magnetic characteristics at the nanoscale and may be used in a wide range of applications, including electronics and medicine. Nanomaterials are distinguished by their large surface area (Bayda et al., 2019).

Because it encompasses chemistry, physics, engineering, biology, electronics and photonics, materials science, medicine, and other fields, nanotechnology is a multidisciplinary platform. Each of these fields has its own terminology and scientific methodology. In order to use nanotechnology and nanoscience in medicine and healthcare, researchers developed scientific methodologies. The word "nanomedicine" refers to the use of nanotechnologies and nanoscience in medicine and healthcare. In specifically, nanomedicine utilizes nanoscale technology and nano-enabled techniques in disease prevention, diagnostics, monitoring, and therapy. Nanotechnologies offer enormous promise in imaging methods and diagnostic instruments, tissue-engineered constructs, drug delivery systems, implants, and pharmacological therapies. Cancer, musculoskeletal ailments, cardiovascular diseases, mental and neurological diseases, bacterial and viral infections, and

diabetes are among the diseases for which nanotechnologies have advanced therapy (Lombardo et al., 2019).

The integration of nanotechnology with biotechnology to produce biological synthesis and ecologically benign technology for the manufacture of nanomaterials employing green chemistry principles and eco-friendly techniques is referred to as bio-nanotechnology. Although nanoparticles are known to enable cleaner and safer applications of various technologies, the existing physiochemical methods for their synthesis (precipitation, solgel technique, hydrothermal synthesis, chemical vapor deposition, and microemulsion) are time consuming, hazardous, expensive, environmentally unfriendly, and require high temperatures, pH, and/or pressure levels for synthesis (Abdel-Aziz et al., 2018).

1.1. Nanotechnology

According to the National Nanotechnology Initiative (NNI) and the National Science Foundation, the size range of nanomaterials is between 1-100 nm, where unique magnetic, optical, structural, and electrical features are formed. Individual atoms, molecules, and bulk materials have fundamentally different physicochemical and biological properties than materials at this scale. Larger surface area per unit volume of nanoparticles, for example, leads to increased surface activity, which enhances the speed of chemical reactions and catalysis, and hence the efficiency of many processes. Nanostructures include nanorods, nanobelts, nanowires, nanotubes, quantum dots, nanoribbons, nanofibers, nanoparticles, and hollow spheres. To arrange nano-sized things, many classification algorithms have been created. Nanomaterials are classified based on their size and composition. These substances have been proposed in one dimension (e.g., thin films, layers, and surfaces), two dimensions (e.g., graphene sheets and nanowires that may be coiled into nanotubes), and three dimensions (e.g., fullerenes, nanoparticles, fullerenes, graphite sheets, dendrimers, and quantum dots). Based on their composition, nanomaterials are categorized into three types: single phase solids (amorphous particles, crystalline, and layers), multiphase solids (matrix composites, coated particles), and multiphase systems (e.g. aerogels, colloids, ferrofluids). Nano-sized substances include ceramics, polymers, metals, and composite materials (Mushtaq and Pearce, 2018).

1.1.1. Nanofabrication and Nanomanufacturing

The three basic strategies for the synthesis of diverse nanomaterials are bottom-up, top-down, and hybrid methods. The top-down process involves reducing a bulk material to the required nano-sized form by removal, controlled etching, and stacking of the original component. Top-down lithography includes E-beam lithography, optical lithography, block co-polymer lithography, soft and nanoimprint lithography, and scanning probe lithography. Bottom-up materials are built from the nanoscopic scale by directing the arrangement of molecules, atoms, macromolecules, or supramolecules. A multitude of techniques are used in this procedure, including sol-gel nanofabrication, atomic layer deposition, molecular self-assembly, chemical and physical vapor phase deposition, and DNA scaffolding. Combining top-down and bottom-up approaches is an alternative tactic (hybrid method) for dealing with technical challenges that occur when using these two methodologies (Bechelany, 2022).

1.1.2. The Most Common Forms of Nanoparticles

The size, shape, physical, and chemical characteristics of nanoparticles may be utilized to categorize them. The sections that follow discuss some of the most common kinds of nanoparticles (Sim and Wong, 2021):

- Liposomes: Liposomes are spherical vesicles made up of lipid bilayers and have particle sizes ranging from 30 nm to several microns. Liposomes can be utilized as drug delivery vehicles for nutrients and pharmaceutical medications, such as lipid nanoparticles in DNA and mRNA vaccines. Liposomes are made by disrupting biological membranes, which can be done with sonication (Sim and Wong, 2021).
- Micelles: A typical micelle aggregates in water, with the hydrophilic "head" portions in contact with the solvent and the hydrophobic single-tail sections sequestered in the micelle core. Micelles can be utilized to store hydrophobic therapeutic compounds because they spontaneously aggregate and self-assemble into spherical vesicles with a hydrophilic outer monolayer and a hydrophobic core in water. They can be used as contrast agents, imaging agents, delivery agents, and pharmaceutical agents, among other things (Sim and Wong, 2021).

- Dendrimers: Dendrimers are branched polymeric molecules with a high degree of organization. Exterior functional groups, which might be cationic, anionic, or neutral terminals, make up the structure. Dendrimers are highly biodegradable and bioavailable because drugs can be enclosed within the inner space or linked to the surface groups. Dendrimers are a promising imaging and medication delivery method (Sim and Wong, 2021).
- Carbon nanotubes (CNTs): Carbon nanotubes (CNTs) are carbon tubes with a diameter measured in nanometers. Because of their large exterior surface area, these nanotubes can attain significantly high loading capacities as drug carriers. They can be single-walled or multi-walled, or formed of many concentrically interconnected nanotubes. As a result, carbon nanotubes (CNTs) are used as contrast agents in imaging and as biological sensors (Sim and Wong, 2021).
- Quantum dots: Quantum dots (QDs) are semiconductor particles with optical and electrical properties that differ from larger particles because matter obeys quantum physics rather than classical physics at this size. They can be employed in a variety of biomedical applications, including drug administration and imaging (Sim and Wong, 2021).
- Metallic nanoparticles: Silver, iron oxide, and gold nanoparticles are examples of metallic nanoparticles. Metal precursors are used to make these nanoparticles, which can be made chemically, electrochemically, or photochemically. Metallic nanoparticles are employed as drug delivery vehicles, laser-based therapy agents, imaging contrast agents, and optical biosensors, among other applications (Sim and Wong, 2021).

1.1.3. Synthesis of Nanoparticles

Researchers are working toward a safer and cleaner biosynthesis of nanoparticles based on green chemistry principles since physical or chemical approaches for nanoparticle biosynthesis are hazardous. Nanoparticle preparation is both environmentally friendly and time-consuming. For the creation of nanoparticles, physical methods require electrical or thermal energy, whereas chemical methods use extremely dangerous substances. Many chemically manufactured nanoparticles are

also unsuitable for biological uses due to chemical contamination by extremely toxic and harmful substances (Khan et al., 2019).

As a result of the expansion of 'green chemistry,' researchers are focusing on microbial compounds with stabilizing and bio-reducing properties for the safe synthesis of biocompatible and eco-friendly nanoparticles that can be used in gene therapy, cancer therapy, biosensors, and as antibacterial agents, for example using microbial pigments in nanoparticle synthesis. Because of their capacity to produce multicolor hues and their quick development on low-cost medium, microbial pigments are used in a variety of industrial applications. In comparison to synthetic colors, they are also non-hazardous and environmentally beneficial (Venil et al., 2020).

1.1.3.1. Green Synthesis Method of Metallic Nanoparticles

Green nanoparticle (NP) production utilizing live cells is a promising and unique method in bionanotechnology. In order to synthesize NPs, chemical and physical methods are utilized; on the other hand, biological approaches are preferable because of their eco-friendly, clean, safe, cost-efficient, simple, and effective sources of high productivity and purity. The use of poisonous and hazardous compounds, as well as the inclusion of external stabilizing, reducing, or capping agents, are not necessary for the green synthesis of NPs. Various biological organisms, including bacteria, fungus, yeast, algae, actinobacteria, and plants, may synthesize NPs either intracellularly or extracellularly. Several methods for increasing the productivity of nanoparticles of various size, shape, and stability have recently been devised (Salem and Fouda, 2021).

1.1.3.2. Bacterial-Mediated Synthesis of Nanoparticles

Bacteria are favoured for nanoparticle synthesis due to the low conditions required, ease of purification, and high amount of yield. As a result, these prokaryotic microorganisms have become the most frequently researched organisms, so-called "nanomaterials factory." *Bacillus thuringiensis* has recently been employed to produce Ag-NPs with sizes ranging from 43.52 to 142.97 nm (Iqtedar et al., 2019).

1.1.3.3. Synthesis of Nanoparticles by Fungi and Yeasts

Because of the great efficiencies of fungal metabolites in fabricating various NPs, fungi have been widely employed for NPs biosynthesis. Fungi are a recent addition to the list of microorganisms utilized in the production of nanoparticles. The

extensive usage of many fungal species can be due to their capacity to release large amounts of enzymes or proteins, as well as the fact that they are simpler to trade in the laboratory. Fungi have piqued the interest of researchers because they have advantages over other species in the synthesis of metallic NPs. The ease of scaling up and downstream handling, economic feasibility, and the presence of mycelia presenting a greater surface space are all important considerations. Fungi have also gained greater attention in the exploration of biosynthesis of metal or metal oxide nanomaterials because of their tolerance and metal bioaccumulation capability. Because of the breadth of fungi, there is a divide in favor of using them in the synthesis of nanoparticles. Since fungi are particularly effective secretors of extracellular enzymes or proteins, large-scale enzyme manufacturing is feasible. Another advantage of employing biomass for the green method aided by fungal cells or metabolites to manufacture metallic nanomaterials is its economic feasibility and livability (Ramanathan and Aqra, 2019).

1.1.3.4. Synthesis of Nanoparticles by Viruses

The employment of viruses in biological synthesis of nanoparticles is a revolutionary technology that has produced inorganic nanomaterials such as cadmium sulphide (CdS), iron oxide (Fe_2O_3), silicon dioxide (SiO_2), and zinc sulphide (ZnS). Semiconductor nanoparticles such as ZnS and CdS have piqued the interest of the green chemistry and electronics industries, and methods for their manufacture have been extensively researched. Over the preceding decade, the use of entire viruses to generate quantum dots was investigated. For ZnS surfaces, the virus has a precise detection moiety (Ramanathan and Aqra, 2019).

1.1.3.5. Synthesis of Nanoparticles by Plant Extracts

Copper and copper oxide nanoparticles (CuONPs) have been synthesized using plant materials such as soya, *Aloe barbadensis* Miller, and *Tridax procumbens* leaf cell extract. Plant-mediated biological synthesis of ZnO-NPs in *Acalypha indica*, *Parthenium hysterophorus*, *Ficus benghalensis*, *Sapindus rarak*, *Passiflora foetida*, and *Zingiber officinale* has recently been done. Several studies have been published on the biological synthesis of nanoparticles (Au, Ag, ZnO, Fe, and so on) utilizing liquid extracts of various plant sections (Marslin et al., 2018).

1.1.3.6. Algal-Mediated Synthesis of Nanoparticles

Algae are saltwater microorganisms which have been shown to not only absorb heavy metals from their surroundings, but also to create metallic nanoparticles. For example, dried algal cells of *Chlorella vulgaris* were cultivated in the presence of reduced tetrachloroaurate ions to produce gold nanoparticles. The capacity of *Fucus vesiculosus*, a brown alga, to bioreduce and biosorb Au (III) ions is being investigated. Biological reduction with *Fucus vesiculosus* can be expanded as an alternative environmentally acceptable process for recovering Au from microelectronic waste leachates and dilute hydrometallurgical mixtures (Ramanathan and Aqra, 2019).

1.1.3.7. Synthesis of Nanoparticles by *Actinobacteria*

Nanomaterials with outstanding pharmacodynamic and pharmacokinetic properties that are synthesized in an environmentally friendly method and using green chemistry principles have become increasingly important in recent years. Microorganisms have the ability to produce environmentally benign and biocompatible NPs both intracellularly and extracellularly under ambient circumstances without contaminating the environment (Lee and Jun, 2019). Actinobacteria are one of the most important natural product producers for medication development. Actinobacteria are Gram-positive filamentous bacteria with a high G+C DNA content. They are found in both aquatic and terrestrial habitats and are one of the biggest bacterial phyla (Musiol-Kroll et al., 2019).

1.2. General Characteristics of Actinobacteria

They are Gram-positive bacteria with a high guanine and cytosine content (>50%) in their genome. They can be found in large numbers in both aquatic and soil sediments, where they help decompose organic materials. These bacteria are responsible for the characteristic aroma of freshly exposed, damp soil due to the synthesis of volatile geosmin. Geosmin is created in soil by a range of microbes such as *Cyanobacteria* and *Actinobacteria* that break down organic matter. It can be found in a variety of fruits, vegetables, and even seafood. It can enhance the flavor of food in some circumstances, but it can also be an annoyance in others (Hazarika and Thakur, 2020).

Actinobacteria have the most morphological diversity, with filamentous degrees of organization similar to filamentous fungi. Actinobacteria have a wide

range of morphologies, from coccoid, fragmenting hyphal forms to extremely distinct branching mycelium forms. Many of these bacteria develop UV (ultraviolet) light and dehydration resistant outer spores (van Bergeijk et al., 2020).

1.3. Isolation of Actinobacteria from Various Environments

1.3.1. Acidophilic Actinobacteria

According to numerical phenetic data, acidophilic Actinobacteria form two distinct aggregation taxa (strictly acidophilic cluster groups and the neutrotolerant acidophilic); members of both groups share morphological and chemotaxonomic characteristics. Acidotolerant Actinobacteria with antifungal and/or plant growth-promoting activity has a lot of potential as new biofertilizers and/or biocontrol agents. Actinobacteria are interesting bio-agents for sustainable agricultural production since they can survive in various adverse environmental circumstances by generating spores. They grow in a pH range of roughly 3.5 to 6.5, with optimal rates at pH 4.5 to 5.5, and are prevalent in terrestrial settings such as mine drainage soil and acidic woodland (Malik et al., 2020).

1.3.2. Thermophilic Actinobacteria

Thermophiles are microorganisms that flourish in warm settings. Based on their ideal development temperature, they are classed as moderate thermophiles (50–60 °C), severe thermophiles (60–80 °C), and hyperthermophiles (> 80 °C). They can be found in a range of environments, including terrestrial hot springs, deep-sea hydrothermal vents,and other harsh environments including volcanic areas, tectonically active faults, and recycling waste wastes like compost piles and deep organic landfills (Liu et al., 2020).

1.3.3. Endophytic Actinobacteria

Plant-associated bacteria have recently been discovered to create chemicals with significant medicinal potential. Microorganisms live inside plant tissues, most of which are in a symbiotic relationship, can include fungus and bacteria, including actinobacteria. Endophytes are organisms that live in the interior of plants and complete their life cycle within the host plant, usually without causing any harm. They colonize root, stem, petioles, fruit, buds, leaf segments, weed inflorescence, seeds, as well as dead and hollow hyaline cells, to find a unique niche in plants (Singh and Dubey, 2018).

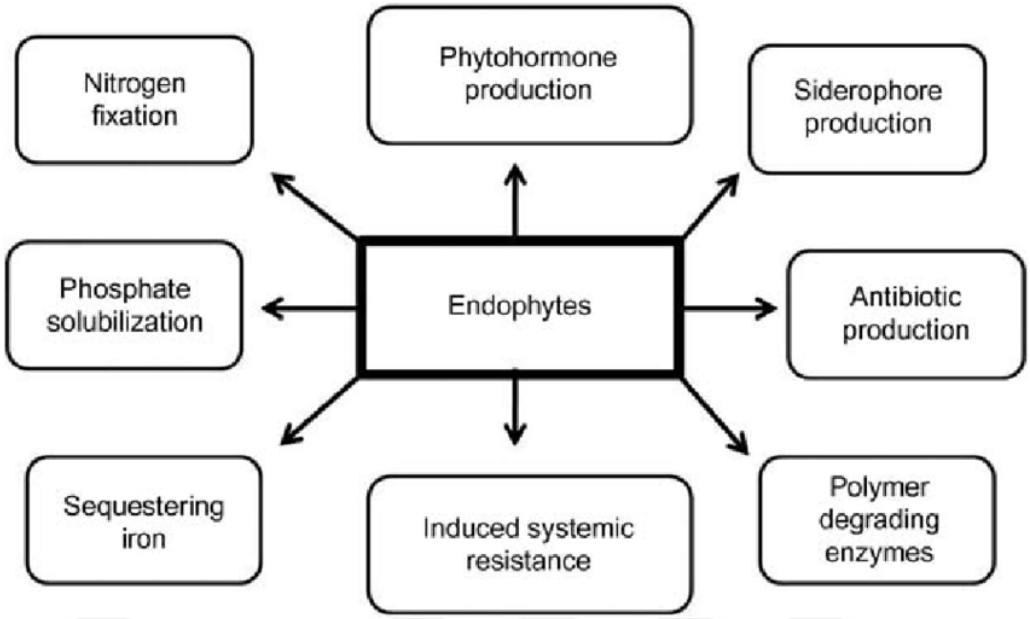

Figure 1.1. Endophytic actinobacteria linked with plants have a high metabolic capacity (Ashok Ganapathy et al., 2018).

1.3.4. Halophilic Actinobacteria

Halophilic Actinobacteria are organisms that can survive in saturated salt habitats; they are salt-loving creatures because of their ability to adjust to the osmotic pressure of the environment. Low temperature, high pressure, a lack of light, and changeable salinity and oxygen concentration characterize the deep sea, making it an extreme and unique environment. Recently, the isolation and utilization of Actinobacteria from typical settings for novel chemicals has resulted in the rediscovery of previously unknown molecules (Abdelshafy Mohamad et al., 2018).

1.3.5. Symbiotic Actinobacteria

Nitrogen (N) is a macro-element that is necessary for plant growth and development. The majority of this requirement is met by legumes' symbiotic relationship with rhizobia, in which air nitrogen is transformed to ammonium in root nodules and transferred within the plants as glutamine. Plant symbionts other than rhizobia have been demonstrated in numerous studies to increase growth and give biotic and abiotic stress tolerance to their host plants. Endophytes, for example, are a vast microbial resource that can colonize plant tissues without creating disease symptoms (Meena et al., 2021).

1.3.6. Endosymbiotic Actinobacteria

Since the beginning of time, the most important survival strategies for living species have been finding food and multiplying. They also had to protect themselves from stressful situations and invading predators. The appearance of eukaryotes and the intracellular establishment of prokaryotes inside eukaryotic cells were crucial events in the prokaryotic world because eukaryotes supplied all their intracellular prokaryotes, predominantly bacteria, required to live a healthy and productive existence. They evolved into endosymbionts in both circumstances. Endosymbiosis is defined as any creature living within the body or cells of another (Kwaśna et al., 2021).

1.3.7. Gut Actinobacteria

The gut microbiota is made up of several commensal microbial species, including quadrillion viruses, 100 trillion bacteria, fungi, parasites, archaea, and yeasts, and has a total biomass of around 1 kg and over 3 million genes. The various gastrointestinal regions are distinguished by distinct bio-compartmentalization and a distinct and stable microbial ecology. The microbiota in the human gut plays a vital role in human health. It helps with metabolic activities, immune system education, and pathogen defence. Actinobacteria are one of the four major phyla of the gut microbiota (Figure 1.2), and despite making up a small percentage of the population, they are critical for gut homeostasis. Symbiotic interactions, or close relationships between Actinobacteria and invertebrates and vertebrates, are critical for life and reproduction, since they aid in nutrition, growth, detoxification of specific substances, and defence against harmful bacteria. The *Actinobacteria* phylum, which has been linked to both chronic and infectious disease and plays a critical role in the metabolism of pharmaceutical, nutritional, and endogenous substances, is found in the distal intestine of 90% of adult people around the world (Binda et al., 2018).

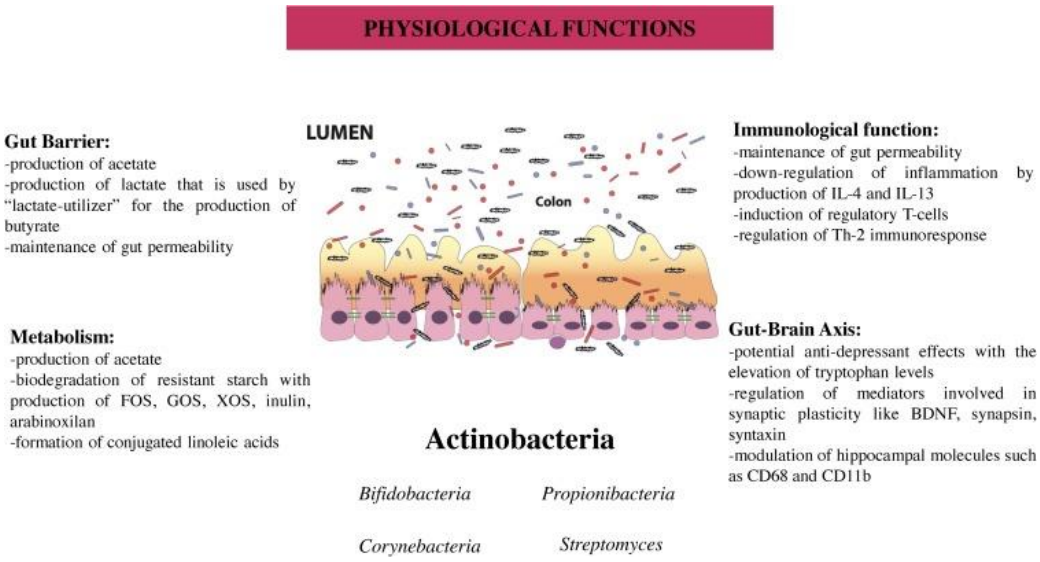

Figure 1.2. The gut Actinobacteria phylum is divided into four major groups: *Propionibacteria, Bifidobacteria, Corynebacteria, Streptomyces* (Binda et al., 2018).

1.4. Applications of Actinobacteria

Actinobacteria are well-known for producing primary and secondary metabolites, which have important applications in a variety of fields. Biotechnology has also been classified according to the service and benefit it provides. Furthermore, the broad metabolic potential of actinobacteria has sparked a lot of interest in these bacteria from a biotechnological standpoint. Aside from antibiotics, for which actinobacteria are well-known, these bacteria have been found to produce a variety of metabolites that are beneficial in a variety of environmental, medical, and industrial concepts. There are some actinobacterial genera provide a significant portion of the antibiotics on the market. They create immunomodulators that boost immune response and enzyme inhibitors that can be used to treat cancer. Pesticides, hydrocarbons, and aliphatic and aromatic compounds can all be degraded by actinobacteria (Figure 1.3) (Enany, 2018).

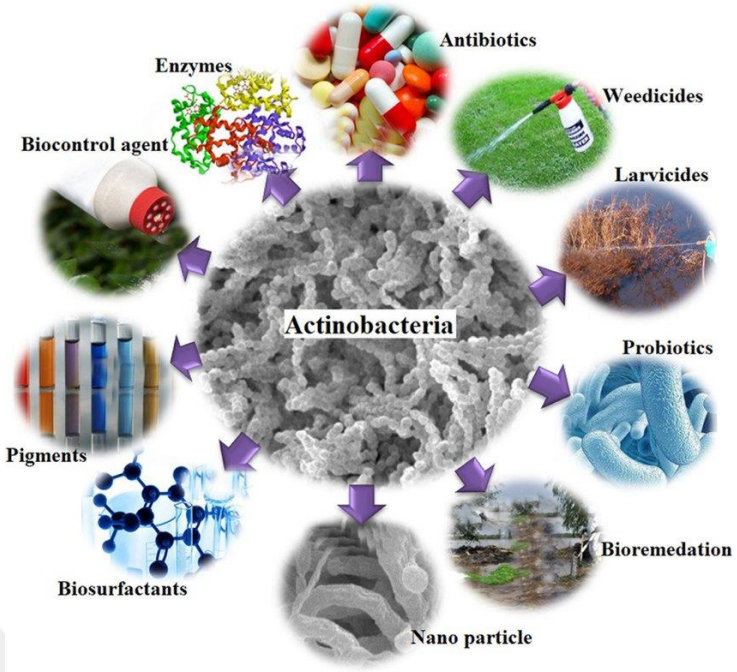

Figure 1.3. Potential application of Actinobacteria in agriculture and industries (Deka et al., 2020)

1.4.1. Antimicrobials

Actinobacteria play an important role in the production of many drugs that are vital to our health and nutrition. Antibiotic production by actinobacteria is well known. The rising global burden of antibiotic resistance, along with a decline in the discovery of new antimicrobial compounds, requires the search for novel and efficient antimicrobial metabolites from previously unknown ecological niches. Recent increases in multidrug-resistant pathogenic microorganisms have necessitated the quest for novel medicines that are effective versus multidrug-resistant pathogens. Actinobacteria, primarily the genera *Micromonospora* and *Streptomyces*, are believed to be the source of over 80% of the world's antibiotics. Actinobacteria have immense potential; there are around 16,000 bioactive chemicals known from actinobacteria today, 14,500 of which are antibiotics. *Streptomyces* is the most prolific actinobacterial genus, with 12,400 identified bioactive compounds (11,000 antibiotics); other actinobacteria have 3600 bioactive compounds. As a result, streptomycetes are the most widely employed antibiotic-producing organisms in the pharmaceutical sector (Verma et al., 2018).

1.4.2. Enzymes

Extracellular enzymes released by actinobacteria are involved in the degradation of chemical compounds and biopolymers such as the ubiquitous aminopolysaccharides, chitosan and chitin. While chitinolytic enzymes may be found in many kingdoms of life, actinobacteria are known to be especially good decomposers of chitinous material, with multiple species possessing outstanding chitin and chitosan degradation gene sets. Actinobacteria play an important role in carbon cycling, especially in the solubilization of insect cuticles and crustacean shells and solubilization of plant and fungal cell walls. They release a diverse spectrum of extracellular proteins, which serve as a source of industrially important enzymes. Actinobacteria may produce vital enzymes such as amylase, protease, cellulase, and lipase. Because of their higher stability and substrate specificity, actinobacterial enzymes have been demonstrated to be more necessary and useful than enzymes from other sources in several investigations. Actinobacterial genera secrete amylases to the cell surface, which assists in extracellular digestion. Lipase is particularly significant in biotechnological applications such as food industry, fermentation, and textile to paper industries due to its ability to breakdown starch. Because of their use in leather tanning, detergent, silk, and food, actinobacterial alkaline and neutral proteases play an important commercial role (Salwan and Sharma, 2018).

1.4.3. Bioherbicides

Weeds are serious agricultural risks because they deplete water, sunlight, soil nutrients, growth area and attract pests. Weeds, if left unchecked, will severely lower yield and cause major losses to farmers. Hand weeding, tillage, and synthetic herbicides are used to control the weed. Manual and mechanical weeding are both time-consuming and expensive. Synthetic herbicides, on the other hand, are more effective, but they also have the potential to affect soil ecology, groundwater, crops, and humans who consume them. Bioherbicides are phytotoxins, phytopathogens, and bacteria that prevent weed development. Bacteria, fungi, and viruses are currently exploited as bioherbicides, although their marketing potential and broad target range of weeds remain unexplored. The actinobacteria are a major genus that has been extensively explored for their bioactive potential. Actinobacterial genera are significant microbial producers of bioherbicides or phytotoxins. Bioherbicides,

particularly microherbicides, are regarded to be effective weed control agents and are becoming increasingly desirable for study and deployment as global environmental awareness rises. *Streptomyces saganonensis* synthesizes herbicidines and herbimycins, which are used to control monocotyledonous and dicotyledonous weeds. Anisomycin has the potential to impair plants' capacity to manufacture chlorophyll. As a herbicide developed from *Streptomyces toyocaensis*, anisomycin formed the chemical basis for the improvement of synthetic commercial herbicides like methoxyphenone. Methoxyphenone and anisomycin have been demonstrated to be effective against barnyard grass and crabgrass, respectively. Anisomycin has the potential to reduce plants' ability to produce chlorophyll (García-Delgado et al., 2019).

1.4.4. Probiotics

Probiotics are living microorganisms providing health advantages when taken, typically through enhancing or replenishing gut flora. Probiotics are typically regarded safe to eat, although in rare circumstances, bacteria-host interactions and unpleasant side effects may occur. There is minimal proof that probiotics provide the health benefits that are promised. Marine actinobacterial genera have received attention for their use as probiotics, despite their importance in a variety of biotechnological applications. Actinobacteria have a high ability to breakdown macromolecules such as starch, proteins, and a variety of polymeric substances, making them effective against shrimp pathogenic *Vibrio* spp. (Menendez and Carro, 2019).

1.4.5. Vitamins

Many vitamins can be effectively produced commercially by microorganisms; microbial production of ascorbic acid, vitamin B12, riboflavin, and β-carotene is thought to be more economically preferable. Vitamin B12 can be generated in nature by Actinobacteria or other bacteria. The separation of vitamin B12 from actinobacteria fermentations prompted significant interest in the prospect of vitamin production through microbial fermentations. Cobalt salts in the medium appear to function as a precursor for all actinobacteria to produce vitamins. However, because cobalt is a potent bactericidal agent, this precursor should be utilized with caution. Actinobacteria have been shown to produce a variety of water-soluble vitamins, with a focus on thiamine and the pteroylglutamic acid derivative that supports the

development of specific strains of *Leuconostoc citrovorum* and coenzyme A (Shah and Dwivedi, 2022).

1.4.6. Pigments

Natural products derived from microorganisms are employed in the creation of pharmaceuticals, food and feed additives, and other commercial things. The actinobacterial pigments are important because chemically manufactured dyes have several limitations, such as the use of extremely toxic chemicals in their manufacturing, which raises worker safety problems and results in the formation of hazardous wastes. Actinobacterial genera have long been known to produce pigments, which may be red, blue, yellow, brownish, distinct brown, orange, pink, greenish brown, or black, depending on the medium used, the strain, and the age of the culture. Actinobacteria are distinguished by the synthesis of diverse extracellular and intracellular colors on synthetic or natural media, which is regarded as a key cultural feature in characterizing the organisms. These actinobacterial pigments are widely employed in a variety of sectors, including food, paper, agricultural processes, cosmetics, water science, research, clothing, and other technologies. *Streptomyces* produce pigments that can be endopigments (those that are attached to specific cell structures) or exopigments (those that are not connected to specific cell structures) (Balagurunathan et al., 2020).

1.4.7. Bioremediation

In the last two decades, ecofriendly approaches for cleaning up contaminated settings employing various microbial species have emerged. This process, known as bioremediation, is typically thought to be less invasive and more beneficial to soil functioning than traditional physicochemical treatments. The bioremediation technique promotes the growth of certain microorganisms that exploit the chemical pollutants that have been released as a source of food and energy. When assessing the high discharge of anthropogenic toxins into the environment, bioremediation as a sustainable method becomes crucial. Among the degraders, actinobacterial genera are the most numerous. They can breakdown high-complexity polymers and play a vital role in organic carbon recycling. According to certain findings, *Streptomyces* flora may play a significant part in the hydrocarbon breakdown process (Usmani et al., 2021).

1.4.8. Nanoparticle Synthesis

Nanoparticles are of great scientific interest because they bridge the gap between solid materials and atomic or molecular structures. Although chemical procedures are inexpensive in terms of volume, they have a number of drawbacks, including the use of toxic solvents, contamination from precursor chemicals, and the generation of hazardous by-products. Natural or biological nanoparticles have a significant role in nature. In contrast to chemical synthesis, nanoparticles produced by biological systems such as fungi, bacteria, and plants are both cost-effective and environmentally beneficial. The uniqueness of actinobacteria as a potential microorganism due to the production of different secondary metabolites provides these organisms widespread attention as effective 'micronanofactories.' Green biosynthesis of actinobacterial nanoparticles, also known as green nano-actinobacteriology, is a new topic of study aimed at generating low-cost, high-quality production of eco-friendly and dependable goods with a wide range of uses. The developing nano-bioscience interaction has yielded promising breakthroughs in the fabrication of nanoscale materials for many purposes. As a result, there is an urgent need to create nontoxic, high-yielding, low-cost, and ecologically friendly technologies for producing metallic nanoparticles. Actinobacteria, in reality, are prolific producers of nanoparticles with a variety of biological activities, including antifungal, antibacterial, anticancer, antimalarial, antibiofouling, antiparasitic, and antioxidant properties. Actinobacterial genera have been examined as prospective "nanofactories" for the production of clean and safe silver and gold nanoparticle production processes (Edison and Pradeep, 2020).

1.5. Synthesis of Silver Nanoparticles

Because of its unique features and applications in several domains, the synthesis of nanoparticles from multiple noble metals such as palladium, tin, copper, silver, and gold, among others, has recently gained increased attention. Furthermore, silver nanoparticles are useful in the pharmaceutical industry because they operate as antibacterial agents with less harmful effects. Silver particles with regular dispersions are useful in the fabrication of various electronic circuits in industrial applications. Various synthetic methods for the manufacture of silver nanoparticles have been described over the years, including physical, chemical, and photochemical processes.

However, the majority of the existing techniques are costly and non-eco-friendly, i.e. damaging to the environment (Behzad et al., 2021).

Various parameters, like synthesis methods, temperature, dispersion agent, surfactant, and so on, have a significant impact on the quality and quantity of generated nanoparticles and, as a result, their properties. It is also worth noting that the primary goal for these silver nanoparticles was not only to synthesize in the nano-range, but also to demand easy, eco-friendly, and cost-effective synthesis of the nanoparticles (Figure 1.4). Because of their appealing physicochemical features, silver nanoparticles are in high demand (Yin et al., 2020).

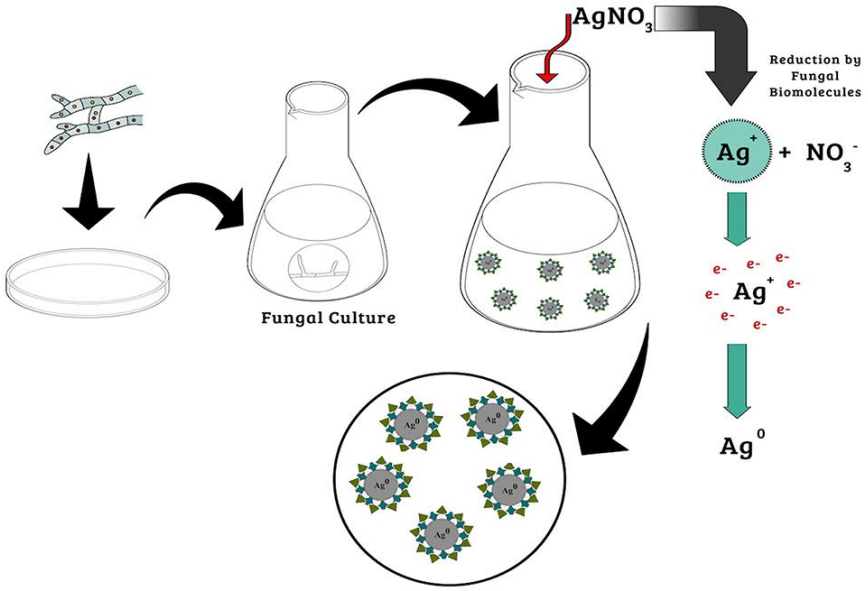

Figure 1.4. Mechanisms of biogenic synthesis of silver nanoparticles (Guilger et al., 2019)

1.6. The History of Using Silver Nanoparticles in Medicine

Although several silver formulations have been prescribed and sold to treat various medical conditions over the last century, the majority of the compounds, including those with outstanding antimicrobial or anticancer properties, are still in the initial phases of evaluation, i.e. *in vitro* studies, and may not make it to human research. Unlike other heavy metals, there is no evidence that silver is a cumulative poison, although its levels in the body can accumulate over time, creating adverse consequences. Silver has a long history of use in a variety of forms and for a variety of purposes. Antibacterial capabilities of silver have been utilized for ages to

fumigate drinking water by storing it in silver vessels. Anecdotal evidence suggests that nanosilver was used in ancient Egypt and Rome (Figure 1.5). Silver plates were employed by the Macedonians to aid wound healing, while Hippocrates used silver to treat ulcers. In 1520, Paracelsus utilized silver orally and silver nitrate as a caustic to cure wounds, a procedure that is still practiced today (Medici et al., 2019).

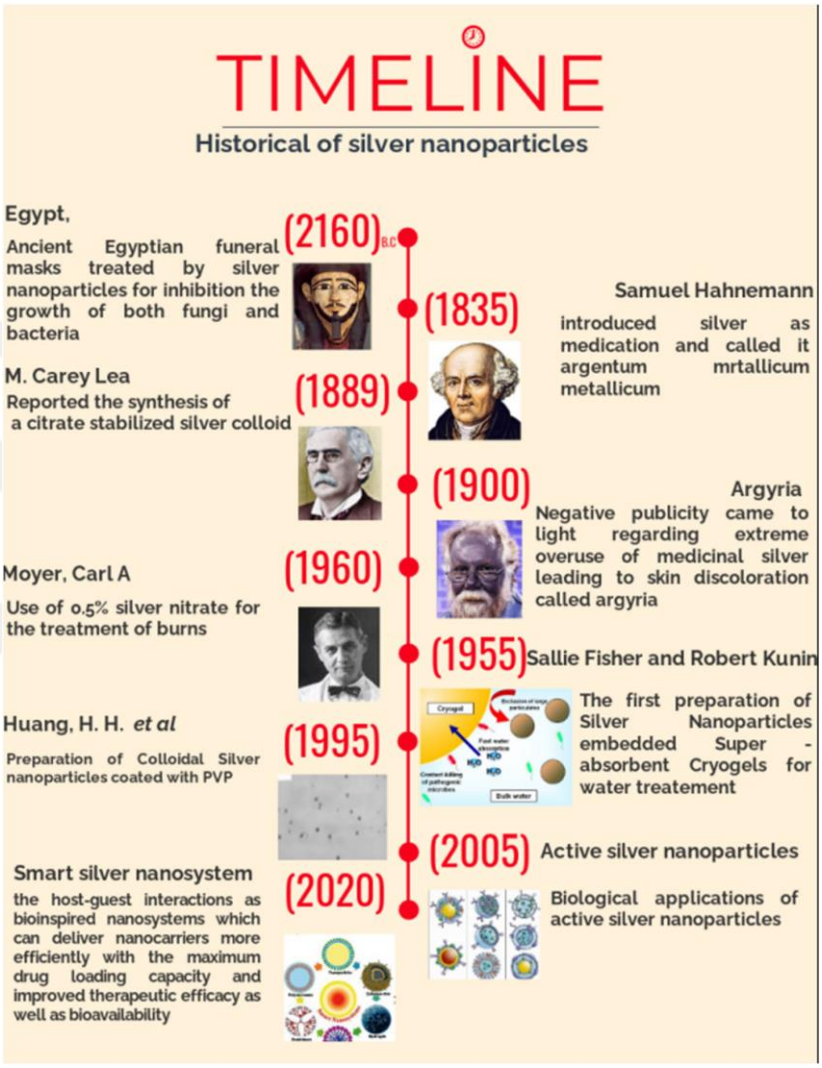

Figure 1.5. Historical timeline of AgNPs (Ghobashy et al., 2021)

1.7. Mechanism of Action of Silver Nanoparticles

Although the particular mechanism of silver nanoparticle antibacterial activity is uncertain, Figure 1.6 depicts many antibacterial effects. Silver nanoparticles have the potential to continually release silver ions, which might be used to destroy microorganisms. Because of electrostatic attraction and affinity for sulphur proteins, silver ions can adhere to the cell wall and cytoplasmic membrane. The connected ions might make the cytoplasmic membrane more permeable, leading the bacterial

envelope to burst. When free silver ions enter cells, they block respiratory enzymes, creating reactive oxygen species but impeding with adenosine triphosphate production. Cell membrane disruption and deoxyribonucleic acid (DNA) modification can be caused by reactive oxygen species. Because sulphur and phosphorus are essential components of DNA, the interaction of silver ions with these elements can cause problems with cell reproduction, DNA replication, and even microorganism death. Furthermore, silver ions can inhibit protein synthesis by denaturing cytoplasmic ribosomes (Salleh et al., 2020).

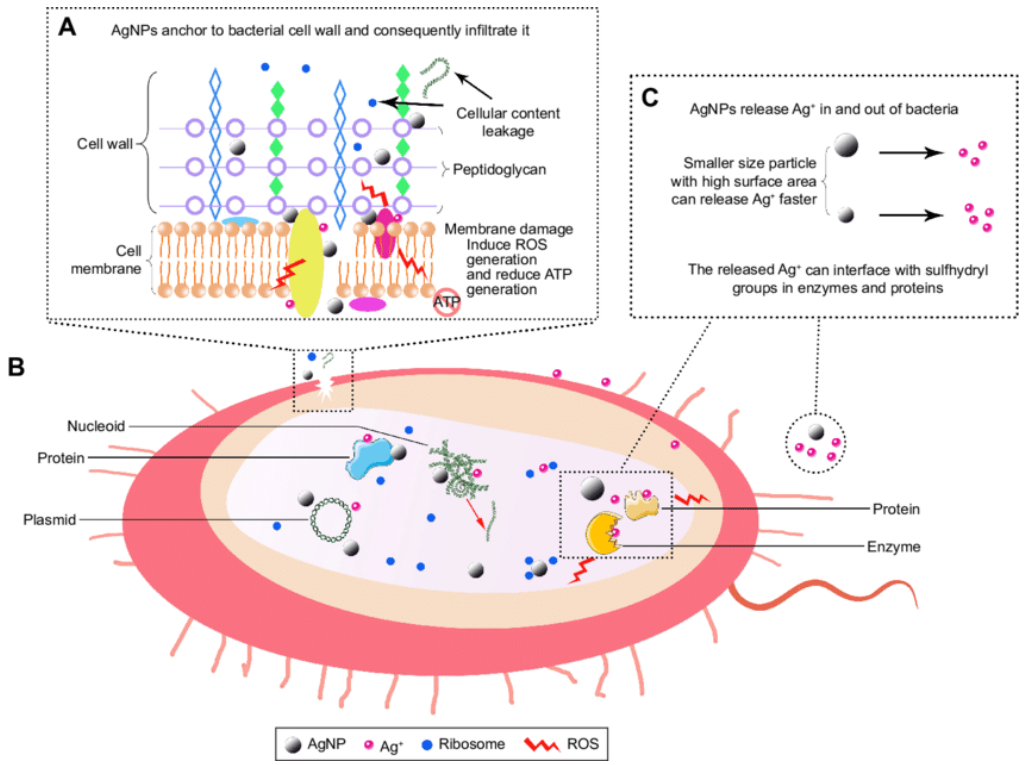

Figure 1.6. Antibacterial mechanisms of AgNPs (Qing et al., 2018)

1.8. Characterization of Nanoparticles

Nanomaterials are polycrystalline solids with particle sizes ranging from 1 to 100 nm. Their tiny size combined with enormous surface area gives the nanoparticles unique features. The use of nanoparticles in electronics, medicine, and agriculture necessitates a thorough understanding of their characteristics.The physicochemical characterisation of produced NPs is a crucial stage that must be carefully studied before using nanoparticles. The size, surface area, shape, uniformity, stability, and other properties of nanoscale systems will provide critical information and insight into nanoparticle production control for commercial applications. Characterization

strategies that are often used include UV–visible spectrometry; Fourier transformation infrared spectroscopy (FT-IR); and high-resolution scanning transmission electron microscopy (HR-TEM), powder X-ray diffraction (XRD); and field-emission scanning (FE-SEM); dynamic light DLS; vibrating thermogravimetric analysis; sample magnetometer (VAM) (TGA); EDX-map (energy-dispersive spectroscopy); and other instruments are the most famous techniques used in characterization of synthesized nanoparticles (Hassanien and Khatoon, 2019).

1.8.1. Scanning Electron Microscopy (SEM)

SEM is a sophisticated analytical equipment that scans an item with a highly focussed beam of electrons. The beam reacts with numerous atoms on the surface of the sample, delivering important information about the surface shape and creating high-quality images with good spatial resolution. The electron beam used for scanning is made up of very energetic electrons ranging in energy from 0.2 to 40 keV, which results in a picture of the material. The picture reveals not only the purity of the sample but also the degree of aggregation. SEM magnification can range from 10 to over 3 milion times (Brodusch et al., 2018).

1.8.2. Transmission Electron Microscopy (TEM)

For effective characterisation of nanomaterials, TEM has been the method of choice. It delivers accurate chemical information as well as high-resolution pictures down to the nanoscale scale. The size, shape, and content of the specimens are all determined by TEM. Knoll and Ruska invented the first TEM in 1931 (winning the Nobel Prize in 1986 for this discovery), and it was commercialized in 1939 (Eswara et al., 2019).

1.8.3. Atomic Force Microscopy (AFM)

AFM, also known as a scanning force microscopy, is a type of high-resolution microscope capable of imaging nanoscale objects. The AFM has been widely employed for nanoscale imaging and material characterisation. The initial equipment consisted of an ultra-small diamond probe tip at the end of a cantilever, with interatomic van der Waals forces providing the essential contact. The noncontact mode was initially presented in 1987, and the first commercially available AFM, the Digital Instruments Nanoscope, debuted in 1989 AFM has shown to be an effective measurement technique in nanobiotechnology. AFM has

established itself as an effective measurement technique in nanobiotechnology and is likely to make significant contributions in the next years. Structures with vertical resolutions of 0.1 nm and X-Y resolutions of roughly 1 nm may be measured with this approach (Krieg et al., 2019).

1.8.4. Ultraviolet-Visible Spectroscopy

UV-visible spectroscopy is a straightforward and inexpensive method for analyzing nanomaterials in the ultraviolet-visible spectral region. This method, which is widely used in analytical chemistry, includes determining the intensity of light reflected by a sample and comparing it to the intensity of light reflected by a reference material. This procedure is carried out using a specialized instrument known as a UV/Vis spectrophotometer. The absorbance is determined by their ratio, which is known as transmittance and is commonly reported in percentage. Nanoparticles have substantially smaller particle sizes than bulk particles and optical characteristics that are susceptible to a variety of parameters such as size, concentration, shape, agglomeration, and refractive index. These characteristics are used in UV-Vis spectroscopy to identify and characterize nanomaterials, as well as to assess the stability of their colloidal solutions. Because of plasmon resonance qualities, the smaller particle size of nanoparticles causes a change in absorption wavelength toward a shorter wavelength. It is measured using a UV-spectrophotometer, which operates in the 200–800 nm range and offers information on a variety of physical characteristics of nanoparticles (Guo et al., 2020).

1.8.5. Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectroscopy is a simple analytical technique that scans test samples for the detection of distinct functional groups contained in nanomaterials using infrared light (within the mid-infrared range of 4000–400 cm^{-1} or 2.5–25 μm wavelength). This approach can quickly identify both organic and inorganic substances in a sample. It operates on the premise that when infrared light passes through a sample, part of it is absorbed and the remainder is transmitted. The resultant signal is the sample's molecular fingerprint (Bahadori et al., 2021).

1.8.6. X-Ray Diffraction (XRD)

XRD is a highly effective non-destructive method for determining whether a material is crystalline or amorphous. The technique includes projecting a

monochromatic beam of X-rays onto a crystal surface, which produces constructive interference as well as a diffracted ray if the criteria are met by Bragg's equation. This technique provides useful information for identifying and characterizing unknown crystalline/semicrystalline structures, the ratio of crystalline to non-crystalline regions, and structural parameters such as particle size (via the Debye Scherer formula), crystal defects and roughness, thickness, and density of thin films (Bahadori et al., 2021).

1.9. The Aims of the Thesis

Bioactive chemicals are considered to be abundant in actinobacteria. These bacteria are especially noteworthy for their extraordinary ability to produce metallic nanoparticles. This thesis work investigated the bioactivity aspects of silver nanoparticles generated by *Micromonospora* sp. CPM1. Additionally, the antimicrobial properties of the nanoparticles generated as well as their structural elucidation were depicted. As a consequence, the project's objectives are as follows:

- Silver nanoparticles synthesis by *Micromonospora* sp. CPM1 culture
- Antimicrobial activity analysis of silver nanoparticles generated
- Physicochemical characterisation of silver nanoparticles
- Whole-genome analysis of *Micromonospora* sp. CPM1

2. LITERATURE REVIEW

Patil and Kumbhar (2017) created silver nanoparticles using *Lantana camara* L. leaf extract and discovered that these NPs have dose-dependent antioxidant activity comparable to that of normal ascorbic acid. AgNPs also demonstrated considerable antibacterial action against Gram-positive *Staphylococcus aureus* compared to Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli* and comparable to ciprofloxacin (Patil and Kumbhar, 2017).

Jha et al. (2017) produced AgNPs from *Ocimum tenuiflorum* L. extract and then tested silver nanoparticles-loaded multi-walled carbon nanotubes (MWCNT) with mammal sperm to determine the increased targeting potential for the development of portable diagnostic tools for infertility therapy. As the surface height of the MWCNT increased from 22 to 32 nm, AFM validated the loading of AgNP within the tube, assuring the encapsulation of 10 nm AgNP within the tube (Jha et al., 2017).

Kumar et al. (2017) showed green synthesis of AgNP by *Jatropha curcas* L. and *Lannea grandis* (Dennst.) Engl., as well as low MIC and minimal biofilm eradication concentrations against *C. albicans* biofilm. The novel formulation was stable and poisonous to goat blood RBC, and it could be used to treat *C. albicans*-related infections in the future (Kumar et al., 2017).

Bilal et al. (2107) created a silver nanoparticles-loaded chitosan-alginate construct from a *Euphorbia helioscopia* L. methanolic extract and tested its antibacterial activity as well as anti-cancer activity against HeLa cells. As a result, the newly created construct may be a viable contender for biomedical applications (Bilal et al., 2017).

Ibrahim and Hassan (2016) created a Ag NPs-functionalized cotton fabric via green synthesis, which demonstrated highly qualitative and quantitative antimicrobial activity against *E. coli* and *S. aureus*, indicating that this characteristic might be used to create antimicrobial finishes and textiles (Ibrahim and Hassan, 2016).

Jadhav et al. (2016) created antibacterial silver nanoparticles from *Ammannia baccifera* L. extract. When compared to commercialized 0.2 % w/w silver nitrate gel, AgNPs gel (0.025 % w/w) demonstrated a same zone of inhibition even against pathogenic microorganisms responsible for burn infections. By encouraging cellular

growth and alleviating pain, the prepared AgNPs gel might be employed as an efficient and better replacement in burns (Jadhav et al., 2016).

Zhang et al. (2010) shown that nanoparticles might be utilized as fluorescent markers in (single particle) imaging studies or bioassays requiring minimal background or tissue penetrating wavelengths (Zhang et al., 2010).

3. MATERIALS AND METHODS

3.1. Isolation of Carotenoid Pigment-Producing Bacteria

The actinobacteria producing carotenoid pigments were isolated from endophytic tissues of plant samples collected from the Blacksea shore in 2017 by Dr. Hilal Ay. The fresh samples of *Polygonum maritimum* L. were collected and underground parts were sterilized as shown by Qin et al. (2009). Briefly, the plant samples were air-dried for 2 days at room temperature and then washed with sterile distilled water for 15 min to thoroughly remove surface soils and adhering epiphytes. After drying, the samples were subjected to a five-step surface sterilization procedure: a 4- to 10-min wash in 5% NaOCl, followed by a 10-min wash in 2.5% $Na_2S_2O_3$, a 5-min wash in 75% ethanol, a wash in sterile water, and a final rinse in 10% $NaHCO_3$ for 10 min. After drying, the underground parts were crumbled into small pieces under aseptic conditions and then 1 g of the smashed plant sample was immersed in in ¼ strength Ringer's liquid (Oxoid) and heated at 60 °C for 20 min. After preparing 10^{-2} and 10^{-3} dilutions, aliquots were dispersed on Czapek–Dox agar medium (sucrose 30 g/l, $NaNO_3$ 3 g/l, $MgSO_4x7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4x7H_2O$ 0.01 g/l, K_2HPO_4 1 g/l, agar 13 g/l, distilled water). The pH of media was adjusted to 7.2 before autoclaving at 121˚C for 25 minutes and the medium was supplemented with nystatin (50 µg/ml) and rifampicin (5 µg/ml) to suppress the growth of fungi and fast-growing bacteria. After three weeks of incubation at 28°C, the orange-colored colonies determined to be carotenoid-producing actinobacteria (Figure 3.1a and b) were kept and purified as glycerol stock solutions (25%, v/v) at -20°C.

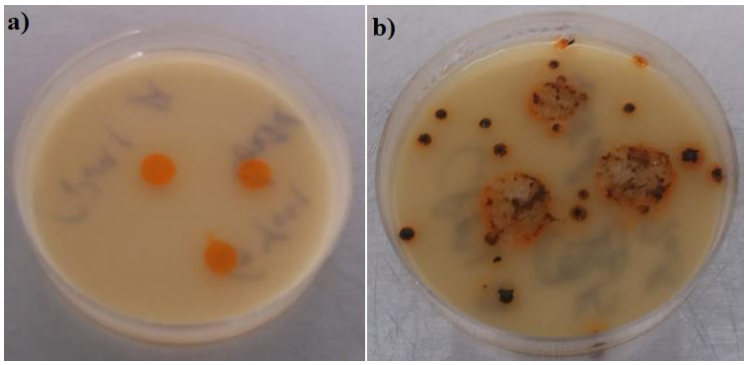

Figure 3.1. The growth of strain CPM1 on GYM medium after three days (a) one month (b) after inoculation

3.2. Synthesis of Silver Nanoparticles

A total of 10 strains were activated on tryptone yeast extract agar (TYG) (tryptone 3 g/l, yeast extract 5 g/l, glucose 5 g/l, 20 g/l, distilled water 1000 ml, pH 7.0) and glucose-yeast extract-malt extract agar (GYM) (glucose 4 g/l, yeast extract 4 g/l, malt extract 10 g/l, $CaCO_3$ 2 g/l, agar 12 g/l, distilled water 1000 ml, pH 7.2) media and tested for their ability to synthesize silver nanoparticles. After activation on agar medium, the colonies were picked and transferred to 30 ml tryptone soya broth (TSB) (Oxoid) media to prepare seed culture. The broth cultures were incubated at 28˚C for 5-7 days in a shaker incubator and then a volume of 5 ml for each strain was used to inoculate 100 ml TSB medium. The inoculated flasks were incubated at 28˚C for 5-7 days in a shaker incubator. After incubation, the culture broths were centrifuged at 10,000 rpm (4˚C) and the supernatants were collected for nanoparticle synthesis. To synthesize silver nanoparticles, 2 mM $AgNO_3$ solution (100 ml) was mixed by same volume of culture supernatants and stirred constantly (Figure 3.2a). Then, the supernatant-$AgNO_3$ solutions were placed in a shaker incubator (150 rpm) and the colour change from yellow to brown-black was observed within a week (Figure 3.2b). The strain CPM1 was the most effective in terms of synthesis time and colour darkness. The silver nanoparticles were collected by centrifugation (13,000 rpm, 4˚C) and washed with sterile distilled water twice. The silver nanoparticles were dried before analyses.

Figure 3.2. Synthesis of silver nanoparticles by strain CPM1 culture broth. (a) culture broth and silver nitrate solution, (b) culture broth and silver nitrate solution after 7 days

3.3. Identification of the Silver Nanoparticle-Synthesizing Actinobacterium

The genome sequence data of strain CPM1 was downloaded from the NCBI GenBank database (accession number JAMHIU000000000) and annotated on the Rapid Annotations Using Subsystems Technology (RAST) server (Aziz et al. 2008). The 16S rRNA gene sequence was extracted from the genome sequence data using the RAST server and uploaded to the EzBioCloud server (https://www.ezbiocloud.net/) (Yoon et al. 2017) to identify the strain CPM1 in the genus level.

3.4. Phylogenetic and Genome Analysis of Strain CPM1

For a whole-genome based taxonomic analysis, the genome sequence of strain CPM1 was uploaded to the Type (Strain) Genome Server (TYGS) available under https://tygs.dsmz.de (Meier-Kolthoff and Göker 2019). The secondary metabolite coding gene clusters of strain CPM1 was determined by uploading the genome sequence to the Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) (https://antismash.secondarymetabolites.org/#!/start) (Blin et al. 2021) and analysed on 'relaxed' detection strictness.

3.5. Characterization of Silver Nanoparticles Synthesized by Strain CPM1

UV-Vis, XRD, FTIR, SEM, and EDX were used to characterize the shape, size, and content of the AgNPs produced under ideal circumstances. In the ultraviolet visible spectral band, a UV-vis spectrophotometer was used for absorption or reflection spectroscopy. The colour change of the AgNP synthesis was monitored visually and the absorbance was measured with a UV-Vis spectrometer (Thermo Scientific Evolution 201/220). Spectral studies were conducted in the range of 200-800 nm at a resolution of 1 nm with a quartz cuvette. The shape, size, and elemental analysis of silver nanoparticles were confirmed by SEM. In addition, EDX analysis was performed to confirm the elemental composition of the silver nanoparticles and to identify signals for silver atoms. To characterize silver nanoparticles, all functional groups likely to be found in nanoparticles obtained from the culture of strain CPM1 were determined by FTIR spectroscopy. FTIR spectra were recorded in the 450-4000 cm^{-1} range at a resolution of 34 cm^{-1}.

3.6. Antibacterial Activity of Synthesized AgNPs

The antimicrobial activity of the synthesized silver nanoparticles was tested against *Bacillus cereus* EMC15, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter faecalis* ATCC 29212 and *Candida albicans* ATCC 10231 using the drop diffusion method. The pathogenic microorganisms were activated on nutrient agar medium at 37°C for 24 hours and then each microorganism was suspended in sterile Ringer's solution (Oxoid) to obtain an optical density between 0.5-1.0, which corresponds to $1.5\text{-}3.0\text{x}10^8$ CFU/ml. A 70 µl aliquot of suspension was streaked onto nutrient agar medium. After complete drying, 25 µl silver nanoparticle suspension (0.05 mg/ml) was dropped onto the agar. Sterile distilled water was used as a negative control. Nystatin and rifampicin were used as positive controls for yeast and bacteria, respectively. The plates were incubated at 37°C for 24 hours and the inhibition zones were calculated. The experiments were carried out in duplicate.

4. RESULTS AND DISCUSSION

4.1. Comparative Genome Analyses

The draft genome sequence of strain CPM1 was annotated on RAST server using RASTtk pipeline (Aziz et al. 2008; Brettin et al. 2015). The size of the genome consisting of 67 contigs is approximately 6.4 Mb and genomic G+C content is 73.2%. The N50 value, numbers of coding sequences and RNAs were calculated as 246252 bp, 6101 and 59, respectively, for the genome of strain CPM1. To identify the strain CPM1 at the genus level, the 16S rRNA gene sequence was downloaded from the genome sequence data using the RAST server and uploaded to the EzBioCloud server (https://www.ezbiocloud.net/) (Yoon et al. 2017).

An EzBiocloud server pairwise sequencing analysis revealed that the CPM1 strain is closely linked to *Micromonospora tulbaghiae* DSM45142^T , *Micromonospora fluminis* A38, *Micromonospora rosaria* DSM 803 and *Micromonospora endolithica* DSM 44398 with pairwise sequence identity scores ranging from 100% to 99.23% (Figure 4.1). To give additional evidence for the strain's uniqueness within the genus *Micromonospora*, a genome-based phylogenomic tree was created using TYGS by analyzing the strain to the most strongly linked genomes in the database. To generate a more complete phylogenomic tree, the genomes of strain CPM1 were submitted to the TYGS. A whole-genome-based phylogenomic analysis conducted on the TYGS server (https://tygs.dsmz.de/) confirmed that *Micromonospora* sp. CPM1 was closely related to *Micromonospora tulbaghiae* DSM 45142^T (Figure 4.2). The TYGS phylogenomic tree (https://tygs.dsmz.de/) revealed that strain CPM1 formed a unique cluster within the clade constituted of its near neighbors as determined by pairwise 16S rRNA gene sequence analysis, including the type species of *Micromonospora tulbaghiae* DSM 45142^T and *Micromonospora purpurea* DSM 43036^T. To confirm strain CPM1's taxonomic position, the digital DNA–DNA hybridization values calculated from whole genome sequences between strain CPM1 and its close phylogenetic relatives were measured and found to be less than the 70% threshold value recommended by Wayne et al. (1987) to clearly define prokaryotic genomic species. The digital DNA–DNA hybridization values between strain CPM1 and *Micromonospora provocatoris* MT25^T, *Micromonospora tulbaghiae* DSM 45142^T and *Micromonospora aurantiaca* ATCC 27029^T were calculated as 69.6%,

68.5% and 62.0%, respectively, values which are below the threshold of 70% but at the borders to clearly define a genomic species (Wayne et al. 1987). So this strain needs for a comprehensive phenotypic analysis to ensure its novelty.

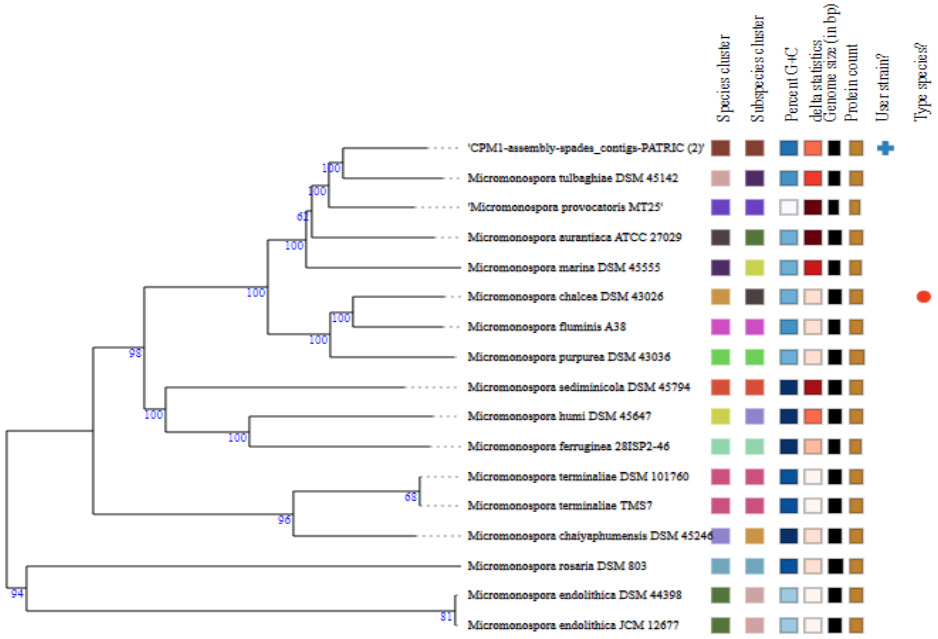

Figure 4.1. A whole-genome-based phylogenomic analysis conducted on the TYGS server (https://tygs.dsmz.de/) confirmed that *Micromonospora* sp. CPM1 was closely related to *Micromonospora tulbaghiae* DSM 45142^{T}

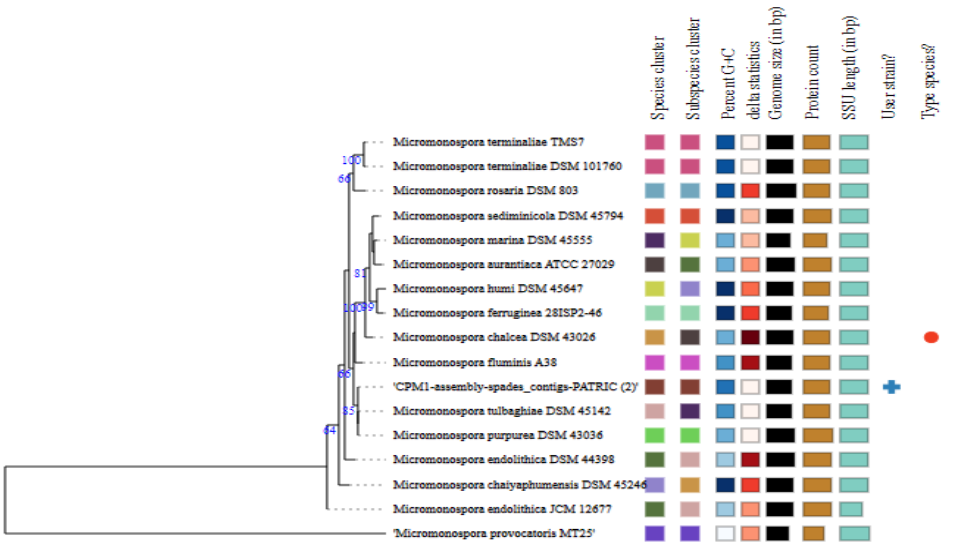

Figure 4.2. The phylogenetic tree inferred from 16S rRNA genes of strain CPM1 and its close phylogenetic neighbours built on TYGS (https://tygs.dsmz.de/)

Various compounds such as amines, amides, carbonyl groups, pigments, proteins, alkaloids and other reducing agents present in microbial cells can trigger nanoparticle synthesis. Chemicals with a strong ability to oxidize or reduce metal ions to produce zero-valent or magnetic nanoparticles are normally excreted by bacteria (Saravanan et al., 2021). The genome annotation of *Micromonospora* sp. CPM1 revealed that the strain many genes responsible for biosynthesis of cofactors, vitamins, prosthetic groups and pigments while the largest proportion of the genome was devoted to biosynthesis and metabolism of aminoacids and derivatives. As reported by Singh et al. (2015), peptides might interact with silver nanoclusters formed previously in solution and create a reducing environment around these clusters. This may cause reduction of silver ions and formation of polydisperse AgNPs. Moreover, peptides containing amino acids such as arginine, cysteine, lysine, methionine, glutamic acid and aspartic acid are involved in the recognition and reduction of silver ions into silver crystals (Nam et al., 2008). The genome of strain *Micromonospora* sp. CPM1 encodes many genes for the synthesis and degradation of these aminoacids, which may have a major role in the biogenic synthesis of silver nanoparticles in the present study. In addition to the genes encoding for aminoacid biosynthesis, strain *Micromonospora* sp. CPM1 have a large number of genes for reductase enzymes such as for oxidoreductases, nitroreductases, alkyl hydroperoxide reductases, NADH-ubiquinone oxidoreductases, aldo/keto reductases and thioredoxin reductases, which might have reduced the silver ions in the supernatant of the strain into silver nanoparticles.

4.2. Biosynthetic Potential of the Strain

A comprehensive annotation for secondary metabolite-coding gene clusters on the antiSMASH server (https://antismash.secondarymetabolites.org/#!/start) revealed that the genome of *Micromonospora* sp. CPM1 encodes for 14 biosynthetic gene (Figure 4.3) clusters coding for terpenes, lantipeptides, polyketides, nonribosomal peptides and a siderophore.

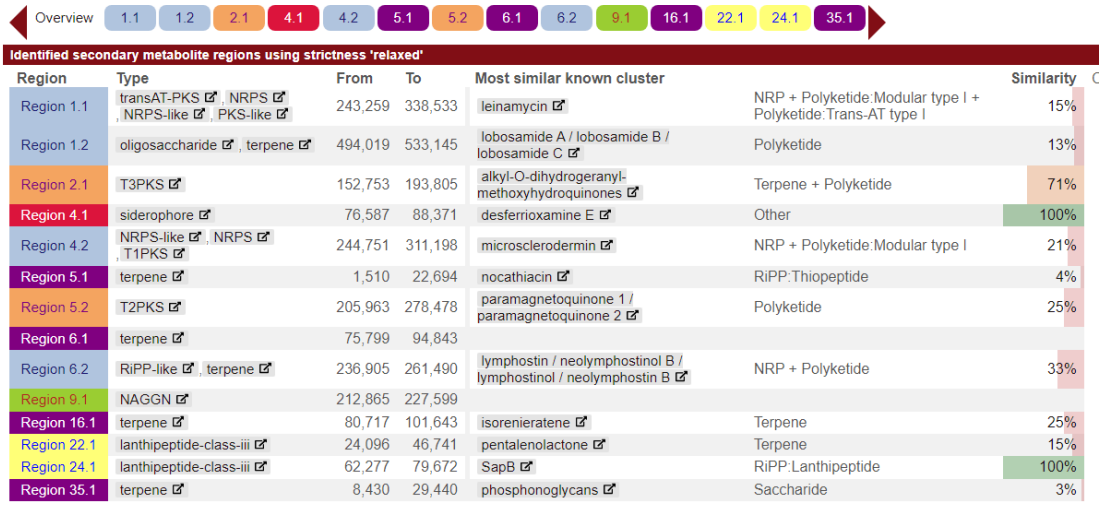

Figure 4.3. Secondary metabolite biosynthetic gene clusters of strain CPM1 identified on the antiSMASH server

4.3. Analysis of UV–vis Spectroscopy

CPM1 culture supernatant treated with $AgNO_3$ solution showed a progressive color shift from yellow to dark brown. After 96 hours, the control without silver nitrate showed no color change. A significant peak at 439 nm was found in the UV–visible spectrum, and surface plasmon resonance (SPR) indicated the successful synthesis of AgNPs. This might have happened because secondary metabolites in the bacterial supernatant reduced the silver ions. Metal silver nanoparticles have unbound electrons, which cause an SPR absorption band to form due to the combined vibration of metal nanoparticle electrons in resonance with the light wave. Silver nanoparticles were found to be stable in solution with negligible aggregation. Furthermore, the plasmon bands are expanded with an absorption tail at the longer wavelengths. This might be attributed to particle size dispersion (Ahmad et al., 2003).

4.4. SEM and EDX Analysis

The form, size, and elemental content of the produced AgNPs were evaluated by SEM examination. SEM examination was used to analyze the shape of AgNPs and confirm their stability (Figure 4.4). SEM analysis revealed that the average particle size of all biosynthesized AgNPs ranged between 15 and 30 nm, with the bulk of synthesized nanoparticles being spherical in form. EDX analysis was used to assess the composition and purity of AgNPs generated during green synthesis. The existence of silver in the sample was confirmed by finding signals of silver at 3.2 keV features of AgNPs in the EDX spectrum displayed in Figure 4.5. The presence

of Na, C, S, and O signals indicates that the AgNPs in the cell free supernatant are capped by organic components such as proteins, flavonoids, carbohydrates, or phenolic compounds. The quantitative elemental content of the produced AgNPs indicated the existence of pure silver.

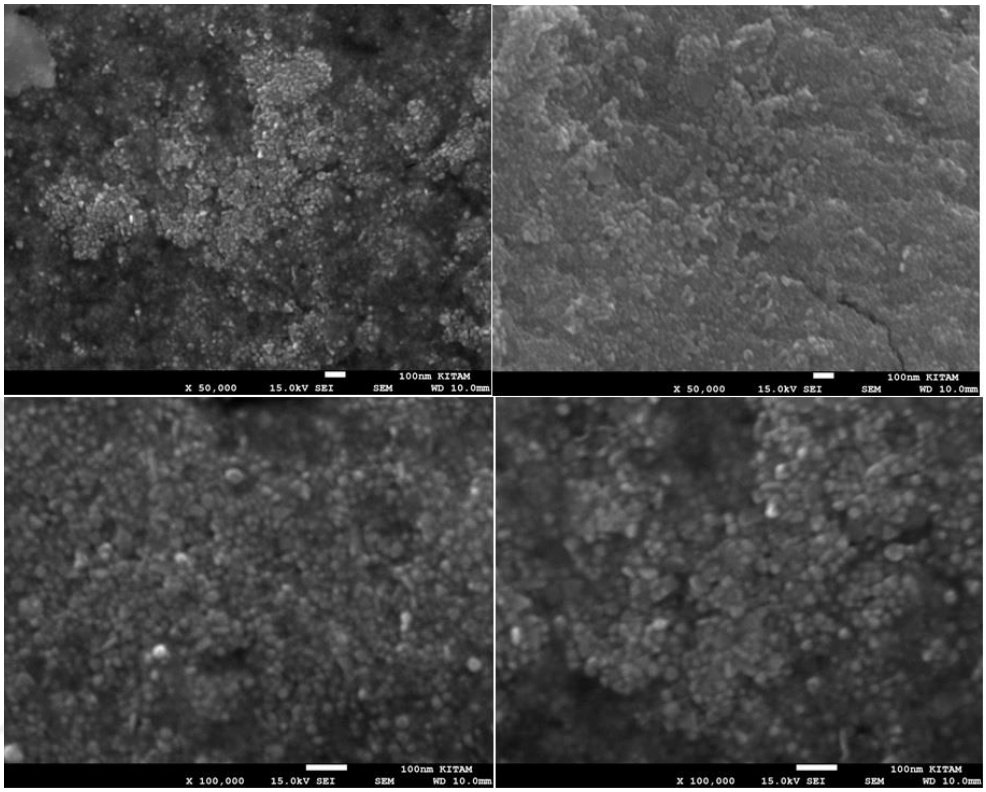

Figure 4.4. SEM images of AgNPs obtained by mixing 2 mM silver nitrate solution with cell free supernatant of strain CPM1

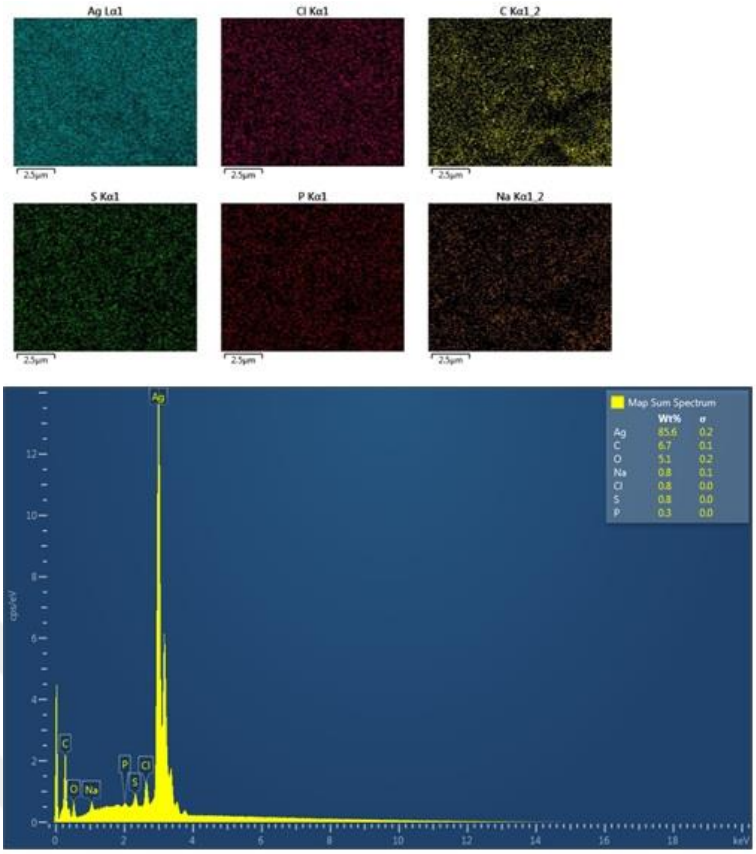

Figure 4.5. EDX profile of silver nanoparticles synthesized by strain CPM1

4.5. FTIR Study

The FTIR spectra of the produced AgNPs (Figure 4.6) revealed absorption peak at 3851.19, 3745.12, 3671.10 and 3646.81cm^{-1}, which represents the strong stretching vibrations of O-H functional group (Lin et al., 2012; Nandiyanto et al., 2019), demonstrating the existence of phenolic chemicals and aromatic alcohols or secondary amide -NH stretch in AgNPs sample. The band at 1539.05 cm^{-1} corresponds to the bending vibrations of the amide bands of the proteins (Otari et al., 2015), correlating to protein binding vibrations, suggesting protein binding with nanoparticles (Khan et al., 2018). The presence of a 701.96 cm^{-1} peak demonstrate for thiol or thioether, CH2-S-(C-S stretch). The 667.61, 628.85, 606.1 cm^{-1} peaks in Figure 4.6 indicates the presence of alcohol, OH out-of-plane bend. These findings show that the functional amino groups interact with the surface of silver nanoparticles, which may have served as a capping region for the stability of the produced AgNPs. The FTIR peaks also show the presence of proteins, phenols, and carboxylic acids in the cell free supernatant, which might have worked as reducing and stabilizing agents during the AgNP formation.

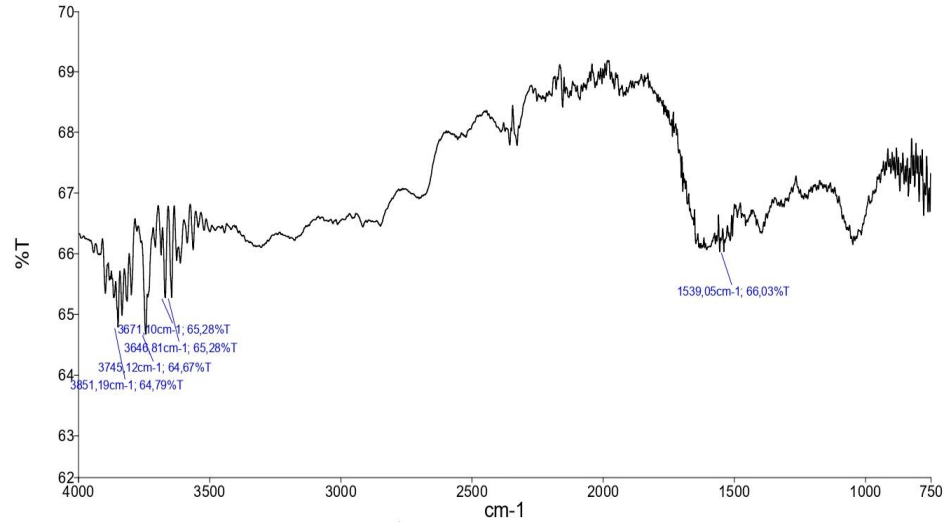

Figure 4.6. FTIR spectra of the produced AgNPs

4.6. Antibacterial Activities of AgNPs

The silver nanoparticles synthesized by *Micromonospora* sp. CPM1 inhibited the growth of *Candida albicans* ATCC 10231 and *Bacillus cereus* EMC15 with inhibition zones of 14.5±0.5 mm and 12±1 mm, respectively (Figure 4.7). No inhibition was observed against *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853 and *Enterobacter faecalis* ATCC 29212.

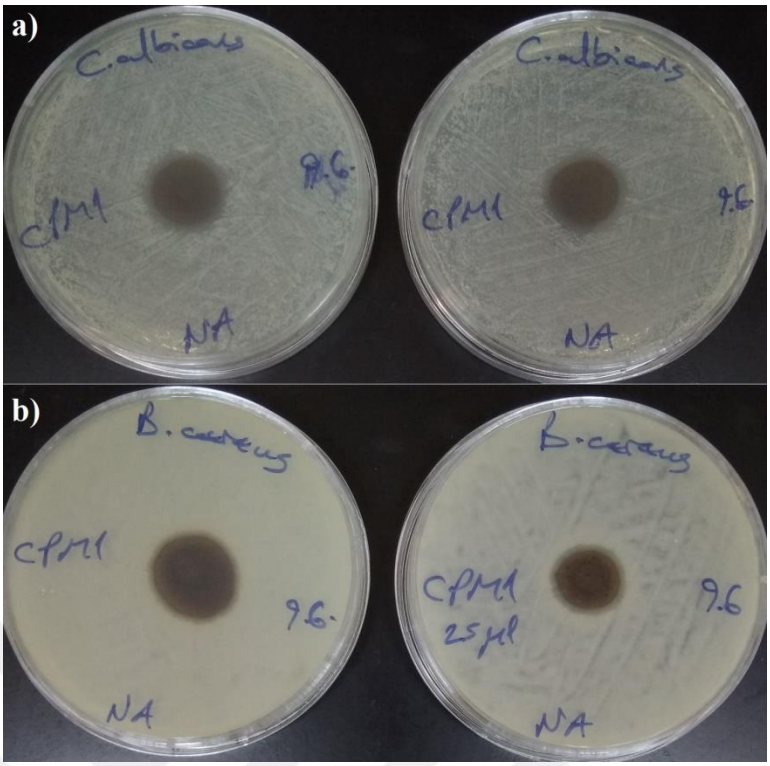

Figure 4.7. Antimicrobial activity of silver nanoparticles synthesized by *Micromonospora* sp. CPM1 against *Candida albicans* ATCC 10231 (a) and *Bacillus cereus* EMC15 (b)

4.7. XRD analysis

The XRD models of the AgNPS showed that the structure of the AgNPs was spherical. It is clear that AgNPs formed were essentially crystalline. In addition, the XRD peaks at 2θ of 27.74°, 32.14°, 38.07°, 44.08°, 46.12°, 64.52°, and 77.48° in the synthesized AgNPs correspond to seven difraction crystallographic AgNPs (Figure 4.8). This standard pattern is compatible with those in the literature. The average crystallite size of AgNPs was computed according to the Debye–Scherrer equation and was found to be 13.4 nm for AgNPs synthesized from CPM1 supernatant. This value is slightly lower compared to the particle size obtained from the SEM image of AgNPs.

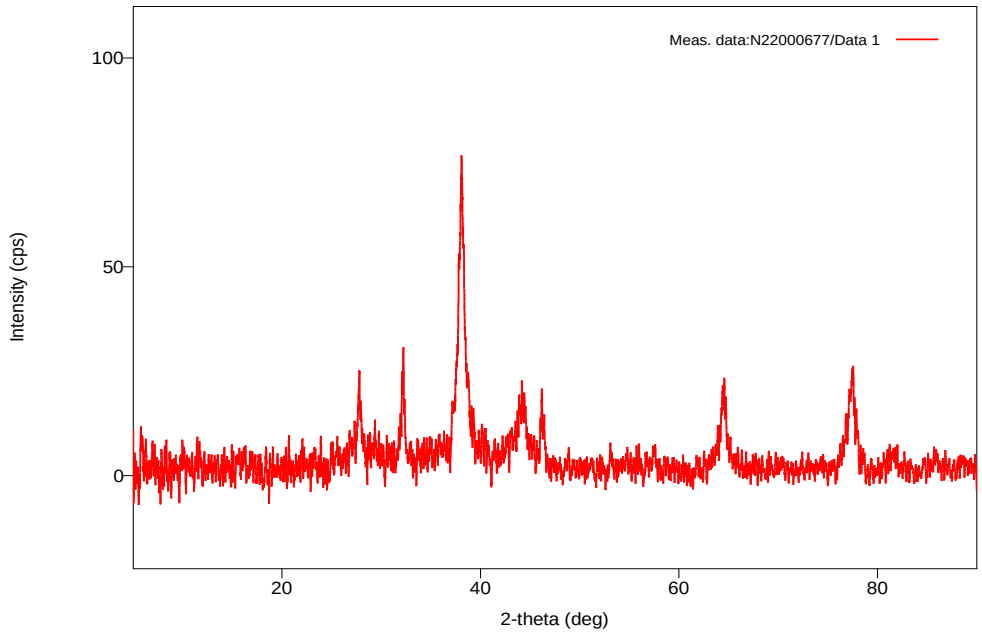

Figure 4.8. XRD pattern of AgNPs synthesized by *Micromonospora* sp. CPM1

5. CONCLUSION

The current work describes an eco-friendly, green extracellular production of AgNPs employing the endophytic actinobacterium *Micromonospora* sp. CPM1. The extract's activity as a capping and stabilizing agent of the produced AgNPs is confirmed by FTIR spectra and SEM pictures. The hydrodynamic diameter of the produced AgNPs reveals that their size and polydispersity are better controlled. Under the studied experimental circumstances, the produced AgNPs show good antimicrobial effectiveness against *Bacillus cereus* and *Candida albicans*. Moreover, biosynthetic potential and taxonomic position of Micromonospora sp. CPM1 were revealed by a comprehensive genome analysis. Exploration and use of *Micromonospora* genus as a source of cheap biomaterial allows us to develop a simple, environmentally friendly, cost-effective, and rapid synthesis of stable and polydispersed AgNPs, which have the potential to be used as promising antimicrobial agents against pathogenic microbes to combat the threat of antibiotic resistance. However, further research is needed to establish biosynthesized AgNPs as a competent treatment modality in the present era of medicine.

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