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**TRANSCRIPTOMIC ANALYSIS OF *KLEBSIELLA*
PNEUMONIAE EXPOSED TO BIOLOGICALLY SYNTHESIZED
SILVER NANOPARTICLES**

Master's Thesis

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ÖZET

BİYOLOJİK OLARAK SENTEZLENEN GÜMÜŞ NANOPARTİKÜLLERİNE MARUZ KALAN *KLEBSIELLA PNEUMONIAE*'NİN TRANSKRİPTOMİK ANALİZİ

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Gümüş nanopartikülleri nanobilim ve nanoteknoloji alanlarında önemli bir rol oynamaktadır. Gümüş nanopartikülleri, özellikle nanotıpta, antibakteriyel ve antifungal aktiviteye sahip yeni ilaçların geliştirilmesinde umut vericidir. Çeşitli fiziksel, kimyasal ve biyolojik yöntemler gümüş nanopartiküllerinin sentezi için kullanılabilmektedir. Bu çalışma, Antarktik bir aktinobakteri olan *Arthrobacter* sp. H14-L1 tarafından sentezlenen gümüş nanopartiküllerine maruz kalan *Klebsiella pneumoniae*'nin transkriptomik analizi için planlanmıştır. Sentezlenen gümüş nanopartikülleri görünür ve ultraviyole ışık spektroskopisi (UV-vis), taramalı elektron mikroskopisi-enerji dağılımlı X-ışını spektroskopisi (SEM-EDX) ve X-ışını kristalografisi ile karakterize edilmiştir. UV-vis analizine göre gümüş nanopartikülleri 419 nm dalgaboyunda maksimum absorbands göstermiştir. SEM-EDX analizi, *Arthrobacter* sp. H14-L1 tarafından sentezlenen gümüş nanopartiküllerinin küresel biçimde ve ortalama 58-68 nm boyutlarında olduğunu göstermiştir. X-ışını kristalografisi analizi, sentezlenen gümüş nanopartiküllerinin kristal yapıda olduğunu doğrulamıştır. Gümüş nanopartikülü sentezinde kullanılan *Arthrobacter* sp. H14-L1'in genomu NCBI GenBank'tan indirilerek biyosentetik gen kümeleri ve filogenetik özellikleri analiz edilmiştir. Sentezlenen gümüş nanopartiküllerinin *Klebsiella pneumoniae* gelişimini inhibe ettiği gösterilmiştir. Gümüş nanopartikülüne maruz kalan *K. pneumoniae* hücrelerinde meydana gelen gen ekspresyon farklılıklarını ortaya çıkarmak amacıyla RNA-Seq analizi yapılmıştır. *Arthrobacter* sp. H14-L1 tarafından sentezlenen gümüş nanopartiküllerine maruz kalan *K. pneumoniae*'da meydana gelen gen ekspresyon farklılıkları, istatistiksel olarak önemli olmamakla birlikte, genel olarak karbohidrat metabolizması, aminoasit biyosentez ve degradasyonu ve stres cevabı ile ilişkili genlerin ekspresyonlarında farklılık gözlenmiştir. Bu çalışma ile elde edilen sonuçlar, gümüş nanopartiküllerinin bakteri hücreleri üzerindeki toksisitesinin moleküler mekanizmalarının anlaşılmasına katkı sunmaktadır.

Anahtar Kelimeler: *Arthrobacter*, Biyojenik gümüş nanopartikülleri, *Klebsiella pneumoniae*, RNA-dizileme, Transkriptomik

ABSTRACT

TRANSCRIPTOMIC ANALYSIS OF *KLEBSIELLA PNEUMONIAE* EXPOSED TO BIOLOGICALLY SYNTHESIZED SILVER NANOPARTICLES

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Silver nanoparticles play an important role in the fields of nanoscience and nanotechnology. Silver nanoparticles are promising in the development of new drugs with antibacterial and antifungal activity, especially in nanomedicine. Various physical, chemical and biological methods can be used for the synthesis of silver nanoparticles. This study was planned for the transcriptomic analysis of *Klebsiella pneumoniae* exposed to silver nanoparticles synthesized by an Antarctic actinobacterium *Arthrobacter* sp. H14-L1. The synthesized silver nanoparticles were characterized by visible and ultraviolet light spectroscopy (UV-vis), scanning electron microscopy – energy dispersive X-ray spectroscopy (SEM-EDX), and X-ray crystallography. According to the UV-vis analysis, silver nanoparticles showed maximum absorbance at 419 nm wavelength. The SEM-EDX analysis showed that the silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1 were spherical in shape and 58-68 nm in size. The X-ray crystallography analysis confirmed that the synthesized silver nanoparticles were in crystalline form. The genome of *Arthrobacter* sp. H14-L1 used in the synthesis of silver nanoparticles was downloaded from the NCBI GenBank, and its biosynthetic gene clusters and phylogenetic characteristics were analyzed. It was shown that synthesized silver nanoparticles inhibit the growth of *Klebsiella pneumoniae*. The RNA-Seq analysis was performed to reveal the gene expression differences in *K. pneumoniae* cells exposed to the silver nanoparticles. Although the gene expression differences in *K. pneumoniae* exposed to silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1 were not statistically significant; some differences were observed in the expressions of genes related to carbohydrate metabolism, amino acid biosynthesis and degradation, and stress response in general. The results of this study contribute to the understanding of the molecular mechanisms of the toxicity of silver nanoparticles on bacterial cells.

Keywords: *Arthrobacter*, Biogenic silver nanoparticles, *Klebsiella pneumoniae*, RNA-sequencing, Transcriptomics

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SYMBOLS AND ABBREVIATIONS

AgNPs	: Silver nanoparticles
NPs	: Nanoparticles
NNI	: National Nanotechnology Initiative
nm	: Nanometer
EDX	: Energy-Dispersive Spectroscopy
SEM	: Scanning Electron Microscopy
XRD	: X-Ray Diffraction
DLS	: Dynamic light scattering
TEM	: Transmission electronic microscopy
AFM	: Atomic Force Microscopy
UV	: Ultraviolet-Visible Spectroscopy
MIC	: Minimum Inhibitory Concentration
FTIR	: Fourier Transform Infra-Red
ESBLS	: Extended spectrum β -lactamases
NGS	: Next generation sequencing

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1. INTRODUCTION

The world we live in is very complex and wide distinct from the lack of knowledge on the various communities and species of organisms. It simplifies the ultimate comprehension of microorganisms. There are about $4\text{--}6 \times 10^{30}$ prokaryotes in the world, in contrast to the approximately 7.7×10^9 humans (Whitman et al., 1998). The human body provides a base for several types of these microorganisms distributed around various body parts. The relationship between the host and the microorganisms is beneficial to each, it is a mutule connection for both, but some of these microorganisms can damage this beneficial relationship by causing infection during the permeation to access a normally sterile site (Altveş et al., 2020).

In order to treat these infections without damaging the host, antimicrobial products have been taken to kill or prevent the infected organism and retrain the host with no harm. Antimicrobial agents include antibiotics, antivirals, antifungals, and antiparasitics. The most commonly used antibiotics are isolated from bacteria and fungi. On the other hand, some bacteria can cause serious infections, such as pneumonia caused by *Klebsiella pneumoniae* a bacterium becoming more resistant to antibiotics over the years. Furthermore, the treatment became complicated as well; it might take a long period; thus, finding new approaches to address this issue is essential (Paczosa et al., 2016).

Researches have created one of the most dynamic fields of science and technology at the confluence of natural sciences, molecular engineering, biology, biotechnology, and medicine. Nanotechnology is considered a practical approach in this matter, it has the potential to work at the atomic, molecular, and supramolecular levels (on a scale of $\sim 1\text{--}100$ nm), to understand, generate, and utilize material structures, devices, and systems with principally new properties and functions resulting from their small structure (Roco et al., 2003).

Integrating nanotechnology with biotechnology to produce biologically-synthesized and environmentally sound technology that produces nanomaterials using green chemistry principles and eco-friendly techniques is called bio-nanotechnology (Abdel-Aziz et al., 2018). Materials with a diameter of 1 to 100 nanometers are called nanomaterials, and nanotechnology uses such materials to change or build innovative items and produce new materials.

The novel features of nanomaterials provide the opportunity to interact with complex biological functions in new ways, such as at the biomolecular level. This fast-growing field offers interdisciplinary researchers the opportunity to design and develop multifunctional nanoparticles which can target, diagnose and treat diseases such as cancer (McNeil et al., 2005).

The term "nanomedicine" points to nanotechnologies and nanoscience in healthcare and medicine. Nanomedicine utilizes nanoscale technology and nano-enabled techniques in diagnostics, therapy, disease prevention, and monitoring. It can offer enormous promise in imaging methods, tissue-engineered constructs, implants, drug delivery systems, diagnostic instruments, and pharmacological therapies. Cardiovascular diseases, mental and musculoskeletal ailments, cancer and neurological diseases, bacterial and viral infections, and diabetes are among the diseases for which nanotechnologies have advanced therapy (Lombardo et al., 2019).

Optimizing the synthesis of nanomaterials has become a rich field of study. Metal nanoparticles are especially unique in nanoscale systems owing to the ease of their synthesis and chemical modifications. It is generally known that the size, shape, and crystal structure of metal nanoparticles have a significant impact on their optical, electrical, and catalytic capabilities. For instance, differently shaped gold (Au) and silver (Ag) nanocrystals exhibit distinctive optical scattering responses (Daniel et al., 2004; Roduner, 2006).

Because of highly localized charge polarizations at corners and edges, anisotropic geometries like rods, triangular prisms, and cubes exhibit many scattering peaks in the visible spectrum as opposed to highly symmetric spherical particles, which only show one (Mie, 1908). As a result, the synthesis of metal nanoparticles with specific morphologies attracted a lot of attention. Metal nanoparticles (MNPs) and nanomaterials have been created using a variety of techniques (Nath et al., 2013).

1.1. Nanotechnology

Any nanoscale technology with various practical applications is considered to be nanotechnology. Literally speaking, nanotechnology entails the development and use of chemical, physical, and biological systems at scales ranging from single molecules or atoms to submicron dimensions, as well as the incorporation of these resulting in nanomaterials into larger systems (Nasrollahzadeh et al., 2019).

Nanomaterials are incredibly tiny, with at least one dimension being 100 nm or less. Nanoscale materials can be one, two, or three dimensional. They can be found in solitary, fused, aggregated, or agglomerated forms, with spherical, tubular, and irregular shapes (Trotta et al., 2019). Nanostructured materials have unique nanoscale optical, electrical, and/or magnetic properties and can be utilized in a variety of applications, such as electronics and medicine. They include nanorods, nanobelts, nanowires, nanotubes, quantum dots, nanoribbons, nanofibers, and hollow spheres. (Bayda et al. 2019).

The multi-agency National Nanotechnology Initiative (NNI) includes the methodical analysis, arrangement, control, and manipulation of matter at the atomic, molecular, and supramolecular scales in the size range of 1 to 100 nanometers (Figure 1.1). The discovery of novel nanoscale materials, phenomena, processes, and new experimental and theoretical research methodologies around the end of the 20th century opened new possibilities for the synthesis of novel nanomaterials and novel nano-systems. Many industries employ this application. This technology promises an edge in the scientific community and could provide novel materials that can help with the challenges facing clinical applications and other areas at the moment (Bhushan, 2017).

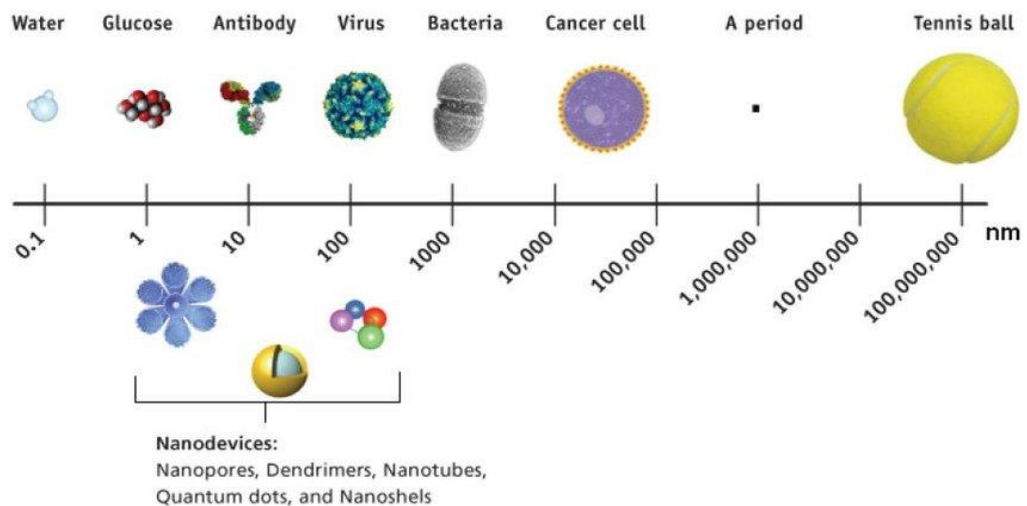


Figure1.1. Scaled objects in nanometer (Jabbar et al., 2018)

1.1.1. Nanofabrication and Nanomanufacturing

Nanofabrication and nanomanufacturing are employed to describe the formation of functional and intricate nanoscale structures in various industries including medicine, photonics, electronics, and energy. Nanomanufacturing is often associated with industrial-scale production methods, that use completely automated assembly lines, while nanofabrication focuses more on the research and advancement of new materials and processes, often performed by skilled artisans (Bechelany, 2022).

The three main ways for creating different nanomaterials are bottom-up, top-down, and hybrid approaches. The top-down method involves removing, carefully etching, and stacking the original component to reduce a bulk material to the required nanoform. E-beam lithography, optical lithography, block copolymer lithography, soft and nanoimprint lithography, and scanning probe lithography are examples of top-down lithography. On the nanoscale scale, bottom-up materials are constructed by manipulating the arrangement of molecules, atoms, macromolecules, or supramolecules (Harish et al., 2022).

1.1.2. Classification and Type of Nanomaterials

According to their dimensions, morphology, condition, and chemical composition, nanomaterials are categorized into two groups as consolidated materials and nano-dispersions (Figure 1.2). Nano-dispersive is a one-dimensional system that is termed nano-powders and nanoparticles while consolidated nanomaterials are further classified into several groups (Gleiter, 2000).

The three-dimensional nanomaterials are called nanoparticles in the nanoscale and there is no significant difference between the longest and shortest axes. Nanofibers have two-dimensions in the nanoscale. Materials such as nanoplates have one dimension in the nanoscale (Pokropivny et al., 2007; Aversa et al., 2018).



Figure 1.2. Classification of nanomaterials based on dimension (Paras et al., 2022).

Nanomaterials come in a variety of forms (Figure 1.3) such as natural nanomaterials occurring naturally in the world. This group includes particles that constitute volcanic ash, smoke, and even some molecules in our bodies, such as hemoglobin in our blood. The vibrant colors of peacock feathers are the consequence of the spacing between nanometer structures on their surface (Hochella et al., 2019).

The majority of artificial nanomaterials that result from human-made processes or objects are made of metals, metal oxides, ceramics, some nonmetals, and other nanostructured materials, whose central core is made up of inorganic materials that give them their magnetic, fluorescent, electrical, and optical properties. In bioimaging, targeted drug delivery, cancer therapies, and other technological fields like biosensing, chemical sensing, electronics, and optical applications, artificial nanomaterials such as gold nanoparticles, nonporous and mesoporous silica nanoparticles, magnetic nanoparticles, and quantum dots have shown great promise (Barhoum et al., 2022).

Unlike simple mixtures of its constituent parts, hybrid nanomaterials are distinct chemical conjugates of organic and/or inorganic materials. For example, fiber-reinforced materials known as engineered ceramics for specific uses (such as the aerospace sector) are examples of ceramic matrix composites. However, hydrogels have easy interactions between polymer chains and nanostructures, making them bioactive and adaptable to specific settings (such as tissue engineering). On the other hand, metal nanocomposites combine two or more intermetallic compounds and alloys

involving banded, core-shell, or nanometal components. The aerospace industry might use carbonaceous compounds that are present in the metal matrix (Saboori et al., 2018).

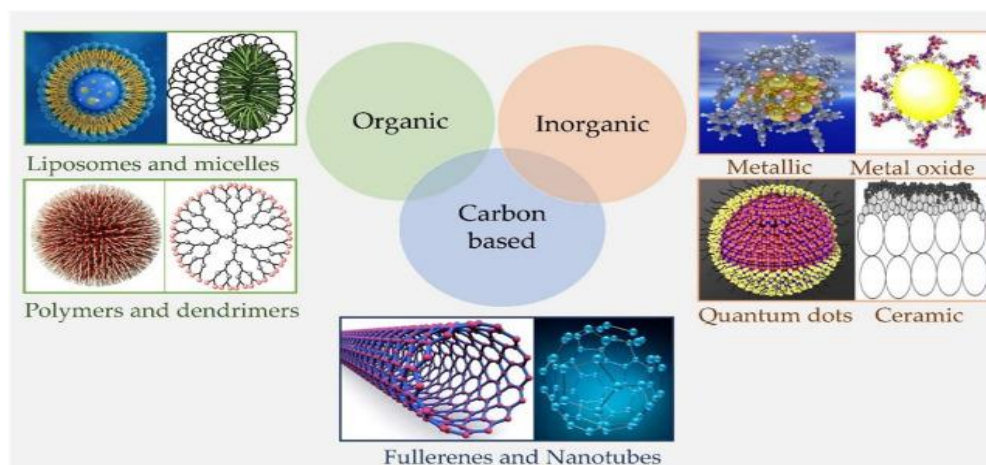


Figure 1.3. Various types of nanomaterials (George et al., 2022).

1.1.3. Nanoparticles (NP)

Nanotechnology has recently developed methods to create particles with sizes between 10 and 500 nm that are applied in the field of biology. Potential applications of nanotechnology in drug delivery systems and noninvasive imaging provides various advantages over conventional pharmaceutical agents (Mody et al., 2009).

Metallic nanoparticles showed a promising ability against multi-drug resistance (Figure 1.4). They are viable alternatives to manage bacterial infections. Recently, researchers determined that the most utilized and extensively researched metallic nanoparticle is silver (Liao et al., 2020).



Figure 1.4. Metallic Nanoparticles against multidrug-resistant bacteria (Mishra et al., 2022)

According to their size, shape, and physical and chemical characteristics, nanoparticles can be divided into a variety of categories, as illustrated in Figure 1.3:

- **Metal nanoparticles:** They are one of the most used nanoparticles now in various fields owing to their well-known localized surface plasmon resonance characteristics. They can be produced from materials that can be generated and altered to be conjugated with a variety of possible uses in biotechnology, magnetic separation, preconcentration of target analytes, targeted medication delivery, vehicles for delivering genes and drugs, and most critically diagnostic imaging (Mody et al., 2010).
- **Carbon-based:** In several sectors, the extensive application of carbon-based nanoparticles is of tremendous interest because of their electrical conductivity, high strength, unique structure, electron affinity, and versatility. Carbon-based nanomaterials include carbon nanotubes (CNT) that have promising properties for use in nanomedicine. The third allotropic form of carbon, known as fullerenes, has carbon atoms arranged in the shape of a regular, hollow sphere or ellipse. Because not all fullerenes are chemically stable enough, they are simply hypothetical. The nanometer-sized diamond crystals known as nano-diamonds (NDs) exhibit extraordinary hardness and great wear resistance. They are made of carbon atoms that are organized in predictable patterns. In the future, nano-diamonds may be used as medication carriers in medicine. Due to their characteristics and the simplicity of surface modification with different chemicals (Lisik et al., 2021).
- **Polymers:** Scientists have developed many techniques to synthesize polymeric nanoparticles with unique properties for a wide range of applications including surface coating, sensor technology, catalysis, and nanomedicine (Pecher and Mecking, 2010).
- **Ceramic:** They have a wide range of applications such as catalysis, photocatalysis, photodegradation of dyes, and imaging applications due to a number of favorable properties, it is an inorganic nonmetallic solid (Thomas et al., 2015).
- **Semiconductors:** They are considered important materials in photocatalysis, photo optics, and electronic devices because of their

properties between metals and non-metals. Nanoparticles can agglomerate and are typically instable; therefore, it is essential to safeguard them. Making core-shell particles or employing capping molecules as surface passivation are two helpful techniques (Bangal, 2005).

- **Lipids:** They are spherical vesicles made of ionizable lipids that are neutral at physiological pH (reducing potential harmful effects, as opposed to positively charged lipids, such as liposomes) and positively charged at low pH (allowing RNA complexation). For instance, lipid nanoparticle-mRNA vaccines are presently being employed in clinical trials against coronavirus illness 2019 (COVID-19), which represents one of their most effective attempts to deliver mRNA to patients (Hou et al., 2021).

1.1.4. Synthesis of Nanoparticles

Top-down and bottom-up are the two basic methods used in nanoparticle synthesis. The milling, lithography, and repeated quenching techniques used in top-down processes do not have adequate control over the particle size and structure. Because it starts from the bottom up and includes creating matter atom by atom, molecule by molecule, and cluster by cluster, the bottom-up method is the one that scientists most frequently utilize to create nanoparticles (Biswal, 2012).

Numerous techniques can be used to create nanoparticles involves both chemical techniques that use highly hazardous materials and physical techniques that call for electrical or thermal energy. Due to the dangers associated with both physical and chemical methods, researchers are concentrating their efforts on developing a safer and cleaner biosynthesis of nanoparticles based on green chemistry principles (Rónavári et al., 2021).

The creation of nanoparticles by bio-synthesis has been proposed as an economical and ecologically responsible alternative to chemical and physical processes. Using plants and microorganisms to synthesize nanoparticles for the use of biomedical applications is a favorable option (Razavi et al., 2015).

1.2. Silver Nanoparticles (AgNPs)

Silver nanoparticles are among the metallic nanoparticles that are mainly utilized due to their low cost and unique properties. The large surface area can change the physical, biological and chemical properties of AgNPs.

Silver nanoparticles are available as nanodots or nano-powder or flakes and other structures. Each existing form is used in a specific field, depending on the properties and the surface area (Husen and Siddiqi, 2014).

They have shown excellent bactericidal properties against a vast range of microorganisms. Numerous methods can be used to create silver nanoparticles so that their shape and physical properties can be examined. The biological procedures exhibit high submission and stability, whereas the physical and chemical methods appear to be very costly and critical. Therefore, because they are straightforward and non-toxic, biological approaches are reliable. The physicochemical parameters of nanoparticles must be accurately determined after synthesis, and this might have a substantial impact on the particles' biological properties (Gurunathan et al., 2015).

1.2.1. Synthesis of Silver Nanoparticles

Silver nanoparticles (AgNPs) can be created by physical, chemical, or biological techniques, respectively.

1.2.1.1. Physical Methods

The manufacture of silver nanoparticles can be accomplished using a variety of techniques, including the matrix isolation approach and the gas flow cold trap method. The synthesis of nanoparticles can be done by using a small ceramic heater with a local heating surface. The small ceramic heater is used to vaporize starting materials. Since the temperature gradient at the heating surface is much steeper than that of a tube furnace, it can therefore cool at a suitable and reasonably quick rate. This physical method can be helpful as a nanoparticle generator for long-term experiments, inhalation toxicity studies, and as a calibration tool for nanoparticle gauges. The concentration of nanoparticles increases with heater surface temperature (Iravaniet al., 2014).

Laser ablation is another method for producing silver nanoparticles. Numerous factors, such as the period of the laser pulses, the wavelength of the laser impinging on the metallic item, the period of ablation, the laser fluence, and the functional liquid

medium, affect the ablation efficiency and characteristics of the nano-silver particles. The lack of chemical reagents in solutions makes laser ablation mechanization superior to other techniques for creating metal colloids (Abou El-Nour et al., 2010).

1.2.1.2. Chemical Methods

The preparation in chemical methods requires water or organic solvents with three essential compounds stabilizing/capping agents, metal precursors, and reducing agents (Zhang et al., 2016).

Chemical reduction utilizing reducing agents (both organic and inorganic) is the process most frequently used to create silver nanoparticles. Numerous reducing agents are used in reducing processes, including sodium borohydride (NaBH_4), sodium citrate, Tollens reagent, ascorbate, elemental hydrogen, N, N-dimethylformamide (DMF), polyol techniques, poly (ethylene glycol), and AgNO_3 (Figure 1.5) (Iravaniet al., 2014).

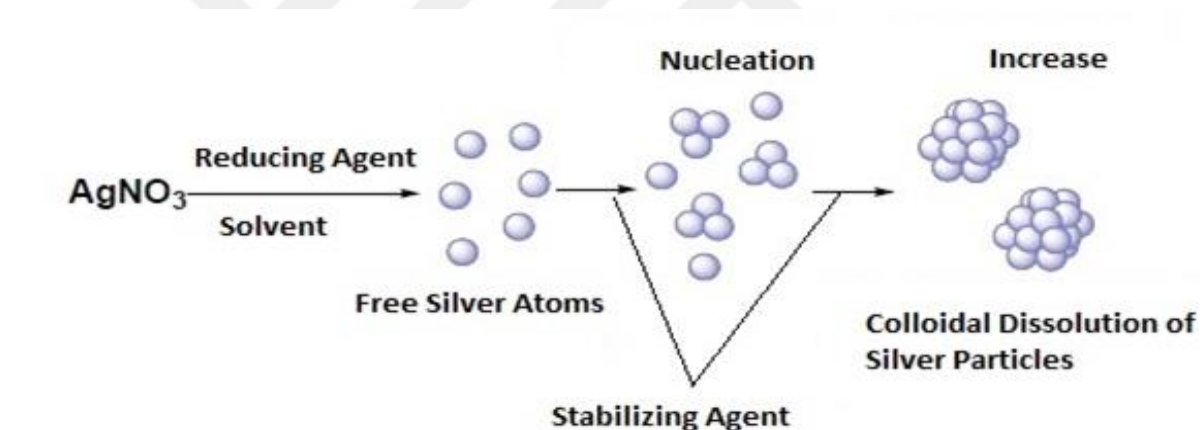


Figure 1.5. Mechanism of the chemical reduction in a solution of AgNO_3 to form silver nanoparticles (Monge, 2009)

1.2.1.3. Biological Synthesis

These methods use a biological system such as fungi, plants, and bacteria, to synthesize nanoparticles, which are alternatives to chemical processes (Figure 1.6). The advantages of these techniques include the generated nanoparticles' high density, stability, diversity of biological resources, and simplicity in water solubility. The shape and structure of silver nanoparticles are reliably influenced by biological activity (Thakkar et al., 2010).

The reducing agent, non-toxic substance, and solvent are just a few of the many components that are necessary for the biological production of nanoparticles. Biological methods can achieve particles that are controlled in size and shape, through the reducing agents by using bacterial proteins or plant extracts, which is a significant factor for several bio-medical applications. Despite the many successful studies of synthesized silver nanoparticles in different shapes and size ranges, certain limitations still remain (Singh et al., 2016).

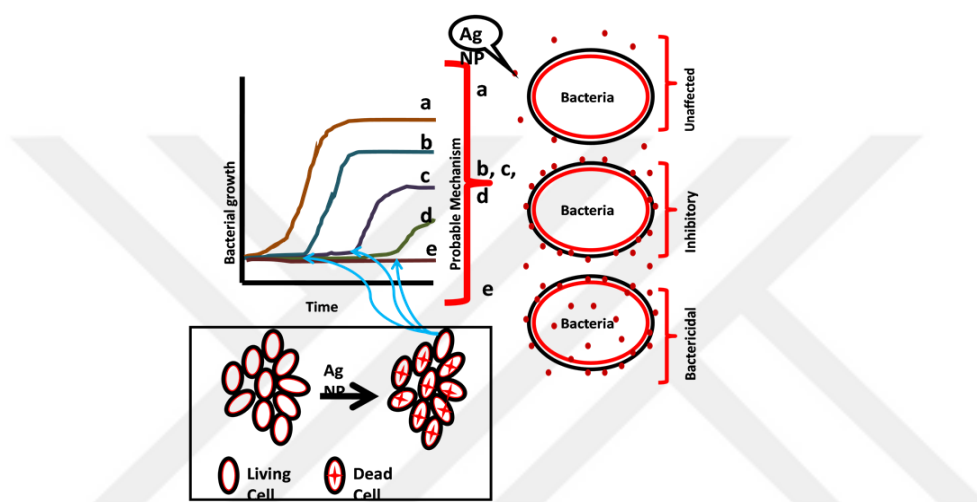


Figure 1.6. Silver nanoparticles synthesized from bacterial cultures by green method (Perni et al., 2014)

1.2.2. Characterization of Silver Nanoparticles

A variety of analytical techniques, including X-ray diffraction (XRD), UV-vis spectroscopy, scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), dynamic light scattering (DLS), atomic force microscopy (AFM), and transmission electron microscopy (TEM), are used to characterize the functional aspects of the synthesized particles (Gurunathan et al., 2016).

1.2.2.1. X-Ray Diffractometry (XRD)

Molecular and crystal structures, quantitative resolution of chemical species, isomorphous substitutions, qualitative identification of diverse compounds, particle sizes, etc. are all examined using XRD as an analytical tool. Any crystal can reflect an X-ray beam's light in a variety of diffraction patterns. It reflects a crystal structure's physical characteristics. The structural characteristics of several materials, including biomolecules, glasses, inorganic catalysts, polymers, and superconductors, can be examined using XRD. The creation of the diffraction patterns plays a major role in the investigation of these materials (Vaia et al., 2012).

1.2.2.2. Fourier Transforms Infrared Spectroscopy (FTIR)

FTIR analysis is frequently used to examine the implicated biomolecules in synthesis. Modest absorbance changes can now be detected, making it possible to do difference spectroscopy and separate the modest uptake bands of the functionally active residue from the substantial background uptake of the entire protein (Kim et al., 2001).

By using FTIR analysis, it is possible to confirm that active molecules have been covalently grafted onto gold nanoparticles, carbon nanotubes, graphene, and interactions between enzyme and substrate during catalysis. Strong signal, good signal-to-noise ratio, quick data capture, and little sample heating are all characteristics of FTIR spectrometers, which are considered as main benefits of FTIR (Gurunathan et al., 2014; Kumar et al., 2010).

1.2.2.3. UV-Vis Spectroscopy

It is a method that is regarded as reliable and advantageous for the initial characterization of synthesized nanoparticles (NPs). It is also used to monitor the stability of the synthesized nanoparticles and the unique optical characteristics of the nanoparticles that strongly interact with particular wavelengths of light. The assimilation of the nanoparticles depends on size, chemical surroundings, and dielectric medium, the peak—assigned on the surface plasmon lies between 200-600 nm in range for different metal nanoparticles within a range size from 2 to 100 nm (Zhang et al., 2016; Henglein, 1993).

1.2.2.4. Scanning Electron Microscopy (SEM)

SEM employs a surface imaging technique that can recognize the distribution sizes of particles, the shape of the nanomaterial, and the surface morphology at the micro and nanoscales of the manufactured particles. SEM is used to evaluate the morphology of nanoparticles smaller than 10 nm, but because of its limitations, it is unable to discern the internal structure. Nevertheless, it can be quite useful for determining the purity and degree of particle aggregation (Fissan et al., 2014).

1.3. Applications of Silver Nanoparticles

Silver nanoparticles possess a privileged use in various applications (Figure 1.7), due to their unique properties. Silver nanoparticles are an excellent choice for a wide range of applications in many different fields, including biology and medical science, due to their special qualities. AgNPs are widely used as antibacterial agents in the sanitary industry, food storage, textile coatings as well as in a number of environmental applications, disinfection of medical devices and household appliances to water treatment (Bosetti, 2002; Cho et al. 2005; Gupta, 1998, Jain et al., 2005; Li, 2008).

In addition, it has been shown that silver nanoparticles are antiviral against HIV-1 in non-cytotoxic concentrations, but the mechanism underlying their HIV-inhibitory activity is still not entirely understood (Kholoud et al., 2010).

Halogenated organic pollutants can be reduced using silver nanoparticles as an excellent heterogeneous catalyst. Additionally, they enhance the ability of organic colours to bleach. In the process of treating water, the biologically produced silver nanoparticles on nitrocellulose membrane filters have the potential to inhibit and inactivate microorganisms like *Escherichia coli* and *Enterococcus faecalis*. Since silver nanoparticles are excellent antibacterial agents, many food and agricultural products contain them as preservatives (Sonika Dawadi et al., 2021).

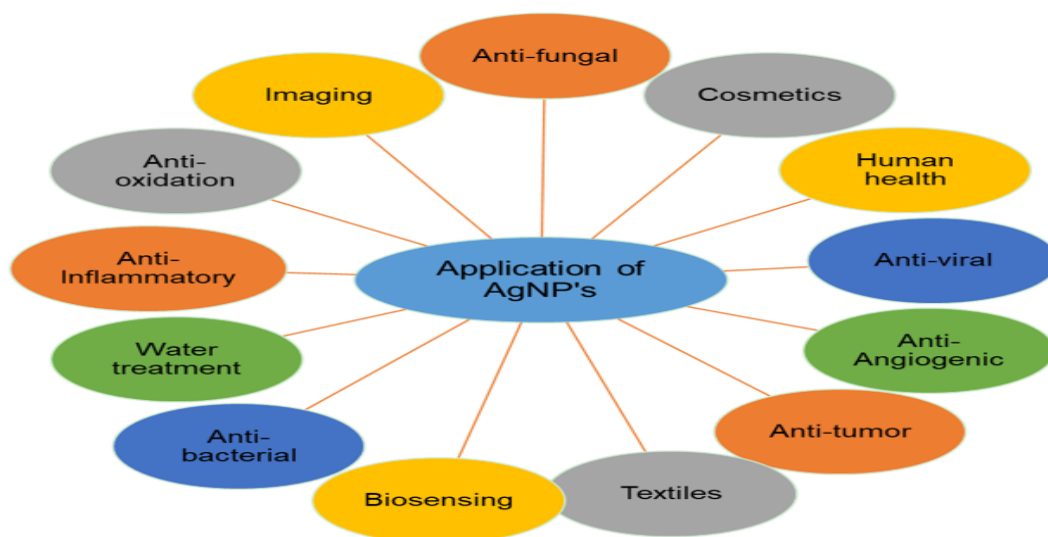


Figure 1.7. Applications of silver nanoparticles (Agrawal et al., 2018)

1.4. Mechanism of Action of Silver Nanoparticles

Although the particular mechanism of the antibacterial activity of silver nanoparticles is uncertain, they exhibit many antibacterial effects. Since silver nanoparticles have the capacity to continuously release silver ions that can be used to kill microorganisms, it is crucial to understand how these particles are produced in different types of cells (bacteria, fungi, and plants), how they interact with different cells, and how they can be used in a variety of different applications (Yin et al., 2020).

Silver nanoparticles can permeate the cell walls of the bacteria or attach to the surface. They can cause a change in the structure of the cell wall membrane, for instance, the death of the cell and the permeability of the cell. The suggested mechanism that has been studied by electron spin resonance spectroscopy mentioned the silver nanoparticles have a formation of free radicals that have a deadly and inhibitory effect on the bacteria (Franci et al., 2015; Rajivgandhi et al., 2020).

A change in the structure of the cell membrane, such as increased cell membrane permeability and cell death, results from silver nanoparticles' ability to anchor in the bacterial cell wall and then enter it (Figure 1.8). AgNPs build up on the cell surface has primarily been seen in Gram-negative bacteria. Through a water-filled channel called a porin found in the outer membrane of Gram-negative bacteria, AgNPs can penetrate bacterial cells (Sondi et al., 2004).

Gram-negative bacteria cell walls contain lipopolysaccharides to help maintain their structural integrity. As a result, the bacteria are more vulnerable to silver

nanoparticles because the lipopolysaccharides negative charge encourages AgNPs adherence. Due to the passage of silver ions into the cytoplasm rather than Gram-positive bacteria's thicker cell wall, the action of AgNPs is more evident in Gram-negative bacteria (Chauhan et al., 2016).

The DNA of the cell contains sulfur and phosphorus as its main components, in which the silver nanoparticles have the ability to act on these soft bases and destroy the DNA which would definitely lead to the death of the cell. Furthermore, silver ions can inhibit protein synthesis by denaturing cytoplasmic ribosome (Mikhailova et al., 2020).

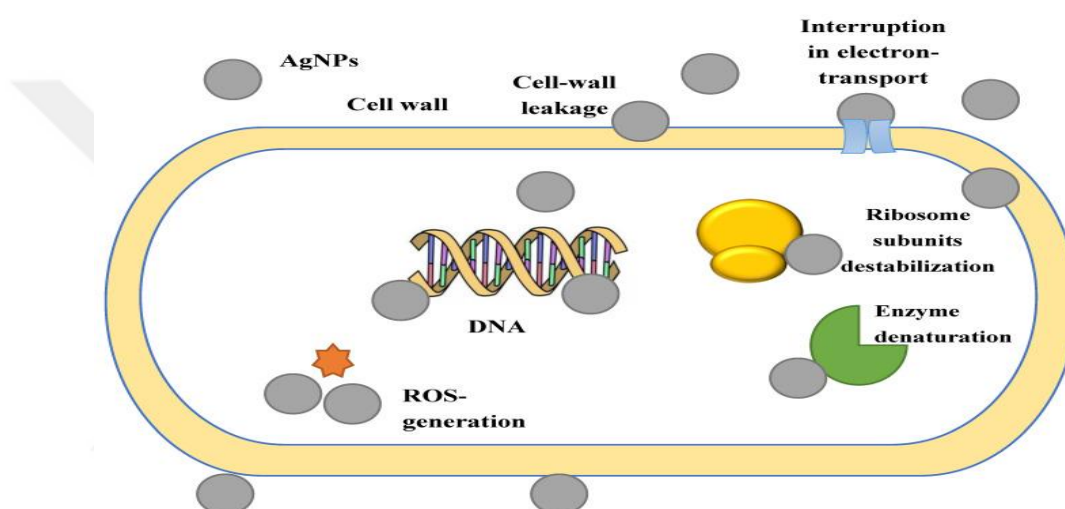


Figure 1.8. Mechanism of action of AgNPs on cell (Mikhailova et al., 2020)

1.4.1. Mechanism of AgNPs Biosynthesis

Numerous investigations have demonstrated that bacteria produce nanoparticles intracellularly. The membrane proteins that carry silver ions into the cell are connected to this process, which is present in Gram-negative bacteria. By acting as an electron shuttle, taking electrons from the nitrate molecule and transferring them to the metal ion to form nanoparticles, as shown for some microorganisms like *Pseudomonas aeruginosa*, *Fusarium oxysporum*, and others.

The reduction of nitrate is necessary for the formation of silver nanoparticles (Figure 1.9) (Kumar et al., 2007).

The proteins and carbohydrates located in the cell wall, which might be implicated in the uptake of silver ions, may be used in the bio-reduction process.

Positively charged silver ions interact electrostatically with a carboxylate group on the surface of the bacterial cell, giving rise to a negative charge that aids in the collection of silver ions. The oxidoreductase enzyme is primarily activated by high pH, which also helps to increase the conversion of tryptophan into a transition tryptophil radical, which reduces silver ions to elementary silver. This impacts the supernatant culture's activation of reducing agents, which releases electrons to decrease Ag^+ to Ag^0 nanoparticles (Nam Lee et al., 2008).

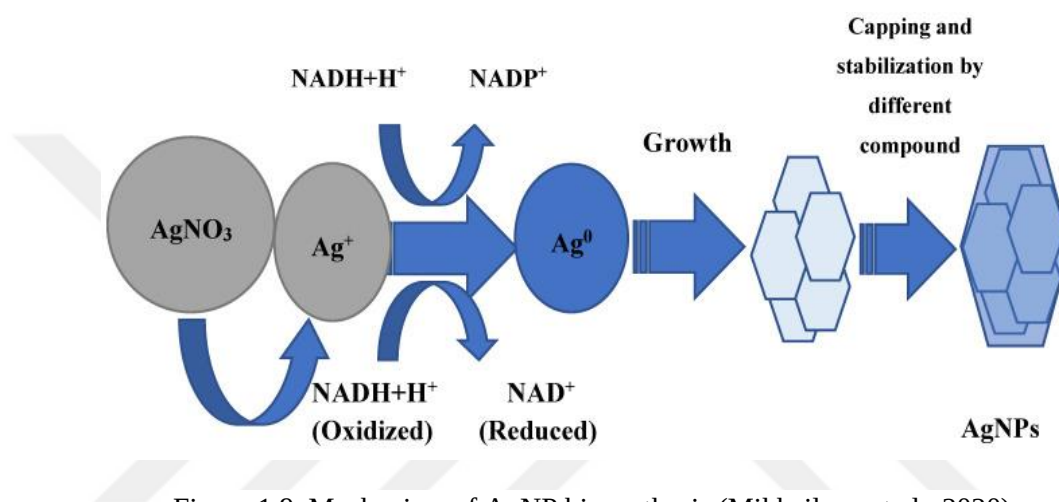


Figure 1.9. Mechanism of AgNP biosynthesis (Mikhailova et al., 2020)

1.5. History of Silver Nanoparticles

Silver nanoparticles are one of the most used nanomaterials currently. The concept of silver nanoparticles is based on the notion that it is something new that has not been seen until recently, and the advances in nanotechnology opened up entirely new areas of application for silver. However, cutlery was used by the Romans and Ancient Greeks to keep water, food, and wine to prevent rotting, but it has also been used for thousands of years for antibacterial and therapeutic purposes. Hippocrates employed silver-based remedies to cure ulcers and expedite the healing of wounds (Alexander et al., 2009).

Sims used delicate silver wires to stitch vesicovaginal fistulas that had formed following childbirth in 1852, greatly reducing infections. Silver treatments were created in the 19th century to heal burns and wound infections. The medical use of silver led to the clinical introduction of antibiotics in the 1940s, since the 1980s the misuse of antibiotics has become a worldwide problem due to bacterial resistance;

therefore, interest in silver has increased particularly since nanotechnology was developed at the beginning of this century (Barillo and David, 2014).

Around 920 AD, Avicenna, a Persian scholar and writer, introduced silver fillings to treat various ailments, from bad breath to irregular heartbeat, and even for blood purification (Wadhera et al., 2005). Paracelsus, a Renaissance alchemist, applied silver nitrate salt as a cautery to treat wounds in 1520. While around 1614, Angelo Sala, an Italian physician and chemist, took silver nitrate solutions as a laxative to treat brain inflammation (Hill and Pillsbury, 1939).

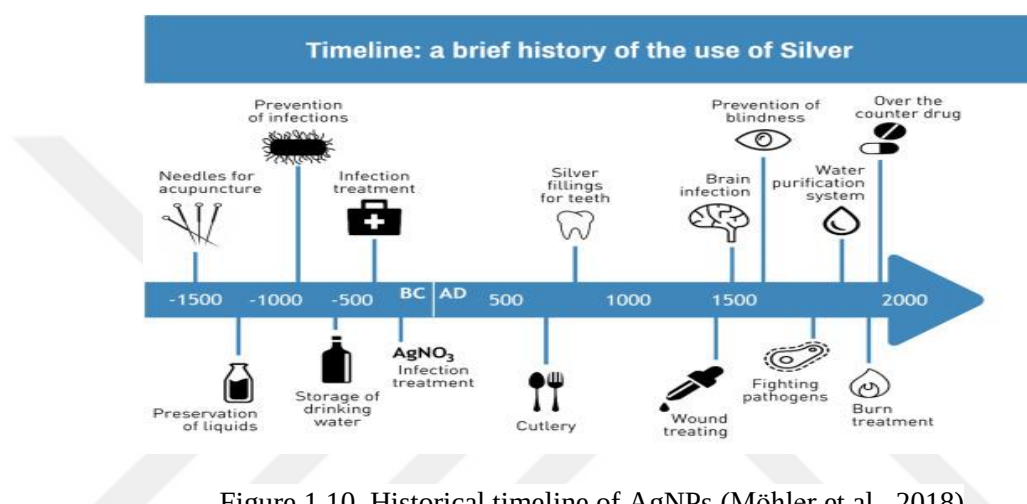


Figure 1.10. Historical timeline of AgNPs (Möhler et al., 2018)

1.6. *Klebsiella pneumoniae*

Klebsiella pneumoniae was identified by German scientist Carl Friedlander who was able to isolate it from a patient with infected lungs in 1882 (Schroeter, 1889).

Klebsiella pneumoniae is a member of *Enterobacteriaceae*. It is a Gram-negative bacterium with a large genome, including plasmids. It can divert into hypervirulent, opportunistic, and multi-drug resistant, causing health issues. *Klebsiella pneumoniae* is a non-motile, encapsulated bacillus. *Klebsiella* species without capsules are more likely to be less malignant and more responsive to be treated with antibiotics without resistance alert (Ashurst et al., 2018). The lipopolysaccharide layer in *Klebsiella pneumoniae* and the outer surface of the bacterium can trigger an inflammatory cascade in the host organism (Bulati et al., 2021).

Resistance to beta-lactams was discovered in 1929 by Alexander Fleming in this Gram-negative organism. Since then, *Klebsiella pneumoniae* has come under intensive

studies to capture gene expression, which is constantly changing to reduce new orientations in resistance (Ashurst et al., 2018).

The dramatic increase in resistance has made *K. pneumoniae* one of the most studied bacteria due to the shortage of antimicrobial therapeutics. Various resistant mechanisms are detected for *K. pneumoniae*, and the resistance to beta-lactams has the utmost influence over functional curing (Jiang et al., 2020).

Three families were identified in the chromosomes of *K. pneumoniae*, SHV, LEN, and OKP, and corresponding ampicillin resistance is a characteristic of the species. The existence of the encoded enzyme in the core genome of *Klebsiella pneumonia* causes resistance to some -lactams, such as SHV, which is consistently found in the chromosome (Babini et al., 2000; Bialek et al., 2014).

Klebsiella species are widespread in nature, including plants, animals, and humans, either as colonization or infection. Despite their presence in humans, significant infections can occur, including urinary tract infections, liver abscesses, bacteremia, and pneumonia. Infections can be either primary, for example, urinary tract infections or pneumonia caused by classic *K. pneumoniae* strains, or secondary infections, caused by bacteremia that develop from secondary spread from primary infection in the lungs or bladder. The risk of *K. pneumoniae* becoming multidrug-resistant in nosocomial infections is higher than in community-acquired infections because many patients have been treated with antibiotics (Chang et al., 2021).

An illness known as pneumonia results in tissue swelling of one or both lungs that may acquire severe symptoms requiring hospital care. Nevertheless, the human body is considered a habitat for *Klebsiella*, which exists in the intestines causing no harm, yet, when it reaches other parts of the body, it could cause disease after the bacterium spreads. It is not airborne; therefore, it minimizes the risk of getting infected by breathing. Nonetheless, direct contact by hand touch, wounds, and the use of personal things of the infected person is likely to cause the infection. People with immune deficiency are more potentially getting infected. Gut colonisation is a significant reservoir for strains that cause nosocomial infections, according to serotyping based on the *Klebsiella* capsule. However, serotyping does not offer enough resolution to distinguish between strains that are closely related. In two recent investigations, the relationship between *K. pneumoniae* colonisation and future

infection was investigated in order to comprehend how *K. pneumoniae* infection develops (Martin et al., 2018; Gorrie et al., 2017).

The complete mechanism of how the infections occurred is not clearly understood yet. The suggested mechanism indicates that bacterial density takes a role in the advancement of the disease; while the risk to health care has been identified, the progression of colonization to infection has not been clarified (Donskey, 2004; Taur et al., 2012).

The frequent changes in *K. pneumoniae* to defend itself with outer layers to surpass the ability of antibiotics result in the bacterial strains becoming more multidrug resistant and hypervirulent (Doorduyn et al., 2016).

1.6.1. Silver Nanoparticles against *Klebsiella pneumoniae*

Broad-spectrum antibacterial agents can be made from silver nanoparticles using a green manufacturing process to fight many microbes. To evaluate the biocidal activity of silver nanoparticles against *Klebsiella pneumoniae* the difference in gene expression was determined by using real-time PCR (RT-PCR) (Foroohimanjili et al., 2020).

In a recent study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was employed as a housekeeping gene control during the quantitative real-time PCR, which was carried out using particular primers. An increase of gene expression was clearly visible in the data. Direct interactions of silver nanoparticles with DNA molecules and proteins, including enzymes, can interfere with bacteria's ability to build biofilms and infiltrate hosts. Indirect effects come from bacterial cells experiencing more oxidative stress. Additionally, a gene mutation brought on by the reduction of silver ions causes disruption of the process of protein synthesis. Silver nanoparticles can decrease protein levels in multidrug-resistant bacteria (Hamida et al., 2020).

1.7. Transcriptome Sequencing and Gene Expression

Gene expression studies and differential gene expression can reveal important aspects of the cell states. In recent years, hybridization-based methods such as microarrays have been the most widely used solutions for gene expression due to their high throughput and relatively low cost. Transcriptome analysis has undergone a radical change thanks to the development of high-throughput next-generation sequencing (NGS) technologies. However, many of the problems that Sanger

sequencing-based microarrays and hybridization, which were previously employed to quantify gene expression, presented, were not taken into consideration during the development of this new technology (Schena et al., 1995; Casneuf et al., 2007; Shendure, 2008).

For the study of differential gene expression (DGE) across the transcriptome, RNA-seq has emerged as a crucial technique. In order to do a DGE study, RNA must first be extracted, followed by mRNA enrichment or ribosomal RNA depletion, cDNA synthesis, and production of an adapter-ligated sequencing library. Following library preparation, the library is sequenced on a high-throughput platform. The identification and control of cells and the processes taking place inside of them are specified by the transcription of a subset of genes into complementary RNA molecules. Understanding the functional components of the genome, as well as how development and illness occur, depends primarily on RNA molecules (Kukurba et al., 2015).

The RNA-seq technology is still under active development and offers a number of benefits in addition to being able to find transcripts that match genetic sequences already in existence. RNA-seq is useful for studying complex transcriptomes because it can detect sequence differences. RNA-Seq has also been shown to be very accurate for measuring expression levels as determined by quantitative PCR (Wang et al., 2009).

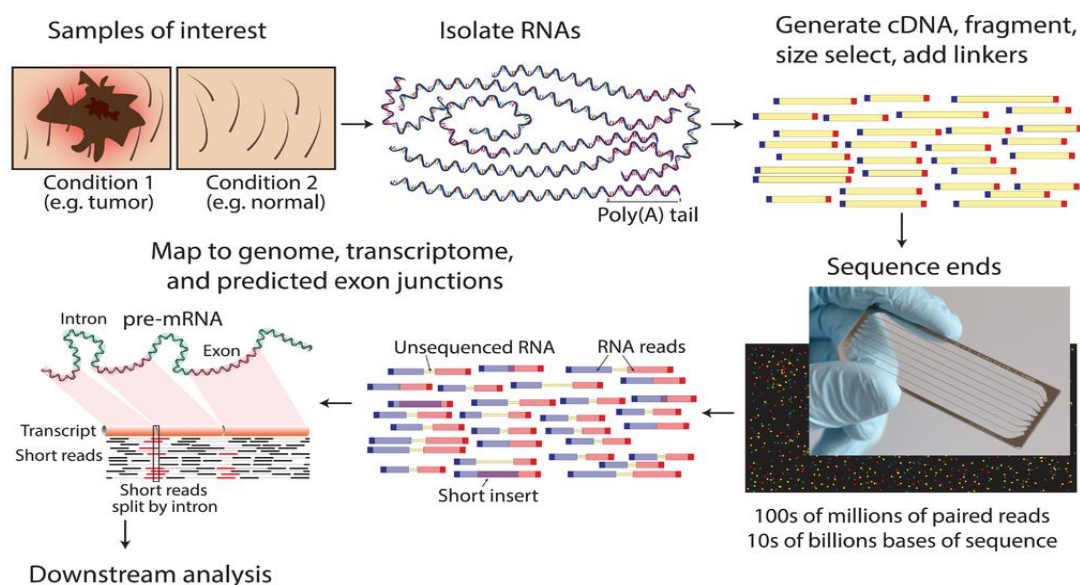


Figure 1.11. RNA converted to cDNA libraries isolated from multiple samples (Griffith et al., 2015)

1.8. The Aims of This Thesis

Actinobacteria are a group of bacteria known for their capability to produce various types of bioactive secondary metabolites. These bacteria can be extracted from a variety of habitats, including terrestrial ecosystems, and exploited to create a variety of materials, including nanoparticles with biotechnological uses. It is generally known that many pathogenic bacteria, including *Klebsiella pneumoniae*, are susceptible to the antimicrobial effects of silver nanoparticles. In this study, it was aimed at synthesis of silver nanoparticles by an actinobacterium isolated from Antarctic soil and exposure of *K. pneumoniae* strain to the synthesized silver nanoparticles to see molecular effects in transcriptomic level. Thus, the objectives of the thesis are as follows:

- Synthesis of silver nanoparticles by *Arthrobacter* sp. H14-L1
- Characterization of silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1
- Genome analysis of *Arthrobacter* sp.H14-L1
- Exposure of *K. pneumoniae* strain with the silver nanoparticles followed by RNA-seq analysis to perform transcriptomic analysis

2. LITERATURE REVIEW

The use of silver nanoparticles against *Klebsiella pneumonia* is taking a wide attention from scientists and researchers.

The effectiveness of silver nanoparticles against the biofilm of strains of *Escherichia coli* and *Klebsiella pneumoniae* that produce β -lactamases demonstrated that they have antibiofilm activity, and as a result, the silver nanoparticles have therapeutic potential in the coating of medical devices and the treatment of infections brought on by high antibiotic resistance (Azam et al., 2014).

It has been reported that *Urtica dioica* Linn. has antibacterial, antifungal, antiviral, and antioxidative properties. In addition, the silver nanoparticles synthesized by *U. dioica* Linn. were shown to have antibacterial activity. These AgNPs have also ability to work in concert with antibiotics to combat a variety of pathogenic bacteria, with potential use in biomedicine made possible by their antibacterial and synergistic activity with traditional antibiotics against a variety of harmful bacteria (Jyoti et al., 2016).

A new field of development has been merged against the resistance of *Klebsiella pneumoniae* and *Staphylococcus aureus* by utilizing AgNPs conjugated with rifampicin. Rifampicin-AgNP showed antibiofilm activity against certain species of bacterial pathogens. They were highly active against *K. pneumoniae* and against *Streptococcus mutans* as well as against methicillin-resistant *Staphylococcus aureus* (MRSA) at low concentrations (Umar et al., 2019).

By creating lysozyme-encapsulated silver nanoparticles (L-Ag NPs), which induce an antibacterial response similar to that of triclosan in multidrug-resistant (MDR) *Klebsiella pneumoniae*, researchers were able to characterize the antibacterial action of these particles against MDR *K. pneumoniae* through chemical analysis, biochemical assays, and deep RNA sequencing. At low concentrations, *K. pneumoniae* reproduction could be prevented by L-Ag NPs. The anti-*K. pneumoniae* impact of Ag-Np was due to the type II fatty acid biosynthesis of Ag^+ in conjunction with oxidative stress that was generated (Sathesh et al., 2021).

After being washed, boiled, and incubated using green synthesis, the silver nanoparticles from the *Acacia* leaves were shown to have antibiofilm, antibacterial, and anti-persister activities against *Klebsiella pneumoniae*. Additionally, the electrochemical cell was used to create graphene nanosheets. The antibacterial activity of the graphene-silver nanocomposite was improved against a range of microbial pathogens. Also, the synergistic effect of graphene-silver nanocomposite was demonstrated to have antibiofilm and anti-persistent cell development in isolates of *K. pneumoniae* (Aziz et al., 2022).



3. MATERIALS AND METHODS

3.1. Synthesis of Silver Nanoparticles

Biogenic silver nanoparticles were synthesized by *Arthrobacter* sp. H14-L1 isolated from Antarctica Horseshoe Island within the scope of the 6th Turkish Antarctic Expedition. *Arthrobacter* sp.H14-L1 was activated in tryptone-yeast extract-glucose agar (TYG) and glucose-yeast extract-malt extract agar (GYM). After growth on agar media, the colonies were transferred into a 30 ml tryptone soy broth (TSB) medium. The broth culture was incubated at 20°C for 3-5 days in a shaker incubator, and then, 5 ml volume was inoculated into 100 ml TSB medium. The inoculated flasks were incubated at 20°C for 3-4 days. After incubation, the culture broths were centrifuged at 8,000 rpm (4°C) for 20 minutes, and the supernatants were collected. For the synthesis of silver nanoparticles, 2 mM AgNO₃ solution (200 ml) was mixed with the same volume of culture supernatants and stirred constantly. Then, the supernatant-AgNO₃ mixture was placed in a shaker incubator (150 rpm, 50°C) for 3-5 days. On the second day, the colour changed from light brown to dark brown, indicating the development of silver nanoparticles. After the incubation, silver nanoparticles were recovered by micro-centrifuging for 20 minutes at 14,000 rpm. The collected silver nanoparticles were twice cleaned with sterile distilled water before being dried on a heat block at 50 °C.

3.2. Characterization of Silver Nanoparticles Synthesized by *Arthrobacter* sp. H14-L1

In order to understand nanoparticle behaviour, safety, biodistribution, and efficacy, it is crucial to characterize its physicochemical properties. Characterization was done using a number of analytical techniques, such as energy dispersive X-ray spectroscopy (EDX), scanning electron microscopy (SEM), X-ray diffraction (XRD), and UV-Vis spectroscopy, to assess the functional aspects of the nanoparticles. Silver nanoparticles' (AgNPs') distinctive optical characteristics enable them to interact with particular wavelengths of light, and their adsorption is influenced by their size and chemical environment. UV-Vis spectroscopy was able to monitor the structure and stability of AgNPs. The spectroscopic analysis was performed in the range of 200-800 nm with a resolution of 1 nm using a quartz cuvette. The XRD analysis was performed after centrifugation, and a powdered sample of synthesized AgNPs was deposited on a diffractometer glass substrate at 40 kV voltage and 30 mA current using Cu-K

radiation (wavelength 1.5418) in the 2 θ range of 5° up to 100° treated at a scan speed of 2°/min. The size, shape and elemental analysis of the silver nanoparticles were analysed by SEM (JEOL JSM-7001f). In addition, EDX analysis was performed to verify the elemental composition of the silver nanoparticles and identify signals for silver atoms.

3.3. Genome Analysis of *Arthrobacter* sp. H14-L1

The genome of strain H14-L1 was downloaded from the NCBI/GenBank using the accession number JAPNLH000000000. The phylogenomic analysis was conducted on Type Strain Genome Server (TYGS) available at <https://tygs.dsmz.de/> (Meier-Kolthoff and Göker, 2019). The secondary metabolite-coding gene clusters were determined on antiSMASH server (<https://antismash.secondarymetabolites.org/#!/start>) (Blin et al., 2023).

3.4. Antimicrobial Activity of Silver Nanoparticles Synthesized by *Arthrobacter* sp. H14-L1 against *Klebsiella pneumoniae*

Antibacterial activity and minimum inhibition concentration of silver nanoparticles against *K. pneumoniae* were determined by microdilution method. The *K. pneumoniae* strain was activated on nutrient agar medium at 37°C for 24 hours, and then, the microorganism was suspended in sterile Ringer's solution (Oxoid) to obtain an optical density between 0.5-1.0 corresponding to $1.5\text{--}3.0 \times 10^8$ CFU/ml. A volume of 100 μ l pathogen solution was added to each well including 100 μ l nutrient broth medium. Then, the diluted solutions of silver nanoparticles were added serially. After 24 h incubation at 37°C, the growth pattern was observed visually.

3.5. Silver Nanoparticle Exposure Test

After determining MIC against *K. pneumoniae*, MIC₂₅ was used for the exposure experiment. Nutrient broth medium including AgNPs at MIC₂₅ concentration was inoculated with *K. pneumoniae* strain suspended in Ringer's solution at McFarland 4.0 density. The culture medium without AgNPs was prepared as the control. After 46 h incubation at 37°C in a shaker incubator (100 rpm), the cultures were centrifuged at 4°C at 8000 rpm for 10 min and suspended in glycerol solution (40%, v/v).

3.6. RNA-Seq Analysis

RNA sequencing for the AgNP-exposed and control groups was performed by the Macrogen Inc. (Korea). Firstly, the RNA extraction was performed and the samples that pass quality assurance requirements moved on to the library development process. The library preparation was performed by NEBNext rRNA Depletion (Bacteria) and TruSeq Stranded Total RNA Library Prep Gold Kit. Briefly, the random fragmentation of the cDNA samples, followed by the ligation of the 5' and 3' adapter, were used to generate the sequencing library. With a PCR primer solution that anneals to the ends of each adapter, fragments that had been adapter-ligated were then amplified. The library templates underwent quality control and quantification process. The library was placed onto a flow cell, where fragments were collected on a lawn of surface-bound oligos that complemented the library adapters. Then, using bridge amplification, each fragment was amplified into a unique clonal cluster. The templates were prepared for sequencing on an Illumina platform once cluster creation was finished. Raw pictures were produced by the Illumina sequencer using integrated primary analysis software called RTA (Real Time Analysis), which also served as the system control and base calling software. The BCL/cBCL (base call) binary files were converted into FASTQ files using bcl2fastq provided by the Illumina. The raw sequence data were submitted to the NCBI Sequence Read Archive (SRA). Differential gene expression analysis was performed on RaNA-Seq platform available at <https://ranaseq.eu/> (Prieto et al., 2020). The samples were quantified by Salmon algorithm (Patro et al., 2017) with default parameters. The differential gene expression analysis was performed with DESeq2 (Love et al., 2014).

4. RESULTS AND DISCUSSION

4.1. Synthesis of Silver Nanoparticles

The silver nanoparticles were synthesized by the culture supernatant of *Arthrobacter* sp. H14-L1 (Figure 4.1).

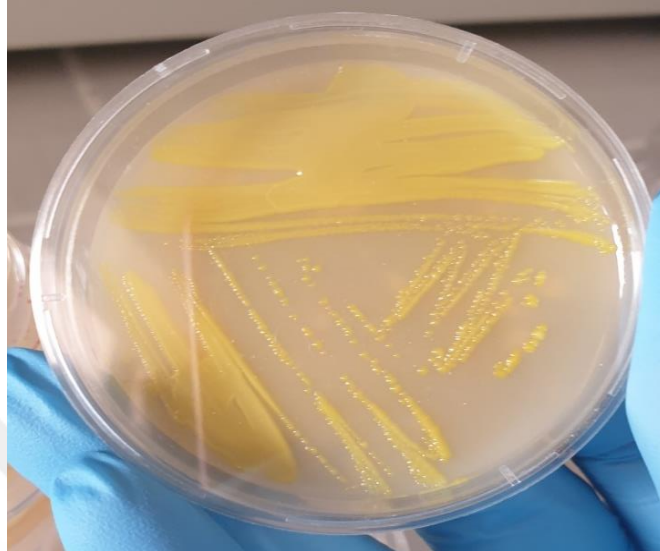


Figure.4.1. The colonies of *Arthrobacter* sp.H14-L1 on GYM medium agar

After incubation of AgNO_3 solution with the culture supernatant of *Arthrobacter* sp. H14-L1, the synthesis of silver nanoparticles was observed by the formation of brown-black colour (Figure 4.2). The silver nanoparticles were collected by centrifuge and dried.



Figure 4.2. Synthesis of silver nanoparticles by strain H14-L1 culture broth (E_1 - E_2)

4.2. Characterization of the Silver Nanoparticles

The UV-Vis spectrum between 400 nm and 450 nm showed a significant peak with a maximum of 419 nm (Fig. 4.3), and surface plasmon resonance (SPR) revealed the successful synthesis of silver nanoparticles (AgNPs). The synthesis might be occurred because the secondary metabolites in the bacterial supernatant reduced silver ions. Due to the unbonded electrons present in the silver nanoparticles (AgNPs), which cause them to collectively vibrate in resonance with the light wave, an SPR absorption band is formed.

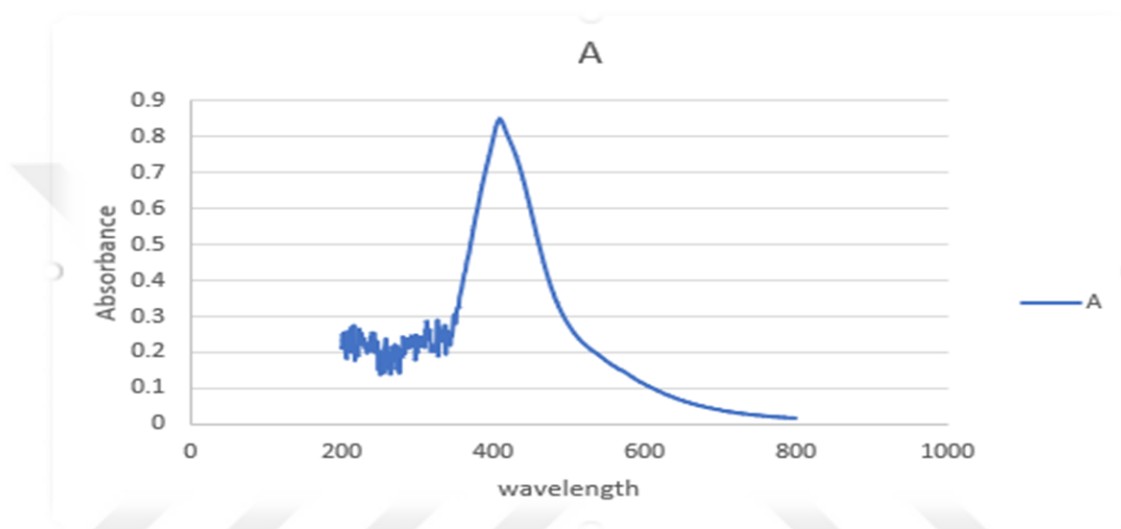


Figure 4.3. UV-vis spectra of silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1

The size, shape and elemental content of the produced AgNPs were evaluated by SEM examination. SEM analysis showed that the average particle size of all biosynthesized AgNPs ranged between 58 and 68 nm, with nanoparticles being spherical in form (Figure 4.4). As for the EDX analysis, the existence of silver in the sample was confirmed by finding signals of silver at 2.5 keV in the EDX spectrum displayed in Figure 4.5. Moreover, the presence of other elements such as carbon C and sulfur S, indicates that the AgNPs in the cell-free supernatant is capped by organic components such as carbohydrates, proteins, phenolic compounds, and flavonoids. The produced AgNPs from the quantitative elemental content indicated the existence of pure silver.

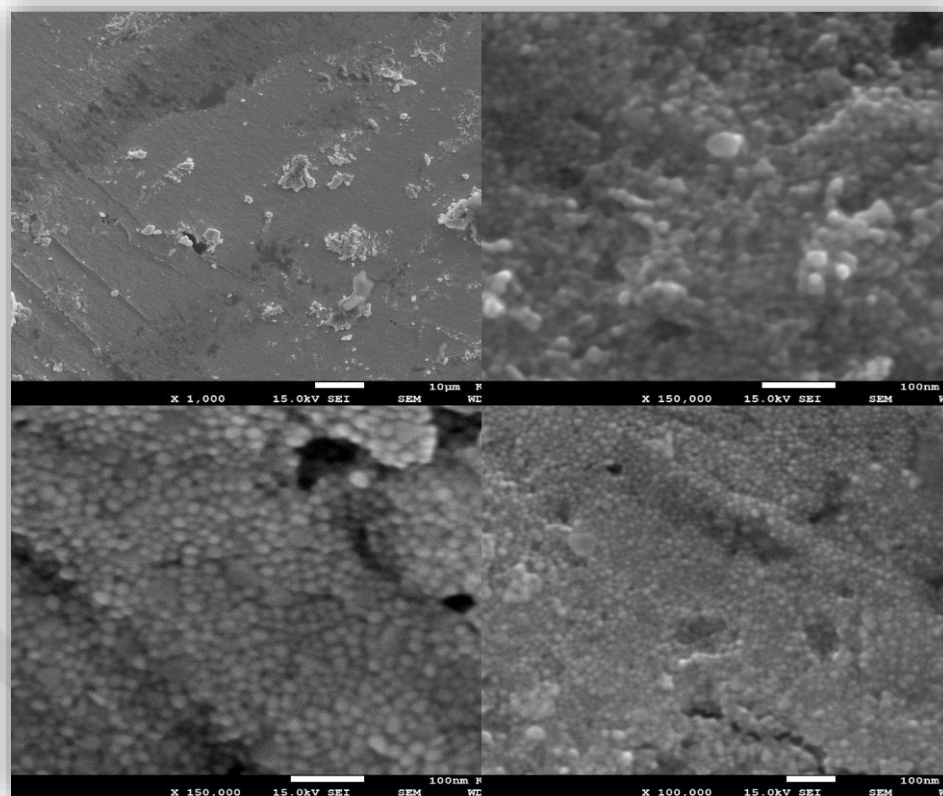


Figure 4.4. SEM images of AgNPs obtained by mixing 2 mM silver nitrate solution with cell free supernatant of *Arthrobacter* sp.H14-L1

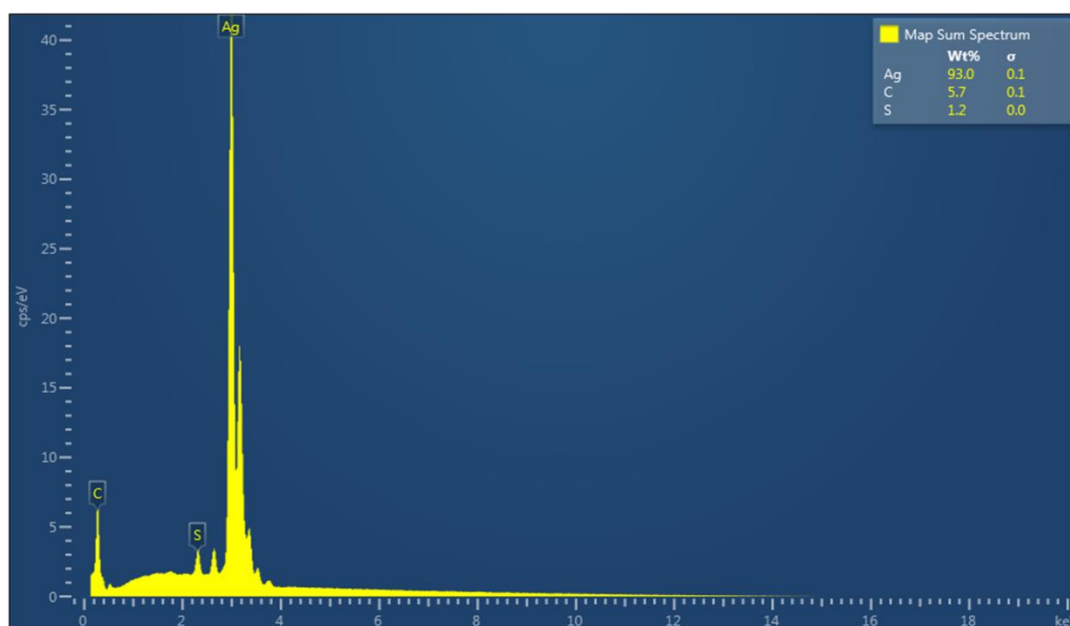


Figure 4.5. EDX profile of silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1

The crystalline structure of the nanoparticles synthesized by *Arthrobacter* sp. H14-L1 was revealed by X-ray diffraction analysis. The XRD analysis was performed after centrifugation and a powdered sample of synthesized AgNPs was deposited on a diffractometer glass substrate and treated at 40 kV voltage and 30 mA current using Cu-K radiation (wavelength of 1.5418) in the 2θ range from 5° operated to 100° at $2^\circ/\text{min}$ scan speed.

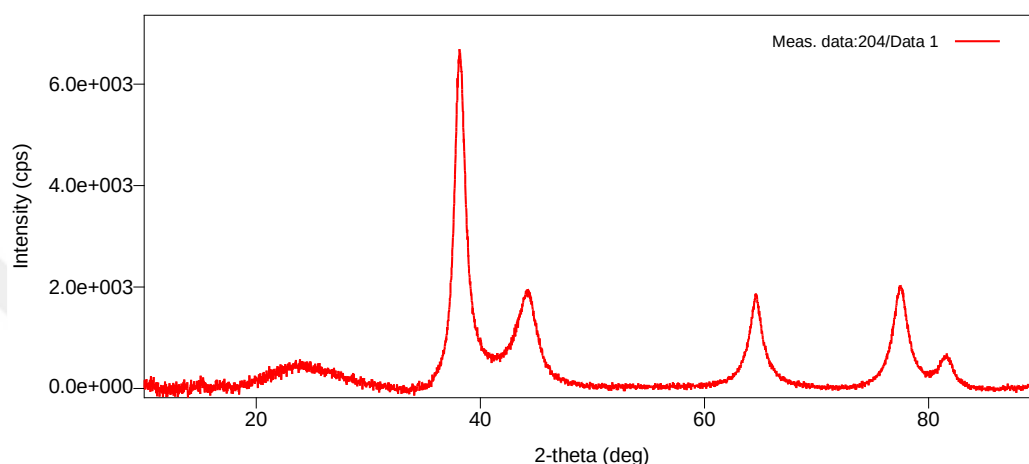


Figure 4.6. XRD pattern of the silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1

The XRD pattern of the silver nanoparticles exhibited the 2θ degrees of 25.50° , 38.146° , 44.393° , 64.587° , 77.564° and 81.78° . The crystal planes correspond to 38.146 (111), 44.393 (200), 64.587 (222) and 77.564 (311) and the data agree well with the Joint Committee on Powder Diffraction Standards (JCPDS: 87-0597) match. The crystal peak at 25.50 might be related to the crystalline molecules in culture broth of *Arthrobacter* sp. H14-L1 (Figure 4.6).

4.3. Genome Analysis of *Arthrobacter* sp. H14-L1

The genome data of *Arthrobacter* sp. H14-L1 was downloaded from the NCBI/GenBank (Accession no: JAPNLH0000000000). The phylogenetic analysis conducted on the TYGS server revealed that *Arthrobacter* sp. H14-L1 represents a novel species within the genus *Arthrobacter* (Figure 4.7).

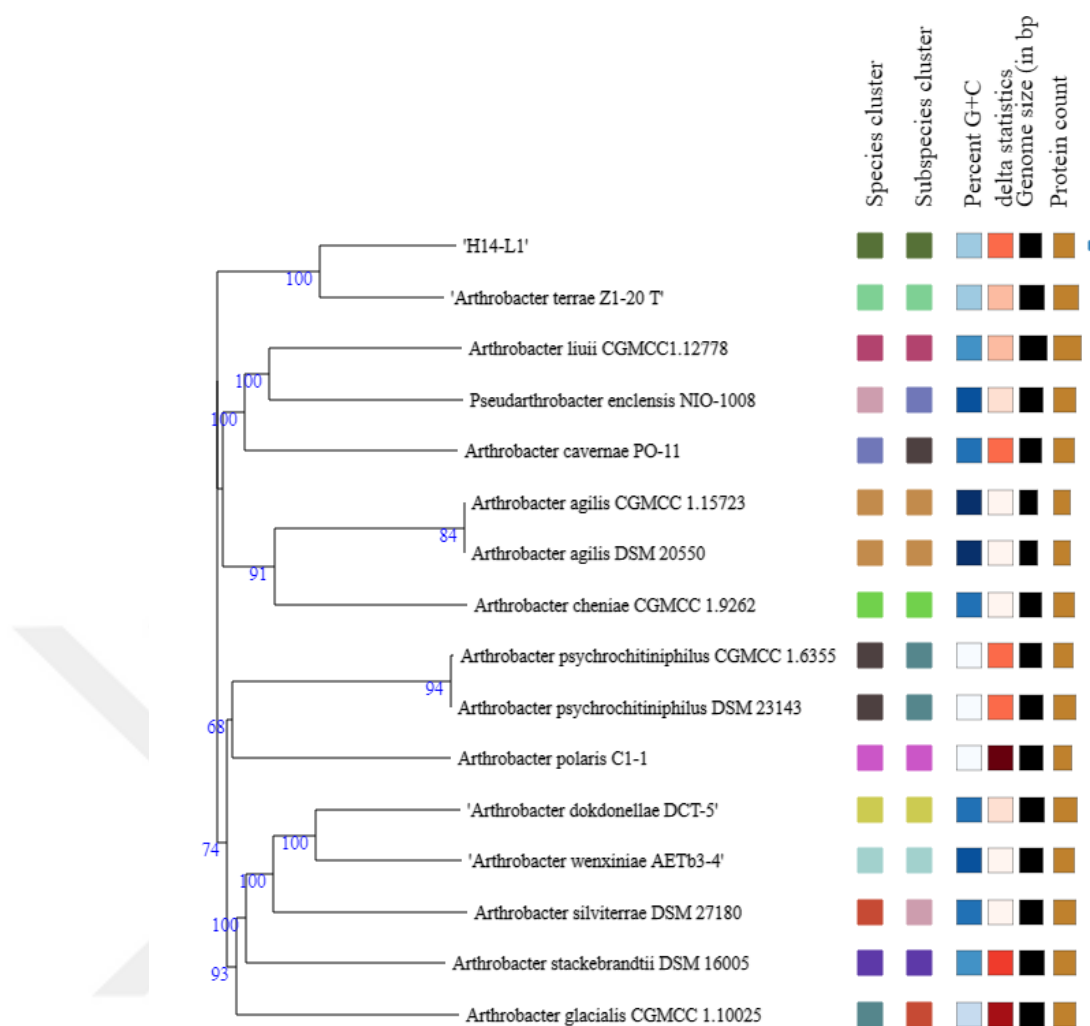


Figure 4.7. A whole genome-based phylogenomic analysis preformed on the TYGS server confirmed that *Arthrobacter* sp. H14-L1 was closely related to *Arthrobacter terrae* Z1-20T

In order to determine secondary metabolite-coding gene clusters within the genome of *Arthrobacter* sp. H14-L1, a universal annotation was performed on the antiSMASH server (<https://antismash.secondarymetabolites.org/#!/start>) (Figure 4.8). The genome of *Arthrobacter* sp. H14-L1 has gene clusters coding for a terpene, type-III polyketide, betalactone and ribosomally synthesized-posttranslationally modified peptide.

Overview 2.1 2.2 4.1 27.1						
identified secondary metabolite regions using strictness 'relaxed'						
Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	terpene	23,797	44,690	carotenoid	Terpene	21%
Region 2.2	T3PKS	247,240	288,475			
Region 4.1	betalactone	33,916	60,826	α,β -epoxyketone	NRP + Polyketide	15%
Region 27.1	RiPP-like	1	5,270			

Figure 4.8. Secondary metabolite biosynthetic gene clusters of *Arthrobacter* sp. H14-L1 identified on the antiSMASH server

4.4. Antimicrobial Activity of Silver Nanoparticles Synthesized by *Arthrobacter* sp. H14-L1 against *Klebsiella pneumoniae*

The antimicrobial activity and determination of minimum inhibition concentration (MIC) were tested by microdilution method. The MIC value of the silver nanoparticles against *K. pneumoniae* was determined as 0.12 mg/ml. A concentration of MIC₂₅ was used to expose *K. pneumoniae* culture for transcriptomic analysis.

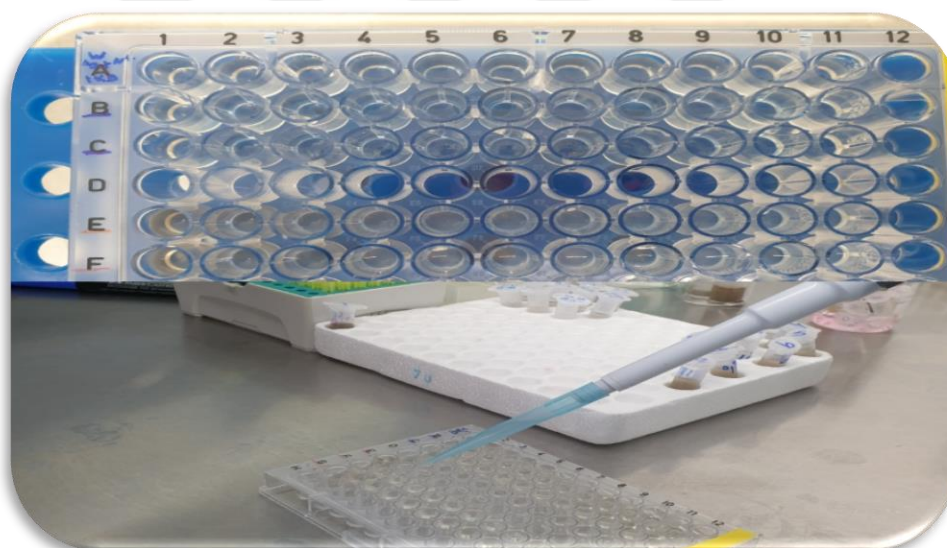


Figure 4.9. The MIC test on well plate

4.5. RNA-Seq Analysis

4.5.1. RNA-Seq Raw Data Statistics

Transcriptome sequencing was performed by the Macrogen Inc. (South Korea) using Illumina paired-end sequencing with TruSeq Stranded Total RNA (NEB Microbe) library type. The library size was checked by running the templates on an Agilent Technologies 2100 Bioanalyzer using a DNA 1000 chip. The accurate

quantification of DNA library templates was acquired using qPCR according to the Illumina qPCR Quantification Protocol Guide. The RNA concentration of the control group (KP) was calculated as 37.5 ng/μl with average size of 301 bp (Figure 4.10), while the concentration and average size for the KP-Nano were calculated as 29.2 ng/μl and 300 bp, respectively (Figure 4.11).

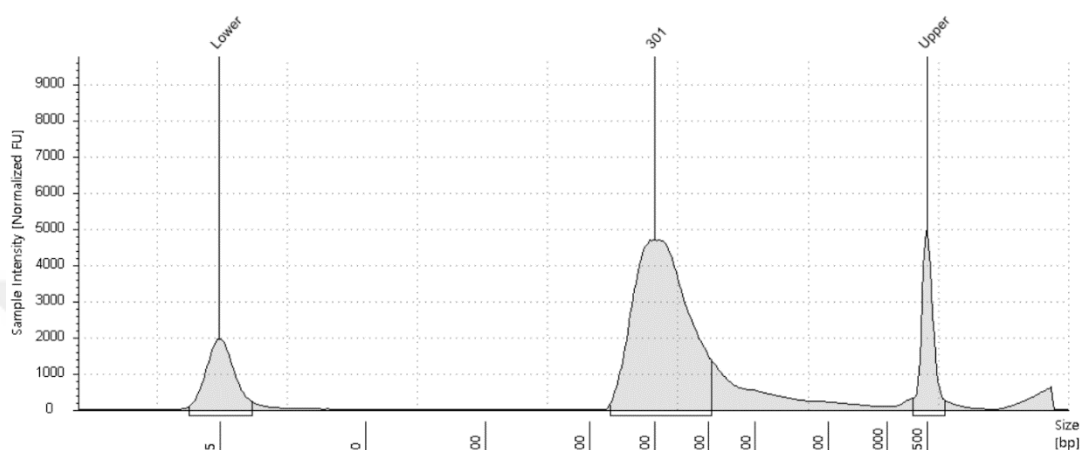


Figure 4.10. Library sample intensity of the control group (KP)

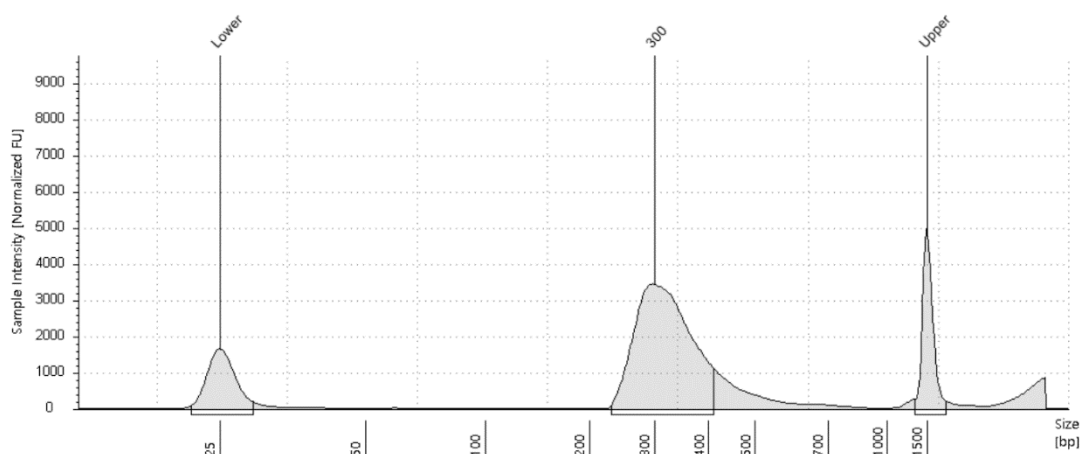


Figure 4.11. Library sample intensity of the AgNP-exposed group (KP-Nano)

The sequencing was performed on Illumina NovaSeq 6000. A total of 83,417,008 reads with 65.9% GC content was generated for the control group (KP) without AgNP exposure. For the AgNP exposed group (KP-Nano), a total of 85,711,048 reads with GC content of 67.1% was obtained. The Q20 and Q30 values of the control group were calculated as 98.0% and 95.2% while those of KP-Nano were 98.2% and 95.3%. The raw sequence data for the control group (KP) and AgNP-

exposed group (KP-Nano) were submitted to the NCBI Sequence Read Archive (SRA) under the accession numbers of SRR24827608 and SRR24906378, respectively.

4.5.2. Differential Gene Expression Analysis

The gene expression analysis was performed on the RaNA-Seq platform by quantifying the reads against the genome of *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884 (GCA_000163455). A total of 18 transcripts were detected in both samples (Table 4.1).

Table 4.1. Differential gene expression results for KP and KP-Nano samples.

Gene Symbol	ExpMean	Exp KP	Exp KP-Nano	log2FC	lfcSE	Stat	p-value	padj
nrdE	62832,25	49627,5	76037	0,616	0,547	1,032	0,302	0,825
hutU	2.0e+5	1.8e+5	2.2e+5	0,314	0,264	1,002	0,316	0,825
hrpA	29431,6	31469	27394,2	-0,2	0,254	-1	0,317	0,825
gltL	3.4e+5	3.7e+5	3.1e+5	-0,237	0,276	-0,989	0,323	0,825
potA	2548,153	5096,27	0,036	-12,264	6,788	-0,866	0,387	0,825
fusA	264,379	528,758	0	-9,049	5,439	-0,748	0,454	0,825
EEW39920	510,745	0	1021,49	9,998	5,133	0,743	0,457	0,825
proA	572,165	0	1144,33	10,162	6,1	0,462	0,644	0,825
opuCA	777,755	1555,51	0	-10,604	6,749	-0,455	0,649	0,825
pstB	407,906	815,811	0	-9,674	6,749	-0,455	0,649	0,825
cspA	3411,935	6823,87	0	-12,737	6,749	-0,455	0,649	0,825
acsA	142,636	285,272	0	-8,161	6,749	-0,455	0,649	0,825
rpoS	202,702	0	405,403	8,667	6,919	0,263	0,793	0,825
EEW39936	125,849	0	251,697	7,981	6,919	0,263	0,793	0,825
aceE	69,119	0	138,237	7,121	6,919	0,263	0,793	0,825
mgtA	67,914	0	135,827	7,096	6,919	0,263	0,793	0,825
carB	65209,85	62616,7	67803	0,115	0,268	0,228	0,819	0,825

glnA	2.9e+5	2.9e+5	2.9e+5	-0,013	0,276	-0,222	0,825	0,825
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- ExpMean: The expression mean of each gene in the input samples. This mean was calculated with the transcripts per million (TPMs) values.
- Exp KP: The expression mean of each gene in the KP sample. This mean was calculated with the transcripts per million (TPMs) values.
- Exp KP-Nano: The expression mean of each gene in the second group samples. This mean was calculated with the transcripts per million (TPMs) values.
- log2FC: The log2 scaled fold change of each gene. This fold change was calculated with the transcripts per million (TPMs) values.
- lfcSE: The standard error of the log2 scaled fold change calculated by Deseq2.
- Stat: The statistic calculated by the selected analysis method for each gene.
- p-value: The unadjusted p value calculated by the selected analysis method for each gene.
- padj: The adjusted p value calculated by the selected analysis method for each gene.

The expressions of *mgtA*, *aceE*, EEW39936, EEW39920, *rpoS* and *proA* genes were induced by the AgNP treatment in *K. pneumoniae* (Table 4.1). Coding for a 4-alpha-glucanotransferase enzyme, the *mgtA* gene is mainly responsible for carbohydrate metabolic processes involving alpha-amylase activity. It also has molecular function as metal ion binding activity. The *aceE* gene encodes for the pyruvate dehydrogenase E1 enzyme, which is necessary for the total conversion of pyruvate to acetyl-CoA and CO₂. The RNA polymerase sigma factor, which is produced by the gene *rpoS* and serves as the principal transcriptional regulator of both the stationary phase and the overall stress response, including the response to organic acid stress, is a product of this stress. Another gene whose expression was triggered by the AgNP treatment, *proA* encodes for a gamma-glutamyl phosphate reductase related to amino acid biosynthesis, mainly L-proline biosynthetic process. On the other hand, the AgNP treatment suppressed the expression of *acsA*, *fusA*, *pstB*, *opuCA* and *cspA*. The *acsA* gene encodes for acetyl-coenzyme A synthetase involving acetyl-CoA biosynthetic process. It has acetate-CoA ligase activity, ATP binding and metal ion binding activities. The *fusA* gene encodes for fructooligosaccharide ABC transporter

substrate-binding protein, which is a component of ABC transporter complex FusABC-MsmK responsible for fructooligosaccharide import. The FusA protein has also metal ion binding activity. Another ABC transporter complex PstSACB involves phosphate import ATP-binding protein coded by *pstB*, which is responsible for energy coupling to the transport system. The *opuCA* gene is also responsible for a protein involving energy coupling to the transport system. Similar to PstB protein, the OpuCA also has ATP binding and ATP hydrolysis activities. Another suppressed gene by AgNP treatment is *cspA* coding for major cold shock protein. The *cspA* gene is mainly related to stress response within the cell.

A volcano plot was created to visualize the log₂ scaled fold change (x axis) and the minus log₁₀ p-value (y axis) of each gene in the differential expression analysis for KP and KP-Nano samples (Figure 4.12). Moreover, an MA plot visualizing the differences between measurements taken in two groups by transforming the data onto M (log ratio) and A (mean average) scales was drawn (Figure 4.13). However, both graphs confirm that there is no statistically significant difference between both samples in terms of gene expression levels.

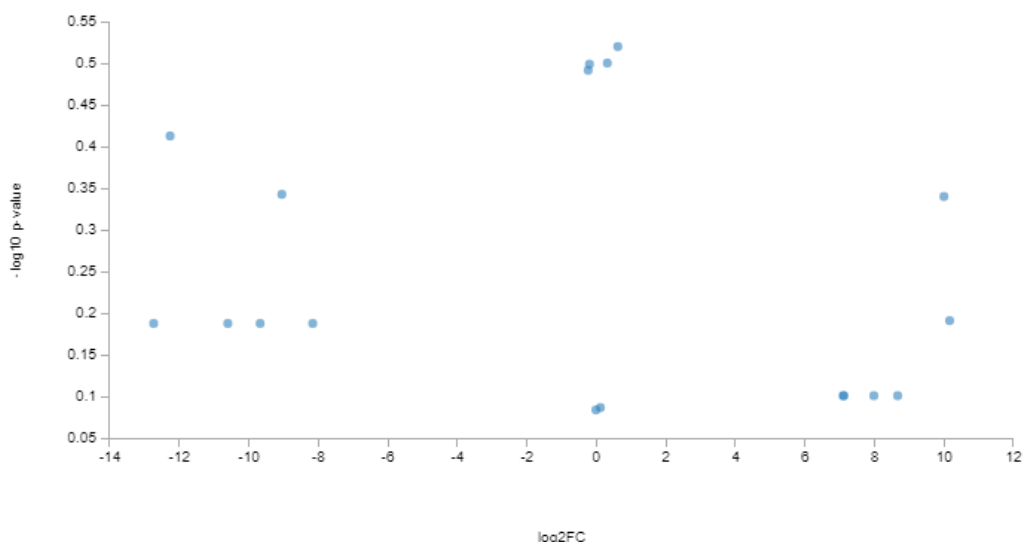


Figure 4.12. The volcano plot showing the log₂ scaled fold change (x axis) and the minus log₁₀ p-value (y axis) of each gene in the differential expression analysis for KP and KP-Nano samples. Genes with significant expression changes are highlighted as red dots

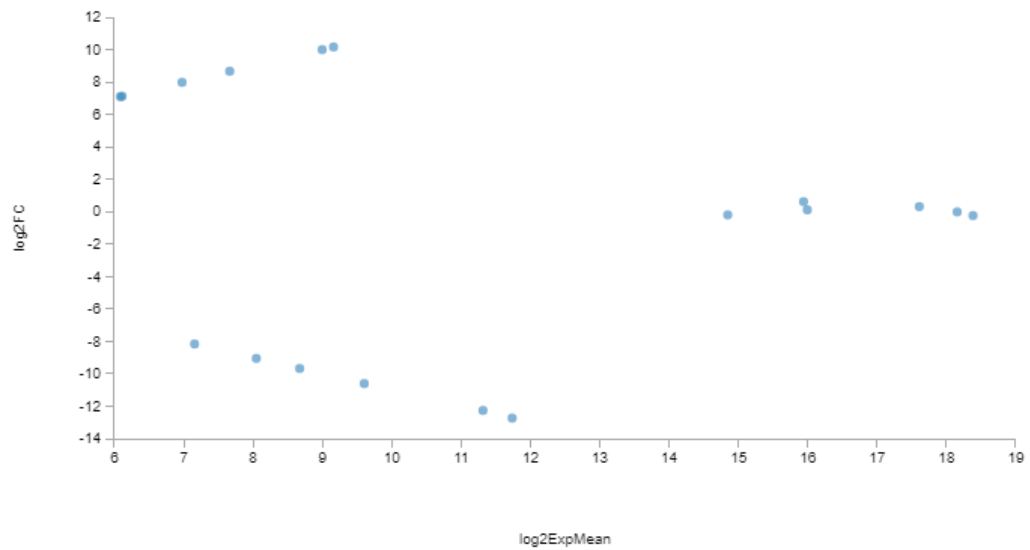


Figure 4.13. The MA plot showing the differences between measurements taken in KP and KP-Nano by transforming the data onto M (log ratio) and A (mean average) scales was drawn. Genes with significant expression changes are highlighted as red dots

The expression values normalized as TPMs (transcripts per million) of the genes *gltL*, *glnA*, *hutU*, *nrdE*, *carB*, *hrpA*, *rpoS* and *cspA* between KP and KP-Nano was compared, and no significant change was found for both groups (Figure 4.14). However, the expression of *gltL* was slightly decreased in KP-Nano while the expression of *hutU* gene was a little increased compared to the control group (KP).

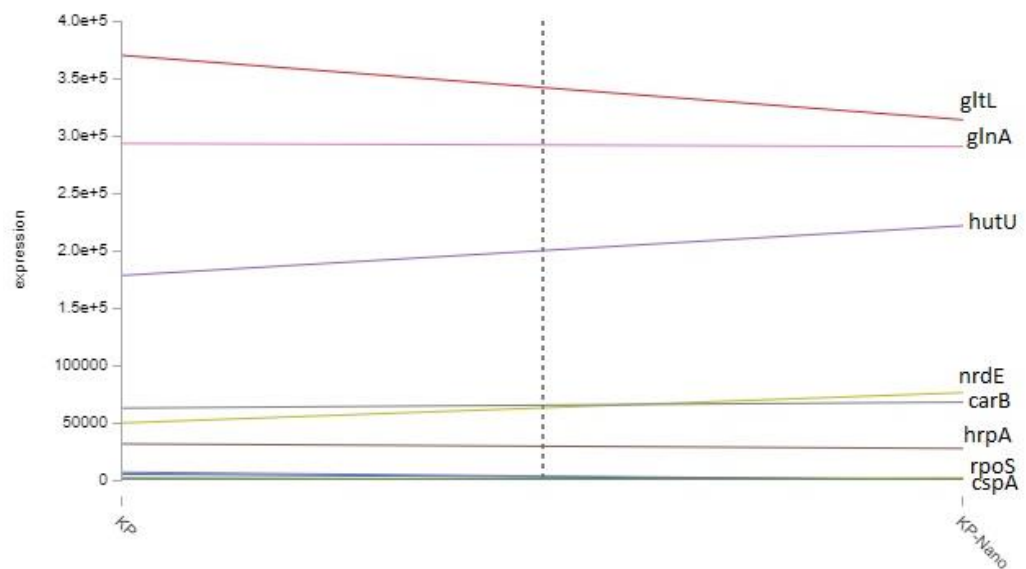


Figure 4.14. The line chart showing the expression value (normalized as TPMs) of selected genes for each sample

The *gltL* gene encodes for glutamate/aspartate import ATP-binding protein, which is part of the ABC transporter complex GltIJKL involved in glutamate and aspartate uptake and is probably responsible for energy coupling to the transport system. The *hutU* gene encodes for urocanate hydratase catalyzing the conversion of urocanate to 4-imidazolone-5-propionate, which is an important process for amino acid degradation. The expression of both genes was affected slightly by the AgNP treatment.



5. CONCLUSION

The present study confirmed the green synthesis and antibacterial properties of AgNPs synthesized by an Antarctic actinobacterium *Arthrobacter* sp. H14-L1. In comparison to nanoparticles produced using chemical and physical methods, biologically produced nanoparticles are considered to be more environmentally benign. The nanoparticles synthesized by actinobacteria have also additional advantages such as having bioactivities against pathogenic bacteria. This study also confirmed the antibacterial activity of silver nanoparticles synthesized by *Arthrobacter* sp. H14-L1 against *K. pneumoniae*. The physicochemical characterizations of the AgNPs confirmed that the synthesized nanoparticles are in nanoscale and have crystalline structure.

Transcriptomic analyses have provided insight into toxicity mechanisms of nanoparticles on the microbial cells. *K. pneumoniae* cells were exposed to AgNPs and, RNA-Seq analysis was used to find the genes that were differentially expressed. Although the observed differences were not important statistically, several genes responsible for primary metabolisms, mainly related to carbohydrate metabolic processes, amino acid biosynthesis and degradation, pyruvate catabolic process and stress response, were affected by silver nanoparticle exposure. The low level of expression difference between the control and the AgNP-exposed group might be related to the concentration of the silver nanoparticles (MIC₂₅) used in the present study. Higher levels of the silver nanoparticles could affect gene expression levels more drastically.

In conclusion, the present study provides insight into biological synthesis of silver nanoparticles by using an Antarctic bacterium as well as possible molecular mechanisms for toxicity of silver nanoparticles on bacterial cells.

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