MICROBIAL NANOFACTORIES FOR PRODUCTION OF SILVER NANOPARTICLES UTILISING LOCALLY-ISOLATED MALAYSIAN BACTERIAL STRAIN AS AN ALTERNATIVE SOURCE OF ANTIMICROBIAL AGENTS

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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The over prescription and misuse of antibiotics contribute to the rise of bacterial resistance to antibiotics, limiting the efficacy of antibiotic treatments for infectious diseases and necessitating the exploration of new strategies. Therefore, silver nanoparticles are proposed as promising candidates for alternative antimicrobial agent due to their superior antimicrobial efficiency, attributed to their excellent surface-to-volume ratio. This study focuses on extracellular synthesis of silver nanoparticles using a locally isolated bacterial strain from Bagan Lalang, Selangor, chosen for its potential cost-effectiveness, fast reduction properties, and leave environmentally friendly residues. The phylogenetic identification of the silver nanoparticles-producing bacteria using 16S rRNA sequencing resulted in a high homology to *Bacillus* genus.

Preliminary screening of bacterial-synthesised silver nanoparticles using UV-vis spectroscopy indicated nanoparticle formation through surface plasmon resonance (SPR), with a distinct absorption peak at 420 nm. Size and polydispersity analysis using dynamic light scattering (DLS) revealed low polydispersity (0.20 value), with an average size of 139.73±13.63 nm. Field emission scanning and transmission electron microscopy images showed spherical and uniformly distributed nanoparticles with the average diameter of less than 25 nm. The extracellular biofilm layer observed in transmission electron microscopy (TEM) suggests a capping agent maintaining nanoparticle integrity. Elemental analysis through elemental dispersive x-ray (EDX) confirmed the presence of 27.41% silver, with minimal biological residues.

Antimicrobial testing against *Escherichia coli* (*E. coli*) DH5α strain using disk diffusion assay demonstrated that bacterial-synthesised silver nanoparticles exhibit inhibitory effects, with a 0.87±0.12 cm inhibition zone, showcasing their potential as antimicrobial agents. Further testing through bacterial growth inhibition assay revealed that a 10% (v/v) concentration of bacterial-synthesised silver nanoparticles inhibits bacterial growth in suspension culture by 50%. Despite exhibiting a lower inhibitory effect compared to other antimicrobial agents, the study systematically compares the antimicrobial properties of bacterial-synthesised silver nanoparticles, revealing an efficiency 1.5 times higher than non-nano silver derived from silver nitrate ($AgNO_3$), with an efficiency rate of 150.5% at a normalised concentration. In conclusion, the locally isolated bacteria serve as a promising biofactory for synthesising silver nanoparticles, offering a scalable and antimicrobially potent solution that may complement or address antibiotic resistance.

Keywords: Antibiotic resistance, bacterial–synthesised silver nanoparticles, extracellular synthesis, antimicrobial efficiency

SDG: GOAL 3: Good Health and Well-Being

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGILANGAN NANO MIKROB UNTUK PENGELUARAN PARTIKEL NANO PERAK MENGGUNAKAN STRAIN BAKTERIA TEMPATAN MALAYSIA SEBAGAI SUMBER ALTERNATIF BAGI EJEN ANTIMIKROB

Oleh

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Penyalahgunaan dan penggunaan berlebihan antibiotik menyumbang kepada peningkatan ketahanan bakteria terhadap antibiotik, menghadkan keberkesanan rawatan antibiotik bagi penyakit berjangkit dan memerlukan penyelidikan strategi baru. Oleh itu, partikel nano perak dicadangkan sebagai calon yang menjanjikan sebagai agen antimikrob alternatif disebabkan kecekapan antimikrob mereka yang unggul, yang dikaitkan dengan nisbah permukaan terhadap isipadu yang cemerlang. Kajian ini memberi tumpuan kepada sintesis luar sel partikel nano perak menggunakan strain bakteria tempatan yang diasingkan dari Bagan Lalang, Selangor, dipilih kerana potensinya yang keberkesanan kos, sifat pengurangan cepat, dan meninggalkan sisa yang mesra alam. Identifikasi filogenetik bakteria penghasil partikel nano perak menggunakan urutan rRNA 16S mendapati homologi tinggi dengan genus *Bacillus.*

Penyaringan awal partikel nano perak yang disintesis bakteria menggunakan spektroskopi UV-vis menunjukkan pembentukan partikel nano melalui resonans plasmon (SPR) permukaan, dengan puncak penyerapan yang jelas pada 420 nm. Analisis saiz dan polidispersiti menggunakan penyebiran cahaya dinamik (DLS) menunjukkan polidispersiti yang rendah (nilai 0.20), dengan saiz purata 139.73 ±13.63 nm. Imej mikroskopi penjanaan medan dan mikroskopi elektron pemindahan menunjukkan nanopartikel yang sfera dan teragih secara seragam dengan diameter purata kurang daripada 25 nm. Lapisan biofilem luar sel yang diperhatikan dalam mikroskopi elektron pemindahan mencadangkan agen penutup yang mengekalkan integriti nanopartikel. Analisis unsur melalui penceraian sinar-X jisim unsur (EDX) mengesahkan kehadiran 27.41% perak, dengan sisa biologi yang minima.

Ujian antimikrob terhadap strain *Escherichia coli* (*E. coli*) DH5α menggunakan ujian difusi cakera menunjukkan bahawa partikel nano perak yang disintesis bakteria menunjukkan kesan penghambatan, dengan zon penghambatan 0.87 ± 0.12 cm, mempamerkan potensi mereka sebagai agen antimikrob. Ujian lanjut melalui ujian penghambatan pertumbuhan bakteria menunjukkan bahawa kepekatan 10% (v/v) nanopartikel perak yang disintesis bakteria menghalang pertumbuhan bakteria dalam kultur ampaian dengan 50%. Walaupun menunjukkan kesan penghambatan yang lebih rendah berbanding agen antimikrob lain, kajian ini secara sistematik membandingkan sifat antimikrob partikel nano perak yang disintesis bakteria, mendedahkan kecekapan 1.5 kali ganda lebih tinggi daripada perak bukan nano yang diperoleh daripada nitrat perak ($AgNO_3$), dengan kadar kecekapan 150.5% pada kepekatan yang dinormalisasikan. Kesimpulannya, bakteria tempatan yang diasingkan berfungsi sebagai bio-kilang yang menjanjikan untuk mensintesis partikel nano perak, menawarkan penyelesaian yang boleh diperluaskan dan berpotensi antimikrob yang mungkin melengkapi atau menangani ketahanan antibiotik.

Kata kunci: Ketahanan antibiotik, partikel nano perak yang disintesis bakteria, sintesis luar sel, kecekapan antimikrob

SDG: Matlamat 3: Kesihatan Baik dan Kesejahteraan

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LIST OF ABBREVIATIONS

A	Angstrom
AFM	Atomic force microscopy
Ag^+	Silver ion
Ag^0	Metallic silver
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
ANOVA	Two-way Analysis of Variance
BLAST	Basic Local Alignment Search Tool
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides triphosphates
ECS®	Electrode Colloidal Silver
FTIR	Fourier Transform Infrared Spectroscopy
GAP	Global Action Plan on Antimicrobial Resistance
h	Hour
HSD	Tukey's Honestly Significant Difference
IDT	Integrated DNA Technologies
ISO	The International Organisation for Standardisation
LB	Luria Bertani
MDR	Multidrug-resistant
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium chloride
MOSTI	Malaysian Ministry of Science, Technology and Innovation
MRSA	Methicillin-resistant *Staphylococcus aureus*

MUSCLE	Multiple Sequence Comparison by Log-Expectation
$NaBH_4$	Sodium borohydride
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NO_3^-	Nitrate ions
OD	Optical density
PCR	Polymerase Chain Reaction
PDI	Polydispersity index
PLGA	Poly-lactic-co-glycolic acid
pN	Pico Newton
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSM	Response Surface Methodology
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SPR	Surface Plasmon Resonance
TEM	Transmission electron microscopy
TEM-EDX	Transmission Electron Microscopy with Energy Dispersive X- ray
USFDA	The US Food and Drug Administration
WHO	World Health Organisation
xg	Relative centrifugal force
XRD	X-ray Diffraction

CHAPTER 1

INTRODUCTION

1.1 Background

Over the past decade, the emergence of antimicrobial resistance has posed an alarming threat to modern healthcare. This phenomenon has primarily arisen due to a few factors, including the overuse and misuse of antibiotics (Zhu et al., 2022), rapid dissemination of resistant strains across the globe due to high mobility, and the intrinsic adaptability of microorganisms to evolve naturally (Ramamurthy et al., 2022). Therefore, this emergence significantly impacts modern treatments, as the antibiotics increasingly less effective, leading to more aggressive and broad-spectrum of antibiotic treatments, causing more expensive healthcare costs, longer hospital stays, and increased morbidity and mortality rates of humans (Serra-Burriel et al., 2020). A post antibiotic era is considered as a global threat that is projected to cause more deaths than all cancers combined by 2050 (Avershina et al., 2021). The World Health Organisation (WHO) declared antibiotic resistance as one of the three most important public health threats of the 21st century (Piergiacomo et al., 2022).

Many infectious microorganisms have evolved to resist the inhibitory effects of these drugs over the years based on the surveillance in different regions of the world indicated that there is an alarming high number of antibiotic-resistant species. For example, bacterial infection called pneumonia has become incurable as the bacteria has been found to be resistant to cephalosporin as well as carbapenems, thereby rendering all available treatment using beta-lactam antibiotics (Fatima et al., 2023). On the other hand, the emergence of resistance to antifungal drugs in invasive yeast infections such as *Candidiasis* has led to worldwide morbidity and mortality, contributing to global economic burden (Lee et al., 2020). The antimicrobial resistance is also the reason why microbes fail to respond to standard drugs, thus, extending the duration of course of treatment further increasing the health care costs (Jindal and Goswami, 2020). Research reports from WHO also shown highly resistance bacteria such as *E. coli* are now against antibiotics as cephalosporin and fluoroquinolones, *Klebsiella pneumoniae* (*K. pneumoniae*) against cephalosporin and carbapenems, *Staphylococcus aureus* (*S. aureus*) against methicillin, *Streptococcus pneumoniae* (*S. pneumoniae*) against penicillin which causes common infections like urinary tract infections, pneumonia, and bloodstream infections and high percentage of hospital-acquired infections (Nimer, 2022). Therefore, the escalated antimicrobial resistance has sparked a growing interest in exploring novel or alternative approaches to address this healthcare crisis which can overcome or complement current antibiotic treatments.

The emergence of antimicrobial resistance is a natural phenomenon that cannot be completely inhibited. Therefore, alternative forms for antimicrobial agents need to be considered to slow down the pace of the rise of antimicrobial resistance. There are many research studies to combat antimicrobial resistance for the past decades as Global Action Plan on Antimicrobial Resistance (GAP) started in 2015, visioning to implement national action plans to limit the progress of antimicrobial resistance as WHO calls for urgent action to avert an antimicrobial resistance crisis and insists on the importance of discovering and developing new antibiotics (Rima et al., 2021). For instance, alternative approach using bacteriophages offer several advantages for infection therapy to treat various ranges of bacteria such as *S. aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*). However, concerns regarding the development of immunogenicity towards bacteriophages have been raised as the release of endotoxins from the bacteria lysed by bacteriophages (Al-Ishaq et al., 2021). Antimicrobial peptides are considered as future antibiotics as they cause disintegration of the cell membrane for broad-spectrum activities. However, antimicrobial peptides develop *in vivo* toxicity and are expensive for large scale production (Wu et al., 2020). Lysins are one of the potential candidates for antimicrobial agents due to their direct antimicrobial action and can be customised according to targeted bacteria using genetic engineering. However, lack of sufficient research makes the lysins difficult for large scale production (Gosh et al., 2019). However, the demand to develop additional bactericidal means has significantly increased due to the growing concern regarding multidrug-resistant bacterial strains and biofilm associated infections despite numerous existing potent antimicrobial agents. Consequently, attention has been especially devoted to new and emerging nanoparticle-based materials in the field of antimicrobial chemotherapy. For instance, antimicrobial properties of silver nanoparticles have been proposed as promising nanomaterials for alternative antimicrobial agents (Gherasim et al., 2020). In nanoscale dimension, silver nanoparticles exhibit superior antimicrobial efficiency due to its excellent surface-to-volume ratio (Patel et al., 2021). The generation of nano-sized silver consequently exhibit stronger antimicrobial activities (Bruna et al., 2021). The exceptional properties of silver nanoparticles have been broadly applied as antimicrobial agents, biomedical device coatings, drug-delivery carriers, imaging probes, and diagnostic and optoelectronic platforms (Lee and Jun, 2019).

Conventional routes of silver nanoparticles production consisting of physical and chemical syntheses cause disadvantages to be employed for scale up and are harmful for human application. Physical methods such as ball milling, laser ablation, vapor condensation and electric arc-discharge cause agglomeration of nanoparticles due to absence of stabilisers or capping agents (Xu et al., 2020). Complex equipment and highly external energy are required to reduce particle size in the process of generating nanoparticles which are not economically friendly to be employed for industrial purposes (Endres et al, 2021). Chemical routes are among popular methods in synthesising silver nanoparticles; however, the routes cause environmental pollution as they produce toxic chemical residues due to excessive use of chemical reagents (Nguyen et al., 2023).

Therefore, green synthesis is suggested to overcome the limitations of non-biological routes by not causing any harmful effects towards humans and the environment. Among green synthesis routes, bacterial-mediated synthesis of silver nanoparticles has gained attention due to its sustainability, biocompatibility, cost effectiveness, non-toxicity, and can be easily used for mass production as some proteins and enzymes are involved in silver metals reduction into silver nanoparticles (Mustapha et al., 2022). In bacterial mediated synthesis, silver nanoparticles can be produced intracellularly and extracellularly (Nag et al., 2021). The drawback of intracellular synthesis requires additional steps to recover the accumulated nanoparticles from inside the cells which bring major concern for industrial perspectives (Zhang et al., 2020). Therefore, the extracellular method is a better alternative for bacterial synthesis of silver nanoparticles to overcome the limitation of the intracellular method due to the ease of recovery of nanoparticles from the solution (Xu et al., 2020).

In Malaysia, the generation of silver nanoparticles has been explored using soil bacteria due to the vast aquatic ecosystem (De Silva et al., 2020). Intensive industrial activities have led to higher concentrations of heavy metals in these ecosystems (Mat Lazim et al., 2023) and therefore led to the emergence of new and varied metabolites, proteins and extracellular polysaccharides (Alotaibi and Shamim, 2021). Exploitation of these metabolic pathways could lead to the discovery of innovative bioactive material, instrumental in breaking down heavy metal pollutants while generating precious metal nanoparticles (Mustapha et al., 2022). Exploration of this ecosystem for marine bacterial strains would therefore contribute to widening the perspective of silver nanoparticles generation using these microbial nanofactories for the development of green bioprocesses.

In this study, extracellular silver nanoparticles synthesis employing bacterium as a nanofactory was chosen over conventional synthesis routes because it is hypothesised to possess similar or superior properties of silver nanoparticles with fast reducing, low-cost production and leave environmentally friendly residues. The study was performed by first identifying the bacterial growth and phylogeny of silver-nanoparticles producing bacterium, followed by physicochemical characterisations of successfully produced bacterial-synthesised silver nanoparticles to determine the nanoparticle formation, average size, polydispersity analysis, morphological and elemental composition. Lastly, the antimicrobial potential of bacterial-synthesised silver nanoparticles was performed to assess the antimicrobial activity towards targeted bacteria in solid agar and suspension forms. Therefore, the establishment of generation and the potential antimicrobial effect of bacterial-synthesised silver nanoparticles from this study can deepen our understanding of the role of nanotechnology in combating infectious diseases and can work towards more sustainable and effective solutions for addressing the global challenge of antimicrobial resistance.

1.2 Statement of Research Problem

Non-biological methods for producing silver nanoparticles suffers from significant challenges including tedious processes, less cost-effective and the production of harmful by-products post-synthesis. Alternatively, there is a pressing need to explore more sustainable routes of silver nanoparticle synthesis through biological systems; by locally isolating bacteria species with ability to extracellularly produce silver nanoparticles with potential antimicrobial properties.

1.3 Hypothesis

Locally-isolated bacterium from Malaysian mangroves will be able to produce silver nanoparticles through extracellular synthesis in aerobic condition and exhibit antimicrobial properties as a candidate for an alternative antimicrobial agent.

1.4 Objectives of study

The study was performed with the current objectives:

1. To identify the bacterial strain with extracellular silver nanoparticles-producing potential under aerobic conditions;

2. To assess the chemical and morphological characteristics of the bacterial-synthesised silver nanoparticles; and

3. To evaluate the antimicrobial properties of bacterial-synthesised silver nanoparticles compared to common antibiotics against gram-negative *E. coli* DH5α strain.

CHAPTER 2

LITERATURE REVIEW

2.1 Nanomaterials and their properties in various applications

The “nanometer” concept was first introduced by the 1925 Nobel Prize Laureate in Chemistry, Richard Zsigmondy when he was the first to measure the gold colloids using a microscope and invented the term of nanometer in characterising the particles (Chari et al., 2022). The “nano” word is derived from a Greek word that describes “dwarf” or “very small” (Fatima et al., 2020). A nanometer (nm) equals one billionth of a meter, or 10^{-9} m or one-millionth of a millimeter pico Newton (pN=10^{-12} N), and Angstrom (A=10^{-10} m) (Madkour, 2019). Then, modern nanotechnology emerged from the lecture of Richard Feynman, the 1965 Nobel Laureate in Physics whereby he presented a lecture titled, “There’s Plenty of Room at the Bottom” during the 1959 American Physical Society meeting at Caltech, in which he introduced the concept of manipulating matter at the atomic level (Bayda et al., 2019). Almost 15 years after Feynman’s lecture, a Japanese scientist, Norio Taniguchi, was the first to use ‘‘nanotechnology’’ to describe semiconductor processes that occurred on the order of a nanometer. He advocated that nanotechnology is the ability to engineer materials precisely at the nanometer level consisting of the processing, separation, consolidation, and deformation of materials by one atom or one molecule (Srivasta et al., 2022).

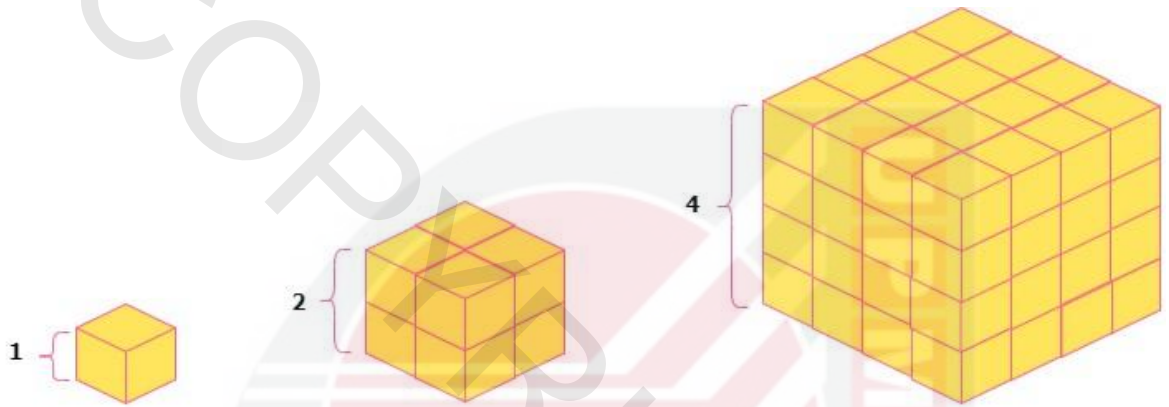
Nanotechnology is defined as the understanding and control of matter at dimensions between one and 100 nm (Xu et al., 2020). The International Organisation for Standardisation (ISO) defined nanomaterials as a material with any external nanoscale dimension or having the internal nanoscale surface structure (Harish et al., 2022). Nanomaterials also have been denoted by The US Food and Drug Administration (USFDA) as materials that have at least one dimension in the range of approximately one to 100 nm and exhibit dimension-dependent phenomena (Banala et al., 2022). As per the European Union Commission nanomaterials means a manufactured or natural material that acquires unbound, aggregated or agglomerated particles where external dimensions are between one to 100 nm size ranges (Patel et al., 2021). Generally, nanoparticles could be defined as materials with at least one dimension less than 100 nm size range (Juárez-Maldonado et al., 2019).

Nanotechnology has drawn attention from researchers due to unique and enhanced properties in miniaturised materials. Nanoscale materials can be present in single, compound, aggregated, or agglomerated structures with

sphere-shaped, cylindrical, and asymmetrical shapes with smaller than 100 nm in one or more dimensions (Patel et al., 2022). Size reduction of materials up to nanoscale range have a higher surface-area-to-volume ratio compared to bulk materials (Asha and Narain, 2020). As shown in Figure 1, the smaller the size, the higher surface-area-to-volume ratio and the greater the likelihood of interactions with the surrounding environment and that is the main reason for their novel properties (Rana et al., 2020; Patel et al., 2021).

Therefore, it is possible to manipulate the basic properties of materials by producing nano-scale range materials according to industrial and research interests as the quantum size affects significantly the properties of particles such as chemical, thermal, mechanical, optical, electrical, and magnetic (Varghese et al., 2019). The distinctive properties and superior performance of nanoscale materials are established by their sizes, surface structures, and inter-particle interactions (Endres et al., 2021). This will establish novel, highly efficient materials that can be produced by simply tuning the particle size that were impracticable in the past (Nakamura et al., 2022; Abdolrahimi et al., 2021). For example, the antibacterial efficacy of silver nanoparticles was significantly enhanced with less than 10 nm size through disk diffusion tests against *E. coli* and *S. aureus* (Wei et al., 2019). In a separate study, copper nanoparticles were successfully synthesised for large-scale production by using a simple synthesis method of copper nanoparticles in water/acetonitrile mixed solvent which resulted in the formation of monodispersed cubic-structured copper nanoparticles with sizes less than 100 nm (Moglie et al., 2019). This method shows excellent stability compared with conventional production via citrate-protected copper nanoparticles, which may be an efficient way to improve the fine tuning of the structure and size of copper nanoparticles (Dero et al., 2022). Other studies reported that selenium nanoparticles have been successfully synthesised with the sizes of 40-100 nm using a simple wet chemical method under ambient conditions (Khandsuren and Prokisch., 2021).

Nanoparticles are defined as particles with the range between one and 100 nanometres in size and are generally classified into the organic, inorganic and carbon based (Ijaz et al., 2020). Organic nanoparticles could be defined as solid particles composed of organic compounds (mainly lipids or polymeric) ranging in diameter from 10nm to 1 µm which serve to encapsulate in soluble materials (Mokhena et al., 2020). By forming very small dispersions of organic compounds, insoluble materials can be made to behave more like truly dissolved molecules without the need to produce new chemicals. This option is a valuable tool in the manufacturing and development of new products such as drug delivery (Lawson et al., 2021). Metal and metal oxide nanoparticles are categorised as inorganic nanoparticles. The only difference between metal based and metal oxide-based nanoparticles is the metal-based nanoparticles are synthesised to modify the properties of their respective metal-based nanoparticles mainly due to their increased reactivity and efficiency (Ijaz et al., 2020). Carbon-based nanoparticles comprise completely carbon elements. Table 2.1 summarises the classification of nanoparticles to suit the targeted applications.



Cubic side (s)	Side area (s^2)	Cubic surface area ($6s^2$)	Volume (s^3)	Surface area to volume ratio
1 cm	1 cm^2	6 x 1 cm^2	1 cm^3	6 / 1
2 cm	4 cm^2	6 x 4 cm^2	8 cm^3	24 /8 = 3 / 1
4 cm	16 cm^2	6 x 16 cm^2	64 cm^3	96 / 64 = 3 / 2

Figure 2.1: The surface-area-to-volume ratio is inversely proportional to the size of a cube. The smaller the size of a cube (object), the higher surface-area-to-volume ratio, which has a key role in interaction of a nanoscale material with its surrounding environment as it enhances the properties (Adapted from Pouran, 2018).

The superior properties of nanomaterials are key for researchers in advancing health care products (Wu et al., 2020) and restoration of polluted environments (Tsekhmistrenko et al., 2020). For example, nanoscale materials played an important role on medical devices such as drug delivery systems, diagnostic biosensors and imaging procedure as the size range is smaller than biological molecules such as proteins, receptors, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) (Mozneb et al., 2020). Nanoparticles with the sizes of 10 – 100 nm can utilise endocytosis processes to enter cells naturally where normal penetration into cells would be difficult for a particular molecule. Therefore, the nanoparticles should be engineered within a specific range to facilitate easy penetration of the cell membrane, accurately target subcellular compartments, and regulate intracellular processes. (Debnath et al., 2021). Poly-lactic-co-glycolic acid (PLGA) nanoparticles are polymers-based nanoparticles which have been exploited in drug delivery formulation such as encapsulating a wide range of bioactive molecules. PLGA functions to protect the bioactive molecules from degradation in the biological environment, providing a sustained and targeted delivery or facilitating intracellular penetration of bioactive compounds and reducing the side effects (Ghitman et al., 2020). For instance, AstraZeneca AB formulated PLGA-based nanoparticles named Bydureon Bcise® PLGA microsphere Exenatide to treat Type II diabetes. In veterinary medicine, the ampicillin loaded to nanoparticles on mice infected with *Salmonella typhimurium*, and result confirmed that mice treated ampicillin joined to nanoparticles confirmed higher survival ratio much like ordinary manage group, required the lower amount of antibiotic required to present the same effects (Youssef et al., 2019).

In environmental applications, inorganic nanoparticles such as magnetic iron oxide nanoparticles have been anticipated for the wastewater treatment and heavy metal removal (Bhateria and Singh, 2019). For example, iron oxide nanoparticles synthesised from *Penicillium pimiteouiense* resulted in the effective removal of chromium ions from synthetic solution for 120 min with maximum adsorption capacity to be 4.62 mg/g (Saravanan et al., 2021). Copper oxide nanoparticles generated from Aloe Vera leaves have been reported to exhibit good photocatalytic efficiency as the nanoparticles degrade methylene blue completely in ten minutes (Akintelu et al., 2020). Biosynthesised silver nanoparticles considered to be an ideal disinfectant to replace chlorine and ozone to eliminate microorganisms to provide hygienic water due to a broad antimicrobial activity at ambient temperature, affordable cost, and able to be safely disposed because no production of harmful by-products that are toxic to the human and environment (Khan et al., 2022). Graphene oxide with silver nanoparticles composite onto cellulose acetate membrane have been proved to inactivate 86% of *E. coli* bacteria within two hours (Zhang et al., 2021). Other than that, silver nanoparticles impregnated sub-micrometer crystalline jute cellulose particles via reduction with *Mussaenda erythrophylla* leaves extract are able to completely degrade congo red and methylene blue dyes within 14 min with 0.005 mg mL^{-1} of nanocomposite concentration (Rabbi et al., 2020).

Table 2.1: Classification of nanoparticles synthesis and its applications

Classification	Size (nm)	Characteristics	Applications	Citations
Organic	10-1000	Biodegradable, non-toxic; micelles and	Targeted drug delivery	Pandey et al., 2022;
Dendrimers, micelles,		liposomes have a hollow core; ideal		
liposomes		choice for drug delivery		
Inorganic	10-100	Stable and easy for functional	Antimicrobial properties,	Sánchez-López et al., 2020;
Metal		modification	diagnostics, bioremediation	Habibullah et al., 2021;
				Kaur and Roy, 2021
Metal oxides	Less than 100	Modification of metal-based	Heavy metal removal,	Bhateria and Singh, 2019;
		nanoparticles in the presence of oxygen	cosmetic products	Gosh et al., 2022;
		to increase reactivity and efficiency		Pan et al., 2021
Carbon- based	1 nm sheet	allotrope of carbon; excellent optical,	Sensors, biomedical,	Tiwari et al., 2020
Graphene	thickness	thermal, and mechanical characteristics;	microelectronics	
		strongest and superior conductive material		
Carbon nanotubes	Less than 100 nm	Large surface area; high electrical	composite fillers in polymers,	Norizan et al., 2020
		conductivity; good chemical inertness	chemical sensors, batteries	
Carbon nanofiber	A diameter of 200 to	High aspect ratio; large surface area; unique	super- capacitors, chemical	Zhou et al., 2020
	300 nm and a length	electrical, and magnetic properties	batteries, fuel/solar cells,	
	of 20 to 200 µm		sensors/biosensors	

2.2 Navigating global and Malaysian challenges in antibiotic resistance: Factors and the importance of alternative antimicrobial agents

The inappropriate usage of antibiotics continues to be a major worldwide issue. Overuse and improper administration, including unnecessary prescriptions, incorrect dosages, and antibiotics self–medication playing a role in the development of antibiotic resistance (Serwecińska, 2020). For instance, a systematic study revealed that antibiotic self-medication among children is more widespread in the Middle East (34 %), Africa (22 %), Asia (20%), South America (17%), while Europe exhibited the lowest prevalence rate at 8%. Factors such as distance from hospitals, low income, and having multiple children were identified as contributors to an increased risk (Bert et al., 2022). In addition, children have a higher likelihood of contracting infections. The prevalence of antibiotic self-medication among children aged zero to five years is notably influenced by the health beliefs of parents (Wu et al., 2021). A study conducted in China revealed that inappropriate antibiotic use could be associated with factors such as insufficient skills and knowledge among physicians and caregivers. Additionally, caregivers exert pressure on physicians to prescribe antibiotics to children. (Guo et al., 2021).

In Malaysia, the issue of antimicrobial resistance persists, driven by the excessive and improper utilisation of antibiotics, endangering global and regional health security (Naeemmudeen et al., 2021). Easy accessibility to antibiotics, self -administration, and incomplete antibiotic regimens are contributing factors to antibiotic misuse in Malaysia, consequently fueling the rise in antimicrobial resistance (Mariappan et al., 2021). Among Malaysian respondents, 56.6% participated in at least one improper practice related to antibiotic usage, particularly in terms of not completing the full antibiotic course (Kong et al., 2021). The rate of self - medication with antibiotics in Malaysia stands at 15.1%, and this behavior is notably influenced by the individual's level of education (Aslam et al., 2021). Research indicates a lack of awareness and a negative perspective on antibiotic use, particularly among residents in rural areas of Malaysia. These individuals displayed moderate knowledge (50%), their attitude towards antibiotic use was predominantly negative (56.9%) (Cq and Cy, 2020). Surprisingly, Malaysian university students lack sufficient knowledge about antibiotics, and approximately one - third hold misconceptions about them. This highlights the need for health education interventions at the university level, considering that this demographic represents the future leadership of the country (Tiong and Chua, 2020).

Therefore, the current issue of antibiotic resistance is critical, emphasising the importance of studying and searching for alternative antibiotics. The WHO repeatedly emphasised the antibiotic resistance is one of the top ten threats to public health, affecting the effectiveness of existing antibiotics and leading to increased morbidity, mortality, and healthcare costs (Agyeman et al, 2022). The bacteria continuously develop new mechanisms of resistance and ways to evade antimicrobial treatments. Pathogens such as *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *Acinetobacter baumannii* (*A. baumannii*) exhibit high levels of antibiotic resistance, limiting the effectiveness of available treatments against them (Morris and Cerceo, 2020). Antibiotic resistance also poses a threat to routine procedures and medical modern healthcare, potentially compromising the safety and effectiveness of surgical procedures and immunosuppressive chemotherapy (Littmann et al., 2020). For instance, patients undergoing cytotoxic chemotherapy for solid cancer tumors commonly experience surgical site infections attributed to *Shewanella algae* (Rodriguez-Vargas et al., 2022). The rising prevalence of antibiotic resistance increases the risk of surgical site infections complicated by resistant bacteria, resulting in poorer surgical outcomes (Menz et al., 2021). Another study reported that effective prevention and treatment of infections were threatened, causing an estimated 4.95 million deaths in 2019 (Cooke, 2022). Furthermore, international travelling influences the resistome in the human gut, leading to the acquisition of antibiotic resistance genes across various drug classes (D'Souza et al., 2021). Approximately 30% of travelers were reported to carry antibiotic-resistant bacteria upon returning to their home country, emphasising the need for coordinated international efforts to combat antibiotic resistance (Bokhary et al., 2021). Tackling antibiotic resistance is not only essential for health but also carries economic implications (Wilson et al., 2020), given the considerable costs associated with prolonged illnesses, heightened hospitalisation rates, and the necessity for developing new drugs (Serra-Burriel et al., 2020). Therefore, it is important to find alternative antimicrobial agents to sustainably combat antibiotic resistance.

2.3 Metallic silver nanoparticles as a potential alternative agent for antimicrobial applications

Silver is a transition metal and it has been known due to its strong antimicrobial effects. Since the 19th century, silver has been practiced to prevent and care for most notably infection diseases (Abram and Fromm, 2020). The antimicrobial characteristics of silver have been employed in the healing of ulcers and wounds, as well as in the preservation of food and water. (Das et al., 2020). In fact, silver and its complexes have long been used as antimicrobial agents due to their lack of silver resistance and effectiveness at low concentrations and low toxicities to human cells (Prencipe et al., 2021). For instance, topical ointments containing silver sulfadiazine, present in Silva-Sorb® Gel and Silvadene® cream, prove to be effective in the treatment of burn injuries and controlling sepsis (Walia and Prasad, 2022). Silver-coated bandages, utilising silver sulfadiazine, are

commonly employed as a more affordable alternative to cover burn wounds and traumatic injuries (Tanaka et al., 2021). Silver-impregnated polymers of medical implants such as silver–coated polyetheretherketone implants effectively prevent bacterial biofilm formation and implant-associated infections, offering a promising alternative to traditional silver-coated implants (Ishihama et al., 2021). Silver also serves as a co-disinfectant in portable water systems like ceramic water filters, enhancing disinfection efficacy and potentially expanding access to safe drinking water. (Venis and Basu, 2020).

However, the effectiveness of elemental silver in bulk sized in killing and inhibiting microorganisms due to lesser contact or penetrate with the targeted microorganisms. Some silver-containing products are reported to be lack of established effectiveness and potential toxicity as the indiscriminate use of silver products can lead to toxicity such as argyria (Li et al., 2023). Therefore, antimicrobial properties of silver in nano-sized dimension have been studied in the defined post-antibiotic era to search for new agents that can help combat pathogenic microorganisms without promoting the appearance of new resistances (Fatima et al. 2020). Silver nanoparticles synthesised using asafoetida gum show potential in inhibiting cancer cell growth and exhibiting significant antimicrobial activity against microbial pathogens (Devanesan et al., 2020). In another study, the antibacterial properties from optimised silver/chitosan nanocomposites were tested against *S. aureus* and *E. coli.* The results indicated that silver nanoparticles enhanced the antibacterial activity of chitosan rather than the chitosan alone (Sun et al., 2020). The study also showed that *E. coli* was more sensitive to the composites than *S. aureus* as the nature of cell walls of the gram-negative *E. coli* bacteria were much thinner compared to gram-positive *S. aureus* bacteria's cell wall. Thus, the penetration of Ag^+ to the thin wall of the gram-negative *E. coli* was more quickly and thus increased antibacterial capability (Leus et al., 2023). Silver nanoparticles generated from bacteria such as *E. coli*, *Exiguobacterium aurantiacumm*, and *Brevundimonas diminuta* with National Center for Biotechnology Information (NCBI) accession number MF754138, MF754139, and MF754140 respectively exhibited great potential as antimicrobial agents against methicillin-resistant *S. aureus* (MRSA) and several other multidrug-resistant (MDR) bacteria with minimum 10 mm zone of inhibition (Saeed et al., 2020).

In another perspective, there are studies comparing with the antimicrobial efficiency between silver nanoparticles and $AgNO_3$ since both are derived from the same elemental silver. Generally, both antimicrobial agents possess killing mechanisms as accumulation of silver nanoparticles to the targeted bacterial cells and penetrate the bacterial cell wall meanwhile the Ag^+ dissociate from $AgNO_3$ when dissolves with water will in contact with negatively-charged bacterial membrane and eventually destroy the bacteria (Mohamed et al., 2020). However, the effectiveness of Ag^+ is limited as the antimicrobial activity may

diminish more quickly once the Ag^+ is released at higher concentration initially (Hamad et al., 2020). Other than that, $AgNO_3$ is typically effective in solution form, which make it less versatile in downstream applications (Fouda et al., 2021). Therefore, these kinds of limitations can be overcome by the use of silver nanoparticles as silver nanoparticles allow for better control over the release of Ag+, which can be adjusted based on the specific requirements of the application (Xu et al., 2020). One study showed that the silver nanoparticles with smaller size (less than 10 nm) possessed higher antimicrobial activity than treated $AgNO_3$ (Cobos et al., 2020). Another study reported the antimicrobial activity of silver nanoparticles was more effective than Ag^+ in $AgNO_3$, as the pellet suspension containing the formed silver nanoparticles had higher activity than the tested $AgNO_3$ in a time kill assay against *E. coli* O157: H7, *P. aeruginosa* and MRSA. (Mohamed et al., 2020).

2.4 Postulated mechanism of killing in metallic silver nanoparticles

Unlike colloidal silver, the small size of silver nanoparticles gives specific physiochemical characteristics compared to those of bulk materials of the same composition, mainly due to the high surface- area-to-volume ratio (Hamad et al., 2020). The smaller the nanoparticles are, the larger the surface available for interaction is, resulting in a higher specific activity (Shrestha et al, 2020). Furthermore, the smaller size of silver nanoparticles releases more Ag^+ and consequently exhibit stronger antimicrobial activities (Singh et al., 2020). The study revealed a reverse relationship between the antibacterial activity and the size of silver nanoparticles. Smaller nanoparticles exhibited a greater dissolution rate in various media, releasing Ag^+ in the process. This phenomenon could significantly contribute to the antibacterial effectiveness of nanoparticles (Bruna et al., 2021).

Nevertheless, the exact mechanism of action for silver nanoparticles towards microbial cells are not well elucidated in literature, even though its antimicrobial effects have been extensively reported. However, there are several postulates towards its mechanism of killing which are: (i) adhesion on the surface of the bacterial cell wall and membrane, (ii) penetration into the cell and disruption of intracellular organelles and biomolecules, (iii) induction of oxidative stress, and (iv) modulation of signal transduction pathways (Tripathi & Goshisht, 2022; Godoy-Gallardo et al., 2021; Mammari et al., 2022).

The accumulation and adhesion of silver nanoparticles on the cell surface for Gram-negative bacteria were due to the presence of lipopolysaccharides which contributed to changes in the structural integrity of the Gram-negative bacteria cell wall. The negatively charged lipopolysaccharides make bacteria more

sensitive to positively charged ions from silver nanoparticles and therefore stimulates silver nanoparticles adhesion (Bezza et al., 2020). Silver nanoparticles also have been reported to increase the probability of the trans/cis ratio of unsaturated membrane fatty acids, which changes the composition of the lipid bilayer (Afridi et al., 2022). It interrupts the membrane structure, causing an increase in permeability and loss of membrane integrity which eventually leads to cell rupture (Mikhailova, 2020). Silver nanoparticles also can cause DNA damage due to entering the cells by the bacterial surface adhesion and changes in the membrane properties (Noronha et al., 2022).

Penetration of silver nanoparticles into bacterial cells occurs through porins in the outer membrane of Gram-negative bacteria. Porins are water-filled channels which are primarily involved in passively transporting hydrophilic molecules of various sizes and charges across the membrane (Lee et al., 2020). For Gram-positive bacteria, the thicker cell wall makes the silver nanoparticles difficult to penetrate into the cytoplasm (Yin et al., 2020). The ability of silver nanoparticles to attach to the bacterial cell wall is due to the electrostatic interaction between positively charged Ag^+ and the negatively charged surface of the cell membrane (presence of carboxyl, phosphate, and amino groups) (Shu et al., 2020). The accumulation of silver nanoparticles and electrostatic interaction lead the silver nanoparticles to penetrate it, therefore causing structural changes in the cell membrane and making the cell membrane more permeable. Thus, membrane destruction occurs and bacterial cell death (Ershov et al., 2022). Silver nanoparticles may also act as a carrier to transport toxic metal Ag^+ to bacterial cells (Ghobashy et al., 2021).

Interaction of silver nanoparticles with bacterial cells resulted in reactive oxygen species (ROS) generation by oxidising disulfide bonds of fatty acids in the membrane and forming free radicals (Kumar et al., 2020). The ROS production leads to bacterial membrane distortion or bacterial cell lysis which ultimately leads to cell death (Kim, 2020). In this case, Gram-positive bacteria benefited in generating less ROS than Gram-negative bacteria due to thicker Gram-positive cell wall restricts silver nanoparticles internalisation (Dyett et al., 2021).

Silver nanoparticles have also been suggested to regulate signal transduction pathways in bacteria by dephosphorylating tyrosine residues on bacterial peptide substrates, which inhibits the growth of bacteria (Kawish et al., 2020). Silver nanoparticles also bind to the 30S ribosomal subunit, deactivate the ribosome complex, and stop protein synthesis. This results in immature precursor protein synthesis involves in the cell membrane formation and subsequently leads to cell death (Mikhailova, 2020). It was also found that antimicrobial properties of silver nanoparticles were associated with the intercalation of silver nanoparticles in cell DNA helix which can break its replication process. Positively charged Ag^+ form

complexes with nucleic acids and break the hydrogen bonds between antiparallel base pairs (Chai et al., 2020). The relaxed form of DNA molecular states changes to condensed form decreases the bacterial replication ability (Kumar et al., 2021). A summary for all these postulated mechanisms of killing silver nanoparticles is as shown in Figure 2.2.

Since silver nanoparticles have been demonstrated to be a potent candidate as antimicrobial agents, the most suitable synthesis approach should be heavily discussed and implemented for commercialisation and industrial applications, specifically for low cost, highly reproducible and environmentally friendly. Moreover, toxicity of silver nanoparticles synthesis should be importantly considered as the silver nanoparticles will be applied for biomedical use.

2.5 Silver nanoparticles synthesis approaches

The synthesis methods of silver nanoparticles can be classified into two processes: top-down and bottom-up. The top-down approach refers to breaking down bulk materials into metal nanoparticles, meanwhile the bottom-up approach involves construction of complex clusters from molecular components to obtain nanoparticles by employing nucleation and growth processes (Abid et al., 2022; Pryshchepa et al., 2020; Banerjee and Rai, 2022).

2.5.1 Top-down approaches for silver nanoparticles synthesis

The top-down approach requires physical forces such as ball milling, laser ablation, vapor condensation and electric arc discharge methods to disintegrate the bulk materials (Selvam et al., 2021). Laser ablation and vacuum sputtering are the common methods in synthesising silver nanoparticles (Slepička et al., 2019). Silver nanoparticles, produced through laser ablation, exhibited average sizes ranging from five to 31 nm and were employed for sensing applications (Kalai Priya et al., 2021). Other than that, synthetic methods such as matrix sputtering has been proved to synthesise silver nanoparticles with precisely controlled diameters as small as two nm with some modification of using mercaptoundecanoic acid (Rossi et al, 2021). Sputtering is one of the methods famous for the simplicity of the process and producing pure nanoparticles as no further chemical reactions or additive stabilisers (Chauvin et al., 2020).

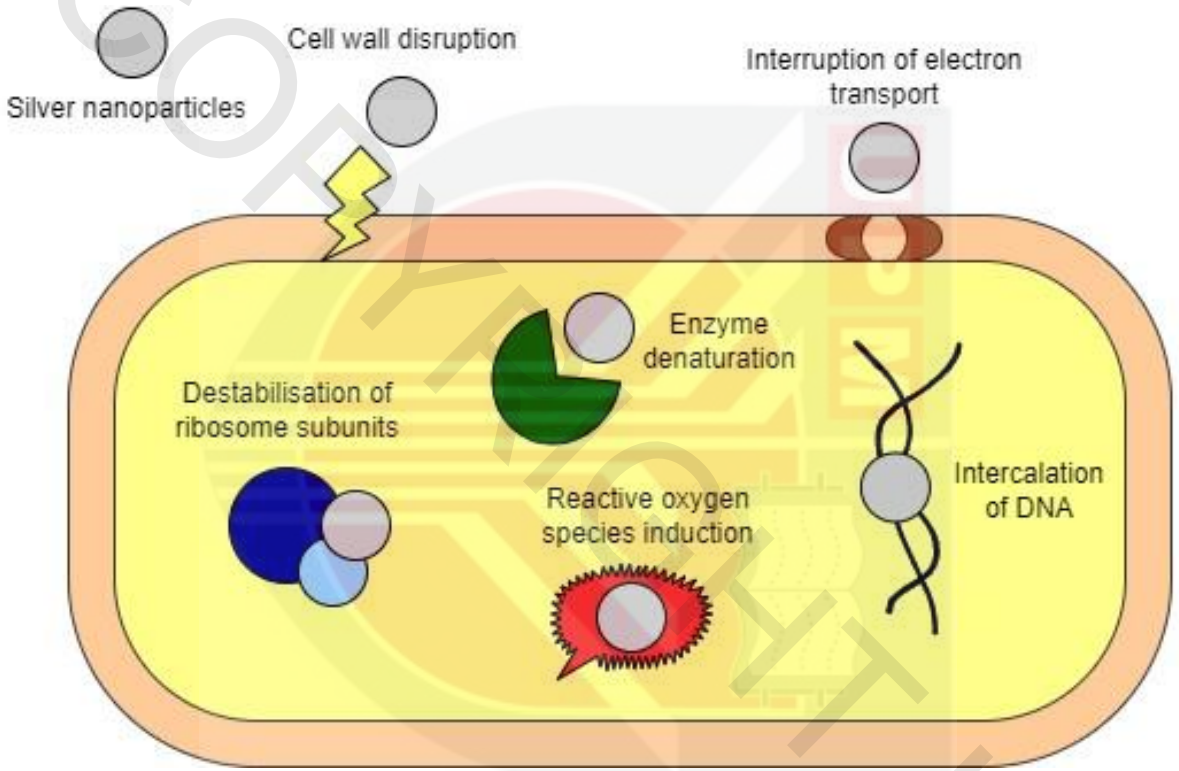


Figure 2.2: Postulated mechanism of actions of silver nanoparticles which are (i) adhesion on the surface of the bacterial cell wall and membrane, (ii) penetration into the cell and disruption of intracellular organelles and biomolecules, (iii) induction of oxidative stress, and (iv) modulation of signal transduction pathways (Adapted from Mikhailova, 2020).

Generally, physical synthesis can massively produce nanoparticles less than 100 nm in size with uniform particle distribution and high purity. However, it brings a great challenge as agglomeration of nanoparticles occurs due to absence of stabilisers or capping agents from chemical reagents which will affect the back-end application (Xu et al., 2020). For example, silver nanoparticles were produced employing a novel, physical, top-down approach of electro exploding wire technique adopted from Siwach and Sen. This method can synthesise small sizes of silver nanoparticles without other salt and chemical contaminants from chemical synthesis of these silver nanoparticles (Mohammed and Naje, 2021). In addition, complex equipment and highly external energy are requisite to reduce particle size in the process of generating nanoparticles which are not economic-friendly to be employed for industrial purposes (Lee et al., 2022). For example, mechanical energy is used in ball milling method as high-speed collisions between rigid balls (ceramics, flint pebbles, and stainless steels) can produce localised high pressures, which grind the metal into very fine powders (Xu et al., 2020). On the other hand, laser ablation method needs high-power laser energy to ablate a metal plate as the metal target absorbs the laser beam energy, followed by nucleation and growth of metal particles and eventually synthesise nanoparticles (Nyabadza et al., 2023).

2.5.2 Bottom-up approaches for silver nanoparticles synthesis

Bottom-up approaches for nanoparticle synthesis involve building nanoparticles atom by atom or molecule by molecule inducing by suitable precursors (Jaji et al., 2020). These methods are often used to precisely control the size, shape, composition, and surface properties of nanoparticles (Khan et al., 2019). Bottom-up approaches comprise of chemical and biological syntheses.

2.5.2.1 Chemical synthesis routes

The bottom-up approaches include chemical and biological syntheses, as both involve the reduction of precursor salt to synthesis nanoparticles (Miu and Dinischiotu, 2022). Silver nanoparticles can be synthesised chemically in organic solutions or water through methods such as photochemical, electrochemical, microwave-assisted and sonochemical methods (Kumar et al., 2021). For example, well-dispersed spherical silver nanoparticles with the size of less than 50 nm range were prepared chemically by reducing silver nitrate with glucose in the presence of protective agent polyvinylpyrrolidone (PVP) (Ghazi et al., 2022). Essential components are needed in this process, which are salt precursor, reducing agent, and stabiliser (Kumar et al., 2020). Silver precursors such as silver nitrate, silver ammonia, silver sulfate, and silver chlorate can be effectively reduced to silver nanoparticles by reducing agents with the presence of

stabilisers for uniform size distribution and good dispersion stability (Xu et al., 2020). For example, chemically-synthesised colloidal silver nanoparticles were also obtained by chemical reduction of silver nitrate in water with sodium borohydride ($NaBH_4$) in the presence of sodium dodecyl sulfate (SDS) as a stabiliser (Rodríguez-Iruretagoiena et al., 2021). In this study, the UV-vis absorption spectra which served as early detection of silver nanoparticles showed that $NaBH_4$ served not only as a reducing agent but also as a stabiliser, which protects the aggregation of silver nanoparticles. Therefore, there was no need for extra stabilising agent use in this silver nanoparticles formation (Noor et al., 2019). TEM images also supported that the particles were dispersed better with increasing the $NaBH_4$ concentration (Lam et al., 2021). The chemically synthesised nanoparticles can be controlled by alternating the components and adjusting the reaction parameters such as the types and ratio of precursors and reducers, with the temperature and pH of the solution, may influence the characteristics of silver nanoparticles (Naik et al., 2019).

Other than that, uniform and well-dispersed silver nanoparticles were prepared by chemical reduction method via reduction of silver nitrate by trisodium citrate and ascorbic acid as a surfactant. The silver nanoparticles were successfully produced ranging from 35 to 80 nm through several characterisation analyses such as scanning electron microscopy (SEM) and X-ray diffraction assay (Barani and Mahltig, 2020). The study emphasised that the increasing concentration of trisodium citrate resulted in decreasing size of silver nanoparticles, while increasing the concentration of ascorbic acid shows the opposite effect (Das et al., 2020). Moreover, the quasi-spherical shape of as synthesised silver nanoparticles is also more uniform with the increase of trisodium citrate. Meanwhile, a slight change in particle shape from quasi-spherical to polygonal was observed as the concentrations of ascorbic acid were increased (Velgosová et al., 2022). Even though chemical reduction method is widely used and the desired sizes and shapes of nanoparticles can be customised by changing the parameters, however the excessive use of harmful chemical reagents producing harmful chemical residues which may limit the use of nanoparticles in medical applications and cause pollution in environment (Xu et al., 2020).

2.5.2.2 Biological synthesis routes

Therefore, in order to overcome shortcomings of the chemical method, the biological method has been suggested as an alternative option for production of silver nanoparticles (Simon et al., 2022). Green method using biological synthesis involves utilisation of plants and plant extracts; together with microorganisms like fungi, yeasts (eukaryotes), bacteria, and actinomycetes (prokaryotes) (Vandana et al., 2020). This method usually relies on macromolecular substances such as exopolysaccharide, cellulose, and

enzymes, and organic components in plant extracts such as enzymes, alcohol, flavonoids, alkaloids, quinines, terpenoids, phenolic compounds which reduces silver salts (Ag^+) to metallic silver, Ag^0 (Xu et al., 2020). Some studies showed that silver nanoparticles have been generated from natural plant extracts *Ocimum tenuiflorum* (Tulsi) (Saha et al., 2019), *Solanum tricobatum* (Thudhuvlai) (Jenifer et al., 2019), *Syzygium cumini* (Naval) (Mittal et al., 2019), *Centella asiatica* (Vallarai) (Eze et al., 2019), and peel of *Citrus sinensis* (Orange) (Mogole et al., 2021) from $AgNO_3$ solution. The aqueous Ag^+ were reduced to silver nanoparticles when added to natural plant extracts. Atomic force microscope (AFM) showed the formation of silver nanoparticle with an average size of 28 nm, 26.5 nm, 65 nm, 22.3 nm and 28.4 nm corresponding to *Ocimum tenuiflorum*, *Syzygium cumini*, *Clonorchis sinensis*, *Solanum trilobatum* and *Centella asiatica*, respectively (Raj et al., 2021). Size and dispersion of silver nanoparticles strongly depend on the bio compounds present in the extract. The presence of a strong reductant in the extract promotes a fast reaction rate and favors the formation of smaller nanoparticles (Mbae and Umesha, 2020). On the other hand, fungi were also utilised for extracellular production nanoparticles of silver as the aqueous Ag^+ exposed to the *Fusarium oxysporum* are reduced in solution (Nair, 2020). The formation of silver nanoparticles synthesised from cell free extracts of edible fungus *Pleurotus florida* in the range of 5-15 nm dimensions and are stabilised in solution by proteins secreted by the fungus (Kaur et al., 2020). Furthermore, biological molecules act as in situ reducing and capping agents. This capping is advantageous as it prevents the agglomeration of the nanoparticles as the bio molecules form a monolayer on the surface of nanoparticles; reduces the toxicity and improves antimicrobial action (Roy et al., 2019). Biosynthesis of silver nanoparticles using yeast extract as reducing and capping agents have been done and showed a uniform distribution and good colloid stability (Skóra et al., 2021). The biomolecules of such as amino acids, alpha-linolenic acid, and carbohydrates in yeast extract have a significant role in the formation of silver nanoparticles, which was proved by the Fourier transform infrared spectroscopy (FTIR) analysis (Bachheti et al., 2021). In addition, amino acids on the surface of silver nanoparticles carry net negative charges which maximise the electrostatic repulsion interactions in alkaline solution, providing favorable stability for more than a year without precipitation (Shu et al., 2020).

Therefore, biological synthesis in general is an economical, environmentally friendly, simple and reliable approach and easily scaled up for large scale synthesis of nanoparticles, furthermore there is no need to use high temperature, pressure, energy and toxic chemicals (Soltys et al., 2021). The summary of frequently used methods for synthesising silver nanoparticles, are visualised in Figure 2.3.

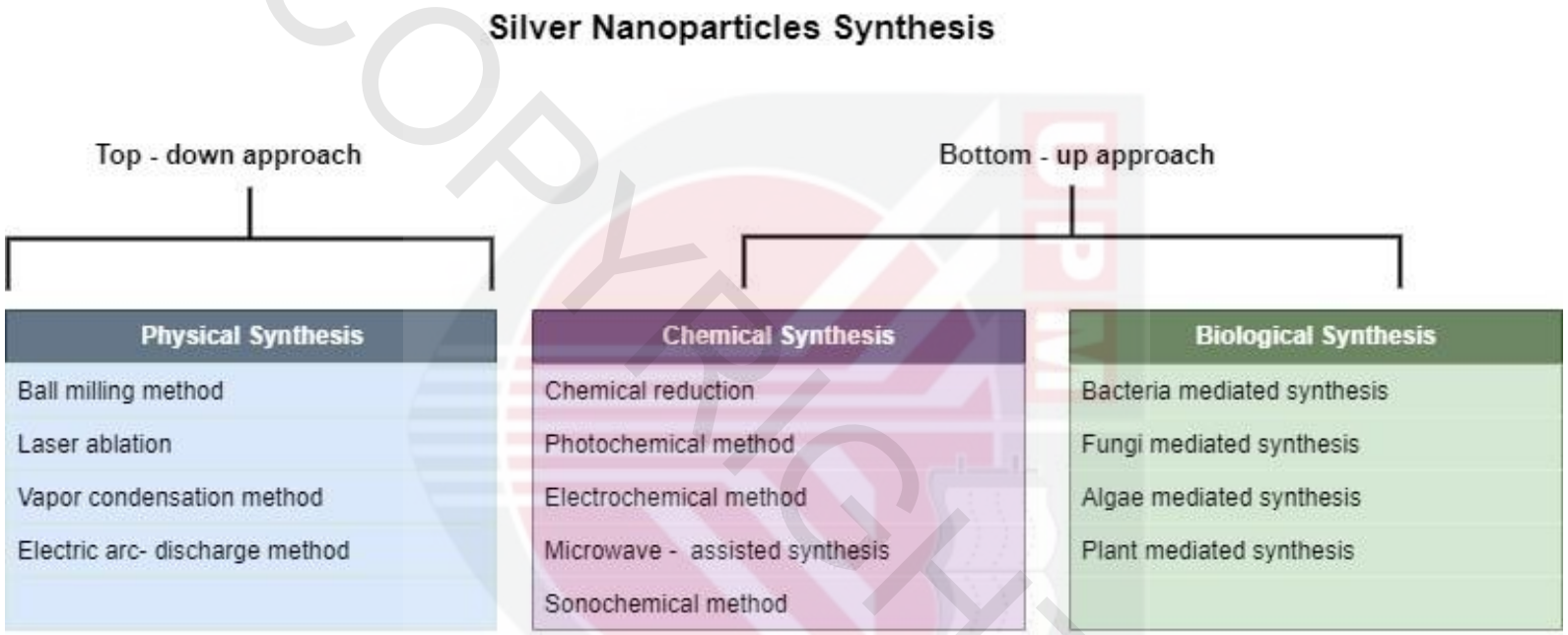


Figure 2.3: The classification of methods in synthesising silver nanoparticles. The top-down approach refers to the breaking down of bulk materials into nanoparticles formation while the bottom-up approach refers to generating of nanoparticles from nucleation and growth processes (Adapted from Xu et al., 2020).

2.6 The advantages of using bacterial cells as a factory to synthesise silver nanoparticles

The first report of bacteria-mediated silver nanoparticles synthesis came in 1999 when the accumulation of silver nanoparticles was discovered inside the cells of *Pseudomonas stutzeri* AG259, a bacterium isolated from a silver mine (Ameen et al., 2020). This bacterium exhibited the property to survive in an extreme silver-rich environment as reduction of metal ions by bacterial cells to its nanoform is one of the survival strategies to render the toxic metal ions, which might be the possible explanation for accumulation of silver nanoparticles (Zhang et al., 2023). After that, a series of bacteria, both Gram-negative and Gram-positive, have been screened for the synthesis of silver nanoparticles. Synthesis of silver nanoparticles using bacteria has gained attention due to its sustainability, biocompatibility, cost effectiveness, non-toxicity, and can be easily used for mass production (Jain et al., 2021). For instance, endophytic bacterium *Bacillus cereus* isolated from the *Garcinia xanthochymus* are capable of synthesising the silver nanoparticles which resulted to be spherical in shape with the size in the range of 20-40 nm with minor aggregation (Sarip et al., 2022). Other than that, spores of *Bacillus athrophaeus* were used as one of the green synthesis methods of producing silver nanoparticles from $AgNO_3$ (Mikhailova, 2020). Enzyme assay revealed that laccase, glucose oxidase, and alkaline phosphatase activities were detected in the spores which are responsible for silver nanoparticles formation (Desai et al., 2024). Newly isolated bacterial strain *Labrenzia* sp. Mab 26, from marine microalgae, *Isochrysis galbana* for efficient biosynthesis of silver nanoparticles extracellularly at room temperature which resulted in average size of 28.9 nm (Sandhya and Vijayan, 2020).

Even though the exact mechanism of silver nanoparticles production by bacteria is not clearly understood yet, several hypotheses have been proposed that some proteins and enzymes are involved in the reduction of silver metals into silver nanoparticles. The extreme environmental conditions such as temperature, pH, and salt concentration force the bacteria to develop some genetic and biochemical mechanisms to withstand environmental conditions and survive in the environment (Matarredona et al., 2020). For instance, *Paracoccus* sp. Arc7-R13, a type of arctic marine bacteria isolated from sediment in the Arctic Ocean, demonstrates the capacity to synthesise silver nanoparticles as a result of exposure to cold temperatures, heightened salt levels, increased seawater oxygen, and UV radiations. These extreme conditions have led to the development of specific adaptive mechanisms enabling the bacteria to withstand such environmental stressors. The bacteria's survival hinges on their ability to effectively regulate intracellular metal ion concentrations. Notably, when confronted with oxidative stress, these Arctic bacteria are more prone to produce antioxidant proteins, particularly those involved in reducing Ag^+, in comparison to other microorganisms (Li et al., 2020). Other than that, some of *E. coli* bacteria resist excessive Ag^+ by effluxing out of the cell (Agrawal et al., 2021). Biogenic reduction of Ag^+ mechanism involves entrapment of Ag^+, reduction, capping, and stabilisation of nanoparticles as depicted in Figure 2.4.

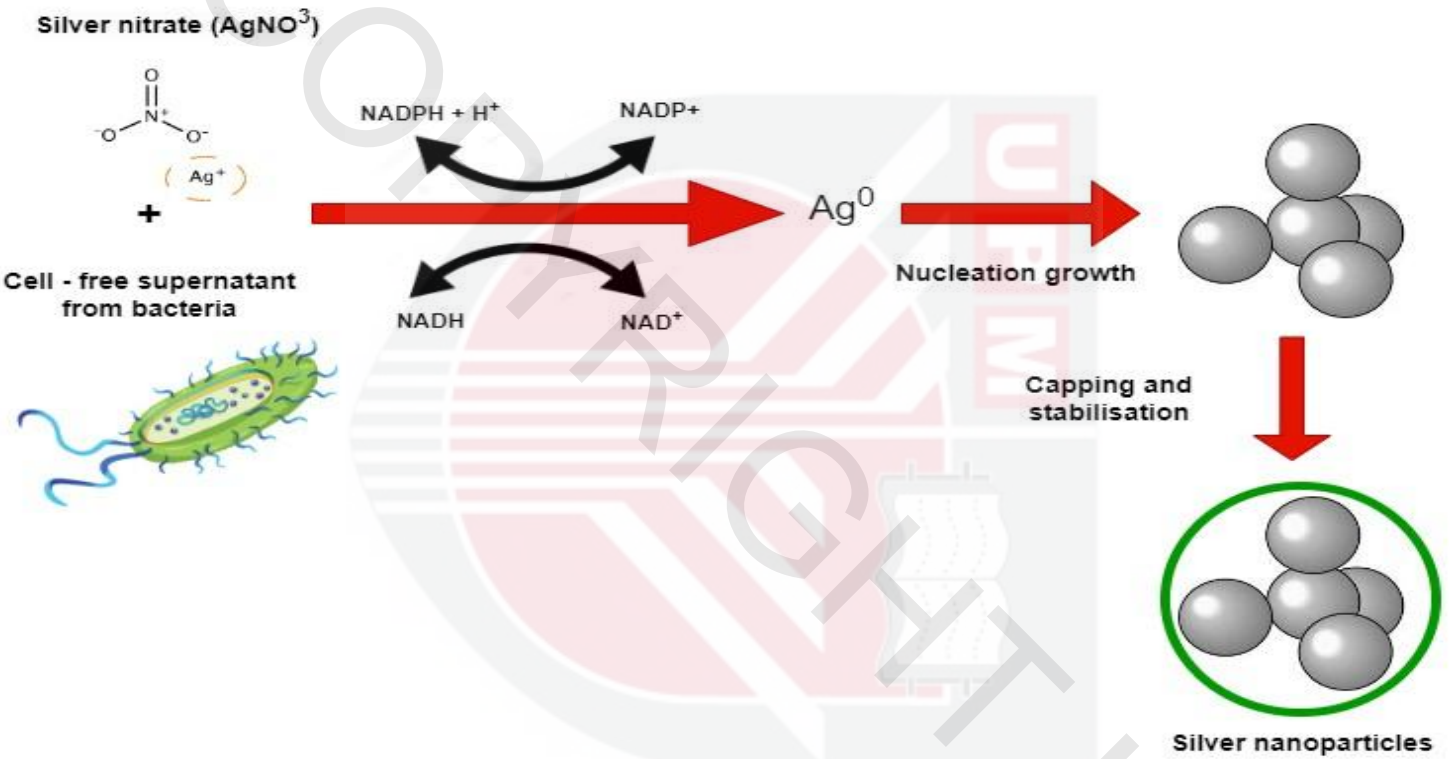


Figure 2.4: The hypothesised mechanism of silver nanoparticles generation by bacteria through NADH-dependent nitrate reductase enzyme. The Ag^+ from $AgNO_3$ is reduced into elemental silver and aggregated in the form of silver nanoparticles (Adapted from Javaid et al., 2018).

The entrapment of Ag^+ happens when electrostatic interaction occurs between the negatively charged carboxylate ions on the cell surface and positively charged The Ag^+ (Yu et al., 2020). In the case of intracellular synthesis, Ag^+ are taken inside the cell for reduction into elemental silver meanwhile extracellular synthesis involves extracellular polymeric substances composed of proteins and polysaccharides capable of adhering and trapping the ions, are secreted by the cell (Garg et al., 2020). After Ag^+ entrapment, nicotinamide adenine dinucleotide (NADH)-dependent reductase plays a significant role in the synthesis of silver nanoparticles whereby NADH-hydrogen ($H^{+)}$ donates its electrons to Ag^+ and Ag^+ gets reduced to their elemental silver form and accumulate in the form of silver nanoparticles (Ahmed and Ogulata, 2021).

In bacterial-mediated synthesis, silver nanoparticles can be produced intracellularly and extracellularly. The Ag^+ is reduced to silver nanoparticles within the cells during intracellular synthesis. As Ag^+ accumulates inside the cells, nucleation occurs, and the synthesis of silver nanoparticles advances alongside microbial growth. The bacterial cell wall, with its negative charge and abundance of enzymes, attracts positively charged, Ag^+ facilitating the reduction of Ag^+ to form stable nanoparticles (Vishwanath and Negi, 2021). Even though the production of using intracellular methods is convenient, the drawback of this method requires additional steps to recover the accumulated nanoparticles from inside the cells. These additional recovery steps bring major concern for industrial perspectives (Rennick et al., 2021). For instance, ultrasonication is the most common technique to recover silver nanoparticles (Pan et al., 2020). Besides that, heat treatment such as autoclaving and the use of detergents and salts can also be employed to lyse the cells (Gomes et al., 2020).

Therefore, the extracellular method is a better alternative for bacterial synthesis of silver nanoparticles to overcome the limitation of the intracellular method (Guleria and Saxena, 2023). In extracellular silver nanoparticles synthesis, there are two possible routes for this: (i) biomolecules released by bacteria into the external medium help in the reduction of silver ions to silver nanoparticles and/or (ii) nanoparticles formed inside the cell are secreted outside (Ghodake et al., 2020). After silver nanoparticles have been generated, the simple recovery step is usually done by high-speed centrifugation up to 10,000–20,000 rpm of the solution containing nanoparticles. These nanoparticles are collected as a pellet, which can be redispersed in the desired solvent (Jadoun et al., 2022). As per mentioned above, extracellular methods of synthesis are advantageous over intracellular synthesis due to the ease of recovery of nanoparticles from the solution (Mukherjee et al., 2020). Table 2.2 below shows the summary of bacterial synthesised synthesis of silver nanoparticles intracellularly or extracellularly.

Table 2.2: Lists of synthesis of bacterial-synthesised silver nanoparticles

Bacteria	Shape	Size (nm)	Location	Reference
Gram-negative				
Acinetobacter baumannii	Spherical	18-50	Extracellular	Abdelhamid et al. (2022)
Escherichia coli	Spherical	6-17	Extracellular	El-Dein et al. (2021)
Pseudomonas aeruginosa	Spherical	30-70	Extracellular	Yang et al. (2020)
Vibrio sp. B4L	Spherical	32	Extracellular	Zamanpour et al. (2021)
Klebsiella pneumoniae	Cubic and irregular	40-80	Extracellular	Sayyid and Zghair, (2021)
Gram-positive				
Bacillus licheniformis	Spherical	2-22	Extracellular	Tan et al. (2021).
Streptomyces hygroscopicus	Spherical	10-30	Extracellular	Alsharif et al. (2020)
Lactobacillus plantarum	Spherical	14-15	Extracellular	Yusof et al. (2020)
Brevibacterium casei	Spherical	5-50	Extracellular	Saeed et al. (2020)
Streptomyces hygroscopicus	Spherical	10-30	Extracellular	Goel et al. (2021)

2.7 Perspective for microbial nanofactories of silver nanoparticles in Malaysia

The huge potential of nanotechnology towards science and innovation has gained interest of the Malaysian government to establish a National Nanotechnology Directorate (NND) under the Malaysian Ministry of Science, Technology and Innovation (MOSTI) (Abidin et al., 2020). The directorate spurs the development of several institutions focusing on nanotechnology invention (Hussain, 2014) such as The Institute of Nano Electronic Engineering (INEE) based at Universiti Malaysia Perlis (UniMAP) is a national research institution focused on nanotechnology, with strong partnerships and collaborations with local and international universities (Hashim et al., 2015). Other than that, there are more nanotechnology institutions such as Ibnu Sina Institute for Fundamental Science Studies (IIS), Universiti Teknologi Malaysia; Institute of Microengineering and Nanotechnology (IMEN), Universiti Kebangsaan Malaysia; Centre of Innovative Nanostructures and Nanodevices (COINN), Universiti Teknologi PETRONAS (UTP); Nanoscience and Nanotechnology Research Group, International Islamic University Malaysia (IIUM); Institute of Advanced Materials (ITMA), Universiti Putra Malaysia (UPM); NanoOpto-Electronics Research Lab (NOR LAB), Universiti Sains Malaysia (USM); and Nanotechnology and Catalysis Research Centre (NANOCAT), Universiti Malaya (UM). In addition, companies such as Nanopac, NanoMalaysia Berhad, and Nano Silver Manufacturing Sdn Bhd (NSM) were rapidly established in Malaysia to support nanoparticle production (Syafiuddin et al., 2017). Since then, silver nanoparticles studies in Malaysia have been increased due to their superior antimicrobial properties in nanoscale dimension. Even though Malaysia as a developing nation is still in the early stages of nanotechnology development compared to the developed nations such as European Union (EU) and the United States (US) (Hasmin et al., 2022), the general public's views on nanotechnology in Malaysia lean toward its advantages rather than risks, and the trust placed in the government, industry, and researchers is instrumental in propelling its advancement (Kamarulzaman et al., 2020).

In Malaysia industrial applications, the antimicrobial silver nanoparticles are employed for plastic, water treatment, and catalyst. NSM, a company focusing on the silver nanoparticles production and application, has launched several products such as Electrode Colloidal Silver (ECS®) for disinfection of water, and products for beauty, health, agriculture, life stock raising, and fish farming. The company has a product called SilverSol™ for antibacterial application. These products clearly show that silver nanoparticles are being heavily utilised for various purposes in Malaysia (Syafiuddin et al., 2017). Nevertheless, while endorsing the utilisation and implementation of nanotechnology, policymakers must exercise caution to ensure the consideration of human health and environmental consequences associated with engineered nanomaterials (Svendsen et al., 2020).

Green synthesis mechanisms especially in bacterial routes have been heavily discussed as it provides the sustainability, biocompatibility, cost effectiveness, non-toxicity, and can be easily used for mass production (Jeevanandam et al., 2022). In Malaysia, the generation of silver nanoparticles has been explored using soil bacteria (Syafiuddin et al., 2017). Malaysia has a vast aquatic ecosystem to include freshwater lakes, freshwater swamps, rivers and peat swamps and marine water bodies such as mangroves, mudflats, intertidal ecosystems and coastal waters encompassing an area about 39,000 km^2 or more than 10% of total land area amounting to 330,000 km2 (Prabhakaran et al., 2017). Industrial activities to aquatic resources have led to increasing toxic discharge, resulting in higher concentrations of heavy metals in these ecosystems (Dehdashti et al., 2020). This led to the emergence of new and varied metabolites, proteins and extracellular polysaccharides. Exploitation of these metabolic pathways could lead to the discovery of innovative bioactive material, instrumental in breaking down heavy metal pollutants while generating precious metal nanoparticles (Pal et al., 2022). Exploration of this ecosystem for nanoparticles - producing bacterial strains would therefore contribute to widening the perspective of silver nanoparticles generation using these microbial nanofactories for the development of green bioprocesses.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

$AgNO_3$ was purchased from Sigma-Aldrich, Germany; Luria Bertani (LB) agar and broth were obtained from Merck Millipore, USA; Universal primers for Polymerase Chain Reaction (PCR): forward primer: (27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer: 1392R (5'-GGTTACCTTGTTACGACTT-3') were synthesised by Integrated DNA Technologies (IDT), Singapore; Deoxynucleotides triphosphates (dNTPs) were purchased from Geneaid, Taiwan; Taq DNA polymerase and 1.5 mM magnesium chloride ($MgCl_2$) were obtained from Qiagen, Germany; Gel extraction and PCR purification kits were procured from Promega, USA; Nuclease-free water was acquired from Amresco, USA; Taq 2X Master Mix was obtained from NEB, USA; Blank, Ampicillin (2 µg/mL) and kanamycin (30 µg/mL) antimicrobial susceptibility disks were procured from Oxoid, USA; All reagents used were of analytical grade and used without further purification.

3.2 General workflow and experimental design

Figure 3.1 shows the general workflow and experimental design based on the study objectives described in Section 1.4.

3.3 Screening for positive isolate of bacterial-synthesised silver nanoparticles from mangrove soil sediment samples

The bacteria samples were previously sampled by Patricia (2019) from Bagan Lalang, Selangor, Malaysia at coordinates 5.4333°N and 100.3833°E. Screening of positive isolates for extracellular silver nanoparticles synthesising bacteria was done using methods previously described by Ghareib et al., (2016) with some modifications. Briefly, a single 24 hour–old colony was inoculated from silver nanoparticles-producing bacteria originated from the mangrove soil sediment samples and cultured in 100 mL LB broth inside a 250 mL Erlenmeyer flask before incubated in an orbital shaker set at 150 rpm for 24 hours at room temperature conditions until the culture became turbid. The culture was then centrifuged at 2600 xg for 30 minutes at room temperature conditions. Approximately 50 mL of cell-free supernatant was then transferred independently into two sterile 50 mL Falcon tubes. After that, the $AgNO_3$ precursor with final concentration of 1 M was freshly prepared prior to addition to cell-free supernatant by adding 0.17 g $AgNO_3$ to 1 mL of sterile deionised

Objective 1

- Screening of silver nanoparticles-producing bacteria
- Colony morphological and bacterial growth curve determination of silver nanoparticles-producing bacteria
- Phylogenetic identification of silver nanoparticles-producing bacteria through 16S rRNA sequencing and molecular phylogeny analysis

Objective 2

- Physicochemical characterisation analyses of bacterial-synthesised silver nanoparticles
- UV-vis spectrophotometry
- DLS
- FESEM
- Transmission Electron Microscopy with Energy Dispersive X-ray (TEM-EDX)

Objective 3

- Disk diffusion assay
- Bacterial growth inhibition assay

Figure 3.1: General methodology and experimental workflow of the study in characterisation of locally produced silver nanoparticles-producing bacteria as well as bacterial-synthesised silver nanoparticles. The successful production of bacterial-synthesised silver nanoparticles has been tested to study and compare the antimicrobial effect towards gram-negative *E. coli* DH5α culture.

water. The 1 mL of 1 M $AgNO_3$ solution was resuspended well and filter-sterilised using 0.45 µm membrane filter. Filter sterilised $AgNO_3$ (Sigma-Aldrich, Germany precursor in deionised water was added to the cell-free supernatant with the final concentration of 10 mM. After that, the fresh cell-free supernatant from two sterile 50 mL Falcon tubes with approximately remaining total of 45 mL each tube will be added with 450 µl of 1 M $AgNO_3$ precursor solution. The 1 M $AgNO_3$ precursor solution was added drop by drop to the cell-free supernatant to achieve optimum reaction for converting into bacterial-synthesised silver nanoparticles. Concurrently, cell-free supernatant only and 10 mM $AgNO_3$ solutions were also prepared as positive and negative controls. All reaction mixtures were incubated at room temperature in dark condition for 72 hours with 150 rpm agitation speed. Preliminary signs of bacterial-synthesised silver nanoparticles formation can be observed in the reaction mixtures color change toward the brown color.

3.3.1 Morphological and growth rate characterisation analyses of silver nanoparticle s-producing bacteria

Morphological characterisation method was performed on positive bacteria isolates that generate silver nanoparticles extracellularly. For morphological characterisation, the silver nanoparticles-producing bacteria was cultured in a freshly prepared LB agar by inoculating a single 24h-old colony from a bacterial stock plate and performing a streaking method. The bacterial plate was incubated at room temperature for 18 hours and morphological characterisation was demonstrated by detailed observation of colony morphology.

Growth rate of silver nanoparticles-producing bacteria was performed from Backlöf, (2011) by inoculating a single 24h-old colony into 100 mL LB broth inside 250 mL Erlenmeyer flask. The growth rate of the silver nanoparticles-producing bacteria was measured at OD 600 nm wavelength for 18 hours with 1 hour gap. The readings were taken and the graph was plotted to determine the doubling time of the silver nanoparticles-producing bacteria.

3.3.2 Molecular identification of silver nanoparticles-producing bacteria using 16S rRNA sequencing

The positive strain was further characterised phylogenetically through 16S rRNA sequencing (Mathivanan et al., 2019) with some modifications. The gene of silver nanoparticles-producing bacteria was isolated and amplified using colony PCR with universal bacterial primers: 27f (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′TACGGTTACCTTGTTACGACTT-3′) to bind with the conserved regions. The PCR reaction mixture contained 50 ng genomic DNA from crude bacterial lysate, 10 pmol of forward and reverse primers, 400 µM of each dNTPs, 0.75 µL Taq DNA polymerase, PCR buffer and 1.5 mM $MgCl_2$. The preparation of the PCR mixture was performed in cold conditions. The PCR program includes initial denaturation at 95 °C for 5 minutes for 1 cycle, 30 cycles for annealing and extension at 95 °C for 45 sec; 51 °C for 15 sec; 72°C for 2 minutes and 1 cycle for final extension at 72 °C for 10 minutes. The amplified DNA was then

visualised on a gel electrophoresis with 1% (w/v) agarose gel along with GeneDirex 1kbDNA ladder under 90 voltages for 30 minutes followed by viewing under ultra-violet. The targeted band in gel was further purified and extracted. The purified PCR products were then sequenced using the forward primer 518F (5'CCAGCAGCCGCGGTAATACG3') and reverse primer 800R (5'TACCAGGGTATCTAATCC3') primers. The amplified 16S rRNA sequences were compared against the GenBank database in NCBI using standard nucleotide Basic Local Alignment Search Tool (BLAST) program to determine similarity percentage. The phylogenetic relationships were constructed using Molecular Evolutionary Genetics Analysis (MEGA) software using the neighbor-joining method.

3.4 Physicochemical characterisation analyses of bacterial-synthesised silver nanoparticles

Bacterial-synthesised silver nanoparticles preparation prior to characterisation are as follows; a single 24 hour-old colony was inoculated from the culture agar plates and cultured in 100 mL LB broth inside 250 mL Erlenmeyer flask and incubated in an orbital shaker set at 150 rpm for 24 hours at room temperature until the culture became turbid. The culture was then centrifuged at 2600 xg for 30 minutes in room temperature condition and cell-free supernatant was added with filter sterilised $AgNO_3$ precursor in deionised water with the final concentration of 10 mM. The mixture was incubated at room temperature for 72 hours with 150 rpm agitation speed in dark condition. The generation of bacterial-synthesised silver nanoparticles were then harvested and purified from Pourali and Yahyaei (2016) with some modifications. Harvesting bacterial-synthesised silver nanoparticles was performed by aliquoting 1 mL of reaction mixture consisting of a cell-free supernatant with $AgNO_3$ precursor solution into a microcentrifuge tube and centrifuged at 2000 xg for 5 minutes. Supernatant part was carefully transferred into a new microcentrifuge tube for another centrifugation step at 14,800 xg for 30 minutes. The bacterial-synthesised silver nanoparticles were then purified by resuspending the pellet from harvesting step in 1 mL of sterile deionised water and centrifuged for another 30 minutes. The purification step was repeated three times to obtain pure bacterial-synthesised silver nanoparticles. The final pellet obtained was then air dried for 48 hours and resuspended in 1 mL sterile deionised water prior to subsequent analyses.

3.4.1 Preliminary screening of bacterial-synthesised silver nanoparticles formation via UV-vis spectrophotometry

Colloidal samples are subjected to an UV-vis spectrophotometer (NanoPhotometer, Implen, USA) as a preliminary evaluation to verify the formation of metal nanoparticles like silver based on the distinct absorbance peaks at 250-600 nm specific wavelength ranges. The dispersion of silver nanoparticles shows intense colors due to plasmon resonance absorption. Therefore, ultraviolet-visible spectroscopy technique is a popular

characterisation method to determine particle formation and its properties (Desai et al., 2012).

An aliquot of 1 mL of cell-free supernatant containing bacterial-synthesised silver nanoparticles until 72 hours incubation into quartz cuvette against LB broth as blank. The optical density (OD) reading was taken from the range of 250 nm until 600 nm with 10 nm gap and the graph was plotted to identify the absorbance peak of bacterial-synthesised silver nanoparticles formation with three biological replicates. The statistical significance of absorbance at the 420 nm wavelength across different time points was assessed using Two-way Analysis of Variance (ANOVA). Subsequently, a Tukey's Honestly Significant Difference (HSD) post-hoc test was conducted to identify significant pairwise differences in nanoparticle formation during various incubation times. All statistical analyses were carried out using GraphPad Prism 9.5.1 (GraphPad Software, U.S.A.).

3.4.2 DLS to determine the size and monodispersity analysis of bacterial-synthesised silver nanoparticles

Particle size distribution and polydispersity analysis of bacterial-synthesised silver nanoparticles in an aqueous solution were determined using DLS (Wani et al., 2010) with the Zetasizer Nano S (Malvern, United Kingdom). DLS was used to determine the average hydrodynamic radius of the microbial generated colloidal nanoparticles representing the average particle size of the nanoparticles in a viscous environment.

The dried pellets of silver nanoparticles were dispersed in 1.5 mL sterile deionised water and sonicated at 30 volts for 30 minutes to obtain a homogenous sample followed by filter-sterilised with a Milipore (Merck, Germany) syringe filter with a pore size of 0.22 µm. The solution was then aliquoted with a total of 1 mL into cuvette prior to analyses including nanoparticles' diameter, z-average and polydispersity index (PDI) using a Malvern Zetasizer NanoZS instrument (Malvern Instrument, UK). The samples were analysed triplicates and average hydrodynamic diameter together with PDI were determined.

3.4.3 Particle morphological analysis of bacterial-synthesised silver nanoparticles formation using Field Emission Scanning Electron Microscopy (FESEM)

FESEM (FEI Model: NOVA NANOSEM 230, USA) was used at Institute of Nanoscience and Nanotechnology (ION2), UPM to probe the size, morphology and elemental composition of bacterial-mediated silver nanoparticles using the method demonstrated by Chandrakanth et al., (2014) with some modifications.

Bacterial-synthesised silver nanoparticles in dried pellet form dispersed in 1 mL of sterilised deionised water, sonicated at 30 volts for 30 minutes, and filter-sterilised with a syringe filter with a pore size of 0.1 µm. The solution was then

diluted in 1:50 ratio by adding 20 µl of nanoparticle solution into new deionised water with a final volume of 1 mL. Then, 10 µL of the diluted nanoparticle solution was dropped onto clean aluminum stubs and dried at 55 °C for 72 hours. A thin layer of gold was then coated on the surface of the sample using ion beam sputtering. The sample was then anchored on a stage and placed in a high vacuum chamber. An ultra-high magnification up to 500000x magnification and 1 nm resolution at 15 kV is used to view the topological landscape of the bacterial-synthesised silver nanoparticles structure.

3.4.4 Size, morphological, and elemental composition analysis of bacterial-synthesised silver nanoparticles using TEM-EDX Spectroscopy

The size, morphology and elemental were further examined using the TEM at IBS, UPM with some modifications (Rauwel et al., 2015). Silver nanoparticles suspension was first filtered with 0.1 µm. filter and sonicated for 15 minutes in an ultrasonic bath to disperse the nanoparticles before being mounted dropwise on a 300 mesh 3 cm carbon coated copper grid and air-dried for 16 hours. Prepared nanoparticles samples were then viewed and the elemental composition was analysed using a TEM-EDX Model JEM-2100F (JEOL, Munich, Germany) operating at 200 kV at 500000x magnification. The degree of conversion of silver formula was calculated with modification as the difference between initial concentration and the final concentration of silver (Dlugosz and Banach, 2019).

$$\frac{Ci\ Ag - Cf\ Ag}{Ci\ Ag}\ x\ 100\%$$

C_i Ag = Atomic weight percentage of Ag in $AgNO_3$ precursor
C_f Ag = Atomic weight percentage of Ag in bacterial-synthesised silver nanoparticles

3.5 Evaluating the antimicrobial potential of bacterial-synthesised silver nanoparticles against gram-negative *E. coli* bacteria

3.5.1 Growth inhibition zones evaluation

The Kirby-Bauer disk diffusion method (Bauer & Kirby et al., 1966) with modifications was used to assay the antimicrobial activity of bacterial-synthesised silver nanoparticles against gram-negative *E. coli* DH5α bacteria strain. One 24 hour-old colony of *E. coli* DH5α bacteria was cultured in LB broth at 37℃ for 24 hours with shaking at 150 rpm. *E. coli* DH5α cultures with approximately 300 uL were spread on sterile LB agar plates and 5 mm of cellulose disks saturated with sonicated bacterial-synthesised silver nanoparticles suspension was placed on the spread microbial culture. The saturation of 5 mm of cellulose discs with bacterial-synthesised silver

nanoparticles suspension was done by immersing 500 uL of sonicated bacterial-synthesised silver nanoparticles suspension for 10 minutes before carefully transferred in the middle of the previous LB agar with *E. coli* DH5α cultures using sterile forceps. Disks saturated with ampicillin and kanamycin at 2 and 30 µg/mL concentrations respectively were tested as positive controls while blank and distilled water saturated disks were used as the negative control. Disk immersed with $AgNO_3$ precursor solution with final concentration of 10 mM was also included in the assay. The assay plates were incubated at 37℃ for 24 hours. After 24 hours of incubation, the diameter of the inhibition zones was measured and recorded. The experiment was conducted with three biological replicates. A Two-way ANOVA was employed to assess the statistical significance of evaluating inhibition zones for antimicrobial agents, comparing them with distilled water as a negative control. All analyses were carried out using GraphPad Prism 9.5.1 (GraphPad Software, USA.). From this data, the rate of efficiency of bacterial synthesised silver nanoparticles comparing to $AgNO_3$ precursor in normalised concentration was calculated as below:

$$\frac{Zone\ of inhibition\ of\ AgNPs\ (Final) - Zone\ of\ inhibition\ of\ AgNO_3(Initial)}{Zone\ of\ inhibition\ of\ AgNO_3\ (Initial)}\ x\ 100\%$$

3.5.2 Bacterial growth inhibition assay

The effect of antimicrobial activity of bacterial-synthesised silver nanoparticles towards the growth rate of *E. coli* DH5α culture was tested with three biological replicates using bacterial growth inhibition assay. A single 24 hour-old colony of *E. coli* DH5α bacteria was inoculated in 100 mL LB broth inside 250 mL Erlenmeyer flask. Then, 10 mL of previous *E. coli* DH5α culture was added in 90 mL of fresh LB broth and incubated at 37℃ with continuous shaking 200 rpm. The absorbance reading at 600 nm wavelength of the incubated culture was measured every 30 minutes and the data was used to construct the growth curve of *E. coli* DH5α strain. Same procedures were repeated with the addition of 1 % (v/v) and 10 % (v/v) sonicated bacterial-synthesised silver nanoparticles at the bacterial lag phase and log phase, respectively. A Two-way ANOVA was employed to assess the statistical significance of the increase in OD within 300 minutes post-treatment. Following this, a Tukey's HSD post-hoc test was executed to identify significant pairwise differences in the growth inhibition activity between the treated and non-treated bacterial cultures. All statistical analyses were conducted using GraphPad Prism 9.5.1 (GraphPad Software, U.S.A.).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Morphological and phylogenetic identification of isolated local bacteria capable of producing silver nanoparticles

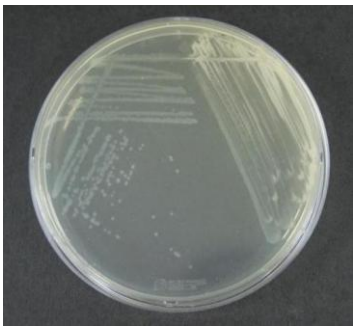
The local bacteria that capable of producing silver nanoparticles have been isolated and further understanding need to be carried out to study the morphology and identify the phylogeny of silver nanoparticles-producing bacteria. Therefore, morphological characterisation has been carried out by culturing and reporting the growth of silver nanoparticles-producing bacteria in agar and broth media meanwhile 16S rRNA sequencing has been performed to specifically identify the phylogeny of silver nanoparticles-producing bacteria. The outcome of these identifications will be the platform to optimise the production of silver-nanoparticles producing bacteria in recommended extensive studies.

4.1.1 Morphological characterisation of silver nanoparticles-producing bacteria

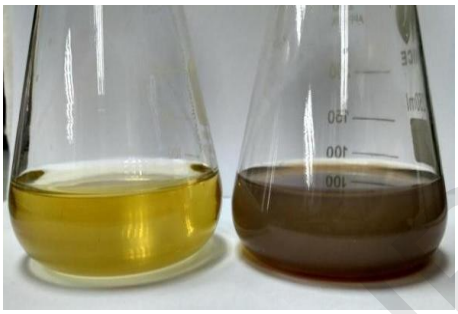
The silver nanoparticles-producing bacteria were isolated from the mangrove soil sediments of Bagan Lalang, Selangor at coordinates 5.4333°N and 100.3833°E and cultured aseptically in LB agar in room temperature condition for 24 hours until pure colonies were grown. The silver nanoparticles-producing bacteria grown in the LB agar was stored at 4°C and subsequently maintained by repeated bacterial plate streak method every two weeks. Figure 4.1 shows the colony morphology of silver nanoparticles-producing bacteria grown in (A) LB agar and (B) the postulated formation of bacterial-synthesised silver nanoparticles after 24 hours incubation with $AgNO_3$ precursor at room temperature, while Figure 4.1 (C) depicts the bacterial growth curve of silver nanoparticles-producing bacteria.

The growth of bacterial-producing silver nanoparticles on agar plate showed in Figure 4.1 (A) after 18 hours of incubation at room temperature. The color of the colony appears as milky-white and opaque, due to the dense accumulation of bacterial cells. The shape of the colonies was observed as round and also irregular shapes. The colony texture was flat but smooth and waxy exterior morphology. Based on visual observation, there are possibilities of bacterial strains possess similar characteristics of colony morphology. For instance, one study reported that four types of bacteria which are *E. coli*, *Lactobacillus rhamus*, *S. aureus*, and *Rhodococcus erythropolis* showed same colony patterns as observed in Figure 4.1 (A). These colonies grew on the LB agar medium and the results were observed after 24 hours of incubation (Badieyan et al., 2018). The growth performance of gram-positive *B. cereus* strain bacteria on LB agar was

A



B



Control 72 hours

C

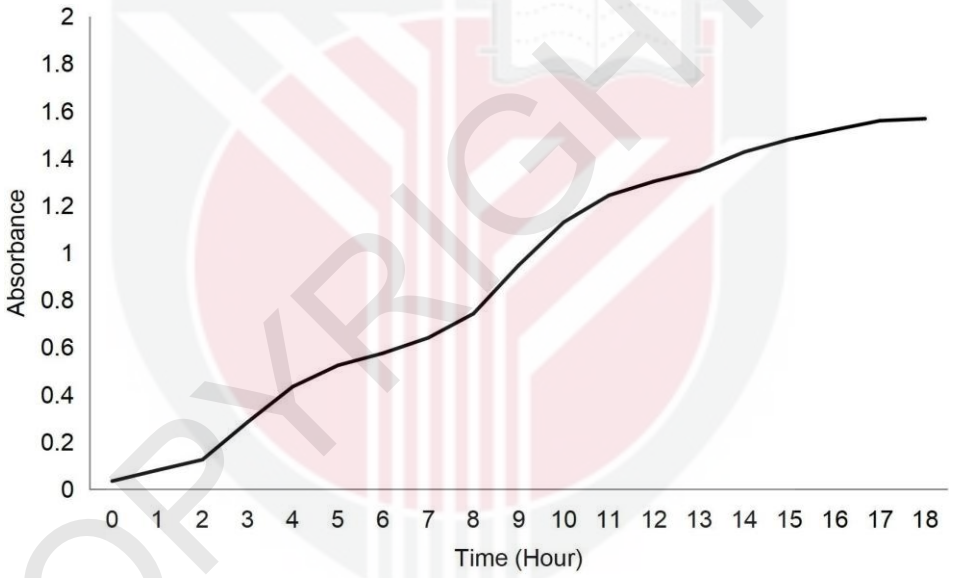


Figure 4.1: Morphological studies of silver nanoparticles-producing bacteria. (A) Colony of silver nanoparticles-producing bacteria after 18 hours of incubation at room temperature produced irregular, milky-white colonies with waxy exterior morphology. (B) Comparison of bacterial-synthesised silver nanoparticles in reaction mixture incubating with 10 mM final concentration of $AgNO_3$ precursor at 72 hours post incubation and cell-free supernatant without precursor as a control. The reaction mixture depicted a change in media color from yellow to dark brown, indicating formation of the nanoparticles. The growth profile of silver nanoparticles-producing bacteria was demonstrated in at room temperature condition.

described as the colony formed was characterised by gray-white, round, opaque, flat with medium-size colony (Lu et al., 2018). On a side note, the colony morphology of the nanoparticles-producing bacteria is not distinctive as compared to common bacteria which are unable to synthesise nanoparticles. This comparison is supported by similar observation of common *Bacillus spp*. (Logan and Vos, 2015) and silver naoparticles-producing *Bacillus spp.* showing a creamy colored, raised colonies with undulated margins (El-Bendary et al., 2021).

The silver nanoparticles-producing bacteria may exhibit particular characteristics specific to their capacity for nanoparticle formation, but these characteristics might not be visible on agar plates. This is often related to specific genetic factors and biochemical pathways in the bacterial system such as nitrate reductase enzymes which play significant roles in reducing metallic silver to silver nanoparticles together with natural capping and stabilising agents for silver nanoparticles to retain the integrity of the nanoparticles formation (Singh et al., 2015). Subsequently, the phylogenetic identification of the silver nanoparticles-producing bacteria needs to be carried out which shall be discussed in the next subsection

However, the presumptive identification of the unknown silver nanoparticles–producing bacteria should be supplemented with other tests such as Gram staining, selective chromogenic agar or BIOLOG system. Gram staining is a fundamental microbiological technique to distinguish bacteria into Gram-positive and Gram-negative based on their cell wall characteristics (Panicker et al., 2022). Selective chromogenic agar is a specialised culture medium designed to selectively isolate particular bacterial groups by targeting characteristics like enzyme activity or resistance to specific antibiotics. Chromogenic substrates within the agar react with specific bacterial enzymes, resulting in the formation of colored colonies. Hence, different bacterial species will produce colonies with distinct colors (Gajic et al., 2022). BIOLOG represents a microbial identification system associated with metabolic fingerprinting employing a range of carbon sources to analyse the metabolic activity of bacteria. In this system, bacteria are introduced to BIOLOG plates featuring diverse carbon substrates. The resulting pattern of substrate utilisation forms a metabolic fingerprint, enabling comparison with a database and aiding in the identification of the bacterium (Luque-Sastre et al., 2020).

Figure 4.1 (B) demonstrates the extracellular aerobic generation of silver nanoparticles from cell-free supernatant as the reaction mixture color was changed from yellow to dark brown after incubation with 10 mM $AgNO_3$ precursor for 72 hours at room temperature under dark condition and there were no changes in color for cell-free supernatant without $AgNO_3$ precursor. This change is an early indicator that bacterial-synthesised silver nanoparticles have been formed due to the SPR phenomenon. This happens when the conduction electrons collectively oscillate on the surface of the nanoparticles in reaction to incident light, absorbing and scattering specific wavelengths which eventually shifting from the initial yellow color towards brown (Kannan et al., 2010). The

color change from yellow to brown is also direct result of the size-dependent optical properties of silver nanoparticles (Link and El-Sayed, 2003). When nanoparticles grow larger, their SPR peak shifts towards longer wavelengths due to changes in the collective oscillation of electrons on the nanoparticle surface. Furthermore, this process also involves intensity concentration dependent as the intensity of the brown color of reaction mixture is increasing over time given by fixed concentration of $AgNO_3$ precursor. However, the intensity of the brown color of reaction mixture indicating formation of bacterial-synthesised silver nanoparticles could be enhanced by increasing the concentration of $AgNO_3$ precursor as substrate (Nayak et al, 2011).

Cell-free supernatant from silver nanoparticles-producing bacteria comprises various bioactive compounds, such as enzymes, proteins, and other reducing agents, that can play a crucial role in the process of synthesis of silver nanoparticles (Saeed et al., 2020). The nature of the bacteria developed a defense mechanism in producing such compounds to survive under extreme high metallic concentration located in mangrove areas. Therefore, this natural defense mechanism developed by silver nanoparticles-producing bacteria was utilised to synthesise silver nanoparticles as reduction of Ag^+ to a harmless form of silver nanoparticles without destroying bacterial cells. One of the bioactive compounds called nitrate reductase enzymes act as reducing agent. This enzyme is induced by nitrate ions (NO^-) from $AgNO_3$ precursor which reduces Ag^+ to its elemental form Ag^0 and results in formation of metallic silver nanoparticles. In this mode of synthesis, the generated silver nanoparticles then accumulated outside the cell without damaging the bacteria. The presence of NO^- from $AgNO_3$ precursor also oxidises NADH to NAD^+, thereby reducing NO^- to NO^{2-} and transferring the electron to Ag^+, converting Ag+ to silver nanoparticles in the process (Kalimuthu et al., 2008). This process also reported in other study as the formation of silver nanoparticles was occurred using cell-free culture supernatants of bacteria and $AgNO_3$ precursor as substrate (Shivaji et al., 2011). This occurrence also coincided with the study of extracellular production of silver nanoparticles originated from soil bacteria *Cupriavidus sp.* when the color changes in the cell-free culture supernatants after stirring with 10 mM $AgNO_3$ precursor at 37°C for 24 hours (Ameen et al., 2020). Other than that, *P. aeruginosa* KUPSB12 has been proven to generate silver nanoparticles extracellularly by the color changes from greenish yellow to brown after incubating at room temperature (Paul and Sinha, 2014).

However, the color change during reduction process is not limited to the production of silver nanoparticles mediated from bacteria only. For instance, the reaction mixture color changes from yellow to brown color for other metallic nanoparticles produced by bacteria such as gold nanoparticles, copper nanoparticles, and platinum nanoparticles as they are also exhibit SPR occurrence. Gold nanoparticles are the most well-known example as they start with yellow color, which eventually shifts towards a vivid pink color (Wen et al., 2009). Copper nanoparticles also exhibit SPR-based color changes as pale-yellow changes to bluish-green over a period of time, suggesting the formation of nanoparticles. (Ramanathan et al., 2013). Platinum nanoparticles can exhibit a change from pale yellow to brown to confirm the nanoparticles have been

synthesised (Riddin et al., 2010). Therefore, the color change from yellow to darker shades of color in bacterial nanoparticle synthesis is due to the growth and aggregation of nanoparticles, driven by the reduction of precursor materials by bioactive compounds produced by bacteria. Other than that, the intensity of the color within reaction mixture could be influenced by the factor of intensity concentration dependent by time as the color was darker at the time point of 72 hours as compared to 0-hour post incubation. The silver reduction process requires a certain length of time for the nucleation process to occur, and for the nanoparticles to grow to the desired size as the time taken for the optimal synthesis of silver nanoparticles from B. cereus isolated from contaminated soil was at 69 hours (Ibrahim et al., 2021).

Extracellular synthesis mechanisms also play a main role in maintaining the integrity of the bacterial-synthesised silver nanoparticles by releasing capping and stabilising biomolecules. It was observed by a study that bacterial-synthesised silver nanoparticles using cell-free supernatants of *Arthrobacter kerguelensis* and *P. antarctica* were stable up to eight months if stored in dark condition (Shivaji et al., 2011). It has also been suggested that stability of the extracellular synthesis of bacterial-synthesised silver nanoparticles using cell-free supernatants could be due to the presence of a proteinaceous capping agent that prevents aggregation of the nanoparticles (Saifuddin et al., 2009). Furthermore, extracellular synthesis favors easy recovery of silver nanoparticles as it only requires high-speed centrifugation. Silver nanoparticles will be pelleted down and air-dried to form powder-like structure, which can be re-suspended in any desired solvent for downstream applications. In another study, the Ag^+ which have been reduced extracellularly using the bacteria of *Vibrio alginolyticus* were centrifuged for 7500 rpm for 15 minutes. Then, the pellet was collected and oven dried in powder form for characterisation and application purposes (Rajeshkumar et al., 2013). Extracellular synthesis of bacterial-synthesised silver nanoparticles from *Pseudomonas sp.* THG-LS1.4 was recovered by high-speed centrifugation at 12,000 g and 25 °C for 10 minutes and washed thoroughly by water to remove the unconverted metal ions or any other constituents. The purified nanoparticles were air dried and obtained in powder form (Singh et al., 2018).

Figure 4.1 (C) demonstrated the growth curve and subsequently determining the doubling time for silver nanoparticles-producing bacteria for 18 hours at room temperature. From this figure, the doubling time of the bacteria was calculated as the log phase was determined from the linear part of a semi- logarithmic plot of absorbance at 600 nm wavelength absorbance over time. The doubling time for silver nanoparticles-producing bacteria was determined by the calculation which is the time it takes for the bacterial population to double in number (Widdel, 2007). The doubling time can be estimated from the steep slope of the exponential phase on the growth curve as referred from Appendix 1. Derived from the graph above, the doubling time for silver nanoparticles-producing bacteria was approximately 53 minutes. Generally, the doubling time of bacteria varies depending on the species or strains, specific growth conditions and environmental factors. However, non- nanoparticles producing bacteria showed faster doubling time than silver nanoparticles-producing bacteria as reported in

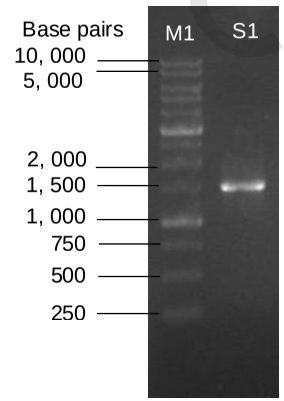
Figure 4.1 (C). For instance, a doubling time for *E. coli* should occur approximately 20 minutes when grown in a rich liquid broth under optimal growth conditions (Son and Taylor, 2021). It can be varied up to 60 minutes depending on growth condition. Other than that, the ultra-fast growing marine bacterium *Vibrio natriegens* holds the record as the fastest growth rate of any known organism as the doubling time of less than 10 minutes (Weinstock et al., 2016). Gram-positive *Bacillus* strain such as *B. subtilis* has range of doubling time starting from 20 to 25 minutes (Armstrong et al., 1970). When comparing with silver nanoparticles-producing bacteria, the slower rate of doubling time might due to the additional biochemical pathway of the bacteria to produce bioactive compounds which are responsible to reduce $AgNO_3$ precursor to generate bacterial-synthesised silver nanoparticles. In summary, these morphological characterisation and bacterial growth rate determination will aid in identification of silver nanoparticles-producing bacteria.

4.1.2 Phylogenetic identification of silver nanoparticles-producing bacteria

Phylogenetic identification of silver nanoparticles-producing bacteria was conducted using 16S rRNA sequencing. The sequence of the 16S rRNA gene has been widely used as a phylogenetic marker to study genetic relationships between different families, genus, species and strains levels of bacteria (Anju and Sarada, 2016). In this study, colony PCR was performed to isolate 16S rRNA gene followed by gel electrophoresis using 1% TAE agarose gel. The purified DNA was then sequenced and phylogenetically characterised. Figure 4.2 showed the results of 16S rRNA sequencing in the phylogenetic identification of silver nanoparticles-producing bacteria with Figure 4.2 (A) representing the PCR amplified 16S rRNA gene of silver nanoparticles- producing bacteria; (B) reported multiple sequence alignment and (C) illustrating the phylogenetic tree depicting *Bacillus* strains with closest similarities to silver nanoparticles-producing bacteria 16S rDNA nucleotide sequences using the Neighbor Joining (Unrooted Tree) through NCBI Blast.

Figure 4.2 (A) depicted that the size of the PCR amplified silver nanoparticles-producing bacteria 16S rRNA fragment lies to 1.5 kilo base pairs (kbp) against 1kb Marker. The data was compared with available literature as it is postulated to belong to *Bacillus sp.* (Anju and Sarada, 2016). As referred to Figure 4.2 (A), the 1.5 kbp 16S size might be also revealed to be grouped as *Morganella sp.* (Parikh et al., 2008); *Pseudomonas* and *Klebsiella spp.* (Athreya et al., 2020). Even though the size of the 16S rRNA gene in bacteria is relatively conserved, the bacteria can differ from 1.5 kbp 16S rRNA fragment size range as 16S rRNA gene can be differ from one bacterial species and strains to another. However, the ability to synthesise silver nanoparticles is due to specific genetic mechanisms and enzymes within the bacteria, but not correlated to the size of 16S rRNA.

A



B

>Bacterial-synthesised silver nanoparticles

CGGACTTGCAGCGTAATATACATGCAGTCGAGCGAATGGATTAAGAGTTCTCTT
ATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGAC
TGGGATAACTCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATG
GTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGC
ATTAGTCTAGTTGGTGCAGCTAACGGCTCACCAAGGCAACCATGCGTAGCCGA
CCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTACGGAATCTACCGCAATGGACGAAAGTCTGACGGAGCAA
CGCCGCGTGAGTGATGAAGGCTATCGGGTCGTAAAACTCTGTTGTTAGGGAAG
AACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCAGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTACTGGGCGTAAAGCGCCGCGCAGCTGGTCTTCTCAAGTCTGATTGT
GAACAGCCCACGGCTCAACCGCTGGAGGGTCATTTGAAACTGGGAGACTTGAA
TGGCAGAAGGAGGAAAGTGGAATTACATGTGTACCGGTGAAATGCCGAAAAGA
TATGGAGGAACCACCAGTAGCGAAGGCGACTTTTCTGGTCTGTAACCGACACT
GAGGCGGCGAAAGCCTGGGGAGCAAACCAGGATTAGGATACCCTGGTACGTC
CACGCCGGAAACGAAGAAGTGCTTAAGTGTAAGAGGGTTTCCGCCCTTTAGTT
GCTGAAGGTTAACCCATTAAGCAATTCCCCCTGGGGAGTACCGCCGGCAAGGC
TGAAAATCCAAAGGAAATAGACCGGGGGCCCGCACAGCCGTGGAACCATGTG
GTTAAATTCGGAAGCAACGCGAAGGAACCTTACCAGGTCTGACATCCTCTGGA
CACCCCAAAAAATAGGCTTCTCCCTCAGGGAGCAGAATGACCAGTGGTGCCAT
GGTTGTCGTTCAGCTCGGGTTCAGTGAGAAGTATGCGTTAAAGTCCTGCCACG
AAGCGCCACCCCTGGATCTTTAGTGACATCATCTAATTGGGCACATCTTAAGGG
GACTCCCGGGACCAACCGGGAGGAAGCGTGGAGAGGACCTCAAATCATCCAT
CACCCCTTATGACCTTGGCTTCACCACCTTGCTCCAAGGACCGGTCCAAAGAG
CTGCACAGAACCCCCAAGTGGGAAGTTATTTTCAAAAACCCTTTTCCATTTGGG
ATGGTAGGTGCCAATTGCCCACCAGGAGGCGGAATTCGTTGGTATTCGGGATT
CACCAGCCCCCGGTAAAAAGTTTCCCGGCCCTGGACACCCCCCCCCTTCCCAC
CCCGGAATTTGTAACCCCCGGAGTTGGTTGGGGAACCCTTTGGAGCCAGCCG
CCTAATATAATCAGAGGTTCG

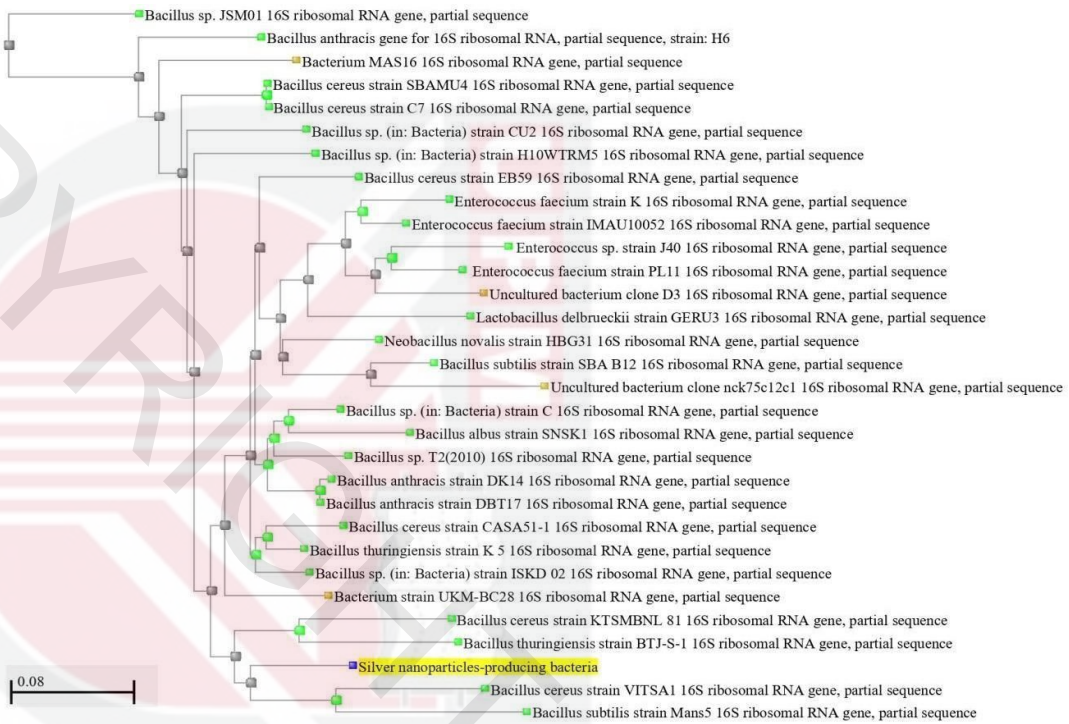


Figure 4.2: Phylogenetic identification of silver nanoparticles-producing bacteria. (A) shows a single solid band of 16S rRNA colony PCR product with 1.5 kb band size, as M1 represents marker and S1 is the genetic product of silver-nanoparticles-producing bacteria. (B) The multiple sequence alignment of silver nanoparticles-producing bacteria (C) Phylogenetic tree construction of silver nanoparticles-producing bacteria as it is most probably belonged to *Bacillus* genus.

The amplified fragment was purified and sequenced as shown in Figure 4.2 (B) comprising of full-length sequence of bacterial-synthesised silver nanoparticles. The sequence obtained was then compared with the NCBI 16S ribosomal RNA sequences database. The sequences hits that demonstrated striking similarity to silver nanoparticles-producing bacterium S1 were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm from the MEGA 11.0 software and a phylogenetic tree constructed using this data as depicted in Figure 4.2 (C). In general, the phylogenetic tree helps us to locate the origin of new genes; detect molecular adaptation; understand morphological character evolution and reconstruct demographic changes in recently diverged species (Kapli et al., 2020). It is shown in phylogenetic tree that silver nanoparticles-producing bacteria are highly homologous to *Bacillus spp*. as they are inherited from the same ancestral gene. The genes are related to many of the *Bacillus* strains such as *B. anthracis*, *B. cereus*, *B. subtilis*, *B. albus*, and *B. thuringiencis*. The silver nanoparticles-producing bacteria also associated with different genera. For example, *Enterococcus faecium* K, IMAU 10052, and PL11 strains, *Lactobacillus delbrueckil* strain GERU3, and *Neobacillus novalis* strain HBG31.

However, the phylogenetic tree construct depicts the silver nanoparticles-producing bacteria are closer to *B. cereus* strain KTSMBNL, *B. thuringiensis* strain BTJ-S-1, *B. cereus* strain UITSAI, and *B. subtilis* strain Mans5. The evidence studies showed a lot of bacterial strains from *Bacillus spp.* are capable to produce silver nanoparticles as two mesophilic bacteria *B. indicus* and *B. cecembensis* have been used to synthesise silver nanoparticles (Shivaji et al., 2011). Interestingly, different bacterial strains that have the ability to synthesise silver nanoparticles naturally are isolated from different habitats rather than mangrove environment. For instance, *B. subtilis* isolated from military hospital was reported to biosynthesise silver nanoparticles (Alsamhary, 2020). Metal resistant *B. cereus* isolates from electroplating industries in India demonstrating a potential generation of silver nanoparticles (Babu and Gunasekaran, 2009). From this observation, it is concluded the bacteria has been evolved with resistance to heavy metal ions or specifically growing in metal ion rich niche can be used for the purpose of factory for nanoparticles production. This conclusion has been supported as non- pathogenic *B. thuringiensis* which are highly resistant to a high concentration of zinc ions were used to synthesise silver nanoparticles (Nayak et al., 2016).

Besides Bacillus genus, plenty of microorganisms have been discovered to biosynthesise silver nanoparticles. For instance, extracellular biosynthesis of silver nanoparticles using a fungus named *Alternaria alternata* has been studied to biosynthesise silver nanoparticles extracellularly with the size range of 20-60 nm (Gajbhiye et al., 2009). Other than that, *Pseudomonas stutzeri* (*P. stutzeri*) AG259 is potentially synthesised silver nanoparticles with the size of 200 nm by reducing silver nitrate precursors (Klaus et al., 1999). Cell-free culture supernatants of five psychrophilic bacteria *P. antarctica*, *P. proteolytica*, *P. meridiana*, *Arthrobacter kerguelensis* (*A. Kerguelensis*) and *A. gangotriensis* are proven to synthesise silver nanoparticles (Shivaji et al., 2011).

Based on the results, the 16S rRNA sequencing successfully narrowed down the identity of the unknown bacterium to the genus level. However, to achieve a more precise identification at the species level, further studies could be undertaken such as conducting whole-genome sequencing of the bacterium. Whole-genome sequencing offers a comprehensive analysis of the organism's entire genetic material and facilitates taxonomic classification by comparing the complete genome to existing databases or reference genomes (Kweon et al., 2020).

4.2 Physicochemical characterisation of bacterial-synthesised silver nanoparticles

Previously, the color of reaction mixture changed when the cell-free supernatant was incubated with $AgNO_3$ precursor from yellow to dark brown indicated the nanoparticles have been formed by the reduction of NO^- to NO^{2-} and transferring the electron to Ag^+, leading to conversion of Ag^+ to silver nanoparticles (Kalimuthu et al., 2008). However, deeper understanding of color changes in reaction mixture needs to be carried out to characterise the nanoparticles mediated by silver nanoparticles-producing bacteria. Therefore, physicochemical characterisations of bacterial-synthesised silver nanoparticles were performed. Physical characterisations such as FESEM and TEM showed morphological view of bacterial-synthesised silver nanoparticles meanwhile co-chemical characterisations such as UV-vis spectroscopy, DLS and EDX analyses were carried out to determine the nanoparticles formation, average size as well as quantification of elemental composition present in bacterial-synthesised silver nanoparticles. Deeper understanding of bacterial- synthesised silver nanoparticles through physicochemical characterisation will help in understanding of antimicrobial potential of bacterial-synthesised silver nanoparticles in the next section.

4.2.1 Preliminary assessment of bacterial-synthesised silver nanoparticles using UV-vis spectroscopy

UV–vis absorption spectroscopy is the common method for preliminary characterisation of nanoparticle structures, as the absorption bands are related to the diameter and aspect ratio of metal and semiconductor nanoparticles (Smitha et al., 2008). Figure 4.3 (A) shows the UV-Vis absorption peak of bacterial-synthesised silver nanoparticles and (B) the time point absorbance peak at the wavelength of 420 nm. The changes in color from yellow to dark brown after the addition of $AgNO_3$ precursor for 72 hours as shown in Figure 4.1(B). The distinct dark brown color arising from tiny dimensions of bacterial-synthesised silver nanoparticles and a result of the absorption of light at a particular frequency due to the oscillation of conduction electrons at the surface of the nanomaterial when in contact with incident light, a phenomenon known as SPR (Aroca et al., 2005). As comparison, absorbance peak of the control is also tested, consisting of LB broth and 10 mM $AgNO_3$ precursor with the same parameter. The generation of the bacterial-synthesised silver nanoparticles

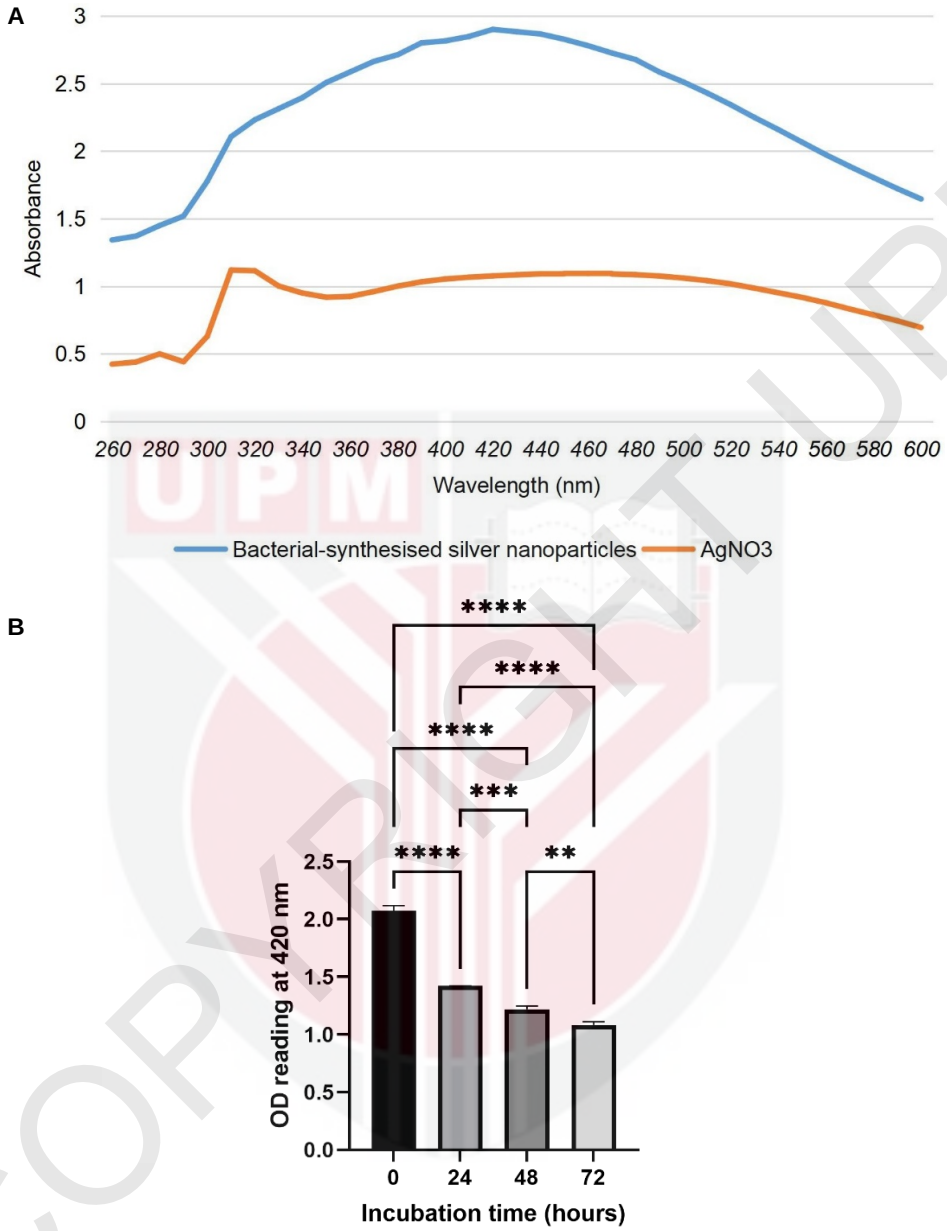


Figure 4.3: Preliminary assessment of bacterial-synthesised silver nanoparticles formation. The line graph shown in (A) UV-vis spectroscopy after the cell-free supernatant of bacterial producing silver nanoparticles incubating with 10 mM $AgNO_3$ precursor after 72 hours in a wavelength range of 300 to 600 nm. The distinguishable absorbance peak was observed at around 420 nm for bacterial-synthesised silver nanoparticles meanwhile positive control comprising LB media incubated with 10 mM $AgNO_3$ precursor showed absorbance peak at 310 nm with lower absorbance reading. (B) is the time point absorbance at the wavelength of 420 nm starting from 0 hour to 72 hours as the trend declines across incubation time. The symbol of "*" indicates significance with $p < 0.05$; "": $p < 0.01$; "***": $p < 0.001$; $p < 0.0001$ and ns: not significant.**

using final concentration of 10 mM $AgNO_3$ precursor was chosen concentration reactants for nanoparticle synthesis as high concentrations can accelerate the nanoparticle formation process and produce controlled and well-defined nanoparticles. However, extremely high concentrations can lead to rapid and uncontrollable reactions, resulting in nanoparticle aggregation or irregular growth. For instance, a study reported that Cyanobacteria system used 10 mM $AgNO_3$ precursor to generate silver nanoparticles (El Semary and Bakir, 2022).

Figure 4.3 (A) showed preliminary determination of bacterial-synthesised silver nanoparticles formation from OD trendline as the cell-free supernatant was incubated with 10 mM $AgNO_3$ precursor and the distinct peak was observed at 420 nm wavelength meanwhile positive control showed strong peak at the wavelength of 310 nm with lower absorbance value. The higher absorbance observed in bacterial-synthesised silver nanoparticles compared to the control is primarily due to the formation, growth, and potential aggregation of silver nanoparticles during the synthesis process. The nanoparticle synthesis process by the silver nanoparticles-producing bacteria may result in a higher concentration of nanoparticles in the solution compared to the control, where only $AgNO_3$ is present which therefore contribute to higher absorbance values in the UV-Vis spectrum. The absorbance reading shifting from positive control to sample indicates the bacterial-synthesised silver nanoparticles have been formed.

A single peak correlates with Mie's theory on the formation of spherical nanoparticles but the broadness of the peak concurs with a wide size distribution or the polydispersity of the biogenerated silver nanoparticles (Sanghi and Verma, 2009). The absorption peak at 420 nm is comparable to the studies of successful generation of silver nanoparticles from *Streptomyces hygroscopicus* as a strong and broad peak was observed between 420 nm and 425 nm, indicating the presence of silver nanoparticles due to the reduction of metal ions by secondary metabolites present in the cells (Sadhasivam et al., 2010). Exploitation of *Aspergillus niger* for extracellular synthesis of silver nanoparticles isolated from the soil has been reported as there was an increase in absorption centered at 420 nm (Gade et al., 2008). Other than that, the bacteria from a deep-sea water sample named *P. aeruginosa* (JQ989348) was isolated and utilised for synthesis of silver nanoparticles. The successful synthesis of silver nanoparticles was confirmed by analysing SPR using UV–visible spectrophotometer at 420 nm (Ramalingam et al., 2014).

The incubation time for silver ions from $AgNO_3$ precursor to be converted into silver nanoparticles was depicted in Figure 4.3 (B). The time point absorbance at the wavelength of 420 nm starting from zero hour to 72 hours as the value trend declines across incubation time. A prolonged incubation time induces nanoparticle aggregation. This phenomenon causes size increment and alters the scattering properties of the nanoparticles, leading to a decrease in the OD reading (Bezrukova, 2004). A study proved the lower OD reading caused by the aggregation of silver nanoparticles (Bu and Lee, 2012). Another study also

demonstrated that an increase in particle size intensifies the scattering mode of the particles (Du et al., 2020). In addition, the reaction kinetics may almost reach a saturation point or equilibrium, causing the rate of silver nanoparticles formation to slow down. This could be reflected in a reduction of OD reading as the reaction progresses. This rate of reduction of Ag^+ ions, when limited, directly impacts the optical density during the reaction process (Tatarchuk et al., 2013). Extending the incubation time silver nanoparticles formation may cause completion of the reduction reaction, reaching a point of equilibrium where the concentration of reactants and products remains relatively constant. This could result in a plateau OD reading in the graph (Kamala Nalini and Vijayaraghavan, 2020). Other than that, beyond a certain timepoint, additional incubation time of the reaction mixture may not significantly contribute to the formation of silver nanoparticles or improve desired properties in terms of nanoparticle yield or quality. This was supported by a study on silver nanoparticle synthesis using *Musa balbisiana* leaf extract. The reaction mixture was incubated for a range of zero to 120 hours, revealing that an incubation time of 72 hours is optimal for both the synthesis and stability of the silver nanoparticles. Over time, the color intensity and absorbance at 420 nm gradually increased until the 72-hour timepoint, after which both parameters remained nearly constant (Dave et al., 2022).

4.2.2 Particle size distribution and polydispersity analysis of bacterial-synthesised silver nanoparticles using DLS

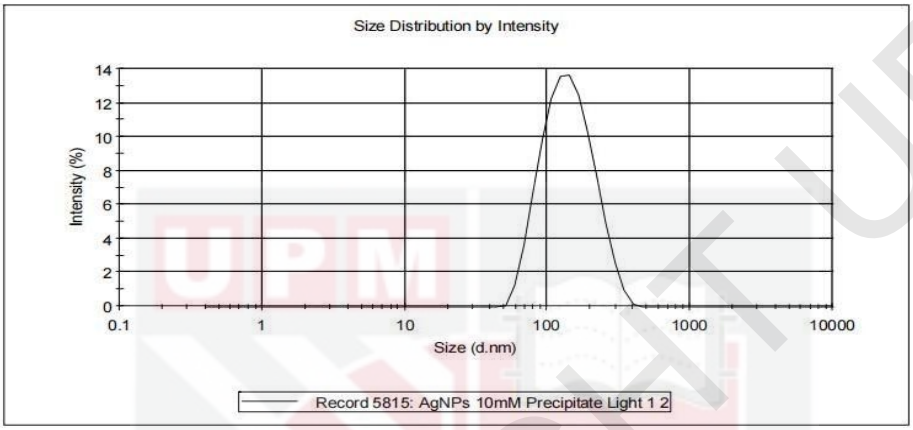
Figure 4.4 (A) is result of the grossion curve of DLS showing silver nanoparticles have been produced. A single and narrow pattern of grossion curve of bacterial-synthesised silver nanoparticles sample indicated a well-defined with less polydispersed nanoparticles. Subsequently, a few replicates were repeated and Figure 4.4 (B) shows the DLS measurements of bacterial-synthesised silver nanoparticles demonstrate particle size and polydispersity in colloidal solution. DLS measurements gave the value of the PDI and hydrodynamic diameter in z-average (nm) value together with standard deviation (SD) for respective data of the bacterial-synthesised silver nanoparticles. The data was obtained from six biological replicates with three technical replicates each.

Interpreting from Figure 4.4 (B), the average hydrodynamic diameter was 139.73+13.63 nm, derived from the diameter of the nanoparticles in highest proportion in the colloidal solution. Low PDI with 0.2±0.05 value refers to uniform particle size distribution revealing homogeneity of nanoparticle size. These findings are comparable to extracellular synthesis of silver nanoparticles by bacteria such as *P. stutzeri* as the silver nanoparticles were built up just about 200 nm in diameter and of a definite composition and shape (Beyene et al., 2017). *Salmonella thypirium* was proven to biosynthesis of silver nanoparticles as the DLS result showed 129.30 nm size with PDI 0.13 value, indicating monodispersed silver nanoparticles have been formed (Ghorbani, 2013). Other than that, some studies showed lower values with less than 100 nm diameter of silver nanoparticles with less than 0.50 PDI values (Modi et al., 2015).

A

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	128.3	Peak 1:	147.6	100.0	59.30
PdI:	0.176	Peak 2:	0.000	0.0	0.000
Intercept:	0.937	Peak 3:	0.000	0.0	0.000
Result quality :	Good				



B

AgNPs	z-average (nm)	PDI
B1	146.03	0.21
B2	147.53	0.21
B3	149.50	0.21
B4	127.30	0.18
B5	118.00	0.15
B6	150.03	0.28
Mean	***139.73***	***0.20***
SD	***13.63***	***0.05***

Figure 4.4: Size and polydispersity of bacterial–synthesised silver nanoparticles analysis in solution via DLS. (A) is the grossion curve of successful formation of bacterial synthesised silver nanoparticles showing size distribution and PDI value and (B) depicted the average of hydrodynamic diameter (nm) and PDI value of bacterial-synthesised silver nanoparticles replicates.

4.2.3 Morphological and particle characterisation of bacterial-synthesised silver nanoparticles using FESEM and TEM

Nanoparticle characterisation using FESEM and TEM are the common methods for analysis of surface morphology of nanoparticles. Figure 4.5 depicts FESEM images of the surface morphology of (A) cell-free supernatant of silver nanoparticles-producing bacteria MA6 while (B) is the $AgNO_3$ precursor diluted with sterile distilled water at 10 mM concentration. Successfully formed of bacterial-synthesised silver nanoparticles has shown in (C) and (D) at two different magnifications.

Interpreting from the Figure 4.5 below, there are significant morphological trends from all samples. Figure 4.4 (A) shows a big clump of irregular shape from a cell-free supernatant of silver nanoparticles-producing bacteria meanwhile (B) is the photo of $AgNO_3$ precursor at 10 mM concentration showing rectangular lattice shape with the size about 1 µm. Figure (C) is the final form of bacterial-synthesised silver nanoparticles with uniform spherical shapes with minor aggregation after incubating the cell-free supernatant with $AgNO_3$ precursor at 10 mM after 72 hours and (D) is the image of bacterial-synthesised silver nanoparticles with higher magnification as we can conclude the size of bacterial-synthesised silver nanoparticles is less than 25 nm with slight differences in range

Ideally, the size of nanoparticles in FESEM should be equivalent with the result of z-average in DLS measurement. However, the FESEM result showed otherwise as the diameter size of bacterial-synthesised silver nanoparticles is smaller than the size depicted in the DLS result. This is due to the aggregation of silver nanoparticles that has occurred in colloidal solution prior to DLS analysis. A closer look at this sample Figure 4.5 (D) at high magnifications of 200 000x postulates smaller, sphere shaped of bacterial-synthesised silver nanoparticles were observed. These findings are similar where the hydrodynamic diameter of silver nanoparticles synthesised by *B. thuringiensis*, *E. coli*, *S. aureus*, and *S. typhimurium* from DLS analysis were larger than FESEM results respectively (Verma et al., 2018).

While FESEM provides crucial information on nanoparticle shape and surface topology, the TEM visualises the cross section of a thin specimen through phase contrast imaging (Egerton, 2005). Figure 4.6 shows TEM micrographs of bacterial-synthesised silver nanoparticles at magnifications of 50 000x and 100 000x using an electron beam accelerating at 100 keV. The TEM micrograph of bacterial-synthesised silver nanoparticles in Figure 4.6 (A) resembled uniform and monodisperse spherical shape bacterial-synthesised silver nanoparticles surrounded by membrane-like structure produced by silver nanoparticles producing bacteria and (B) depicting the size of bacterial-synthesised silver nanoparticles with less than 20 nm, slightly smaller than FESEM images in Figure 4.5 (D).

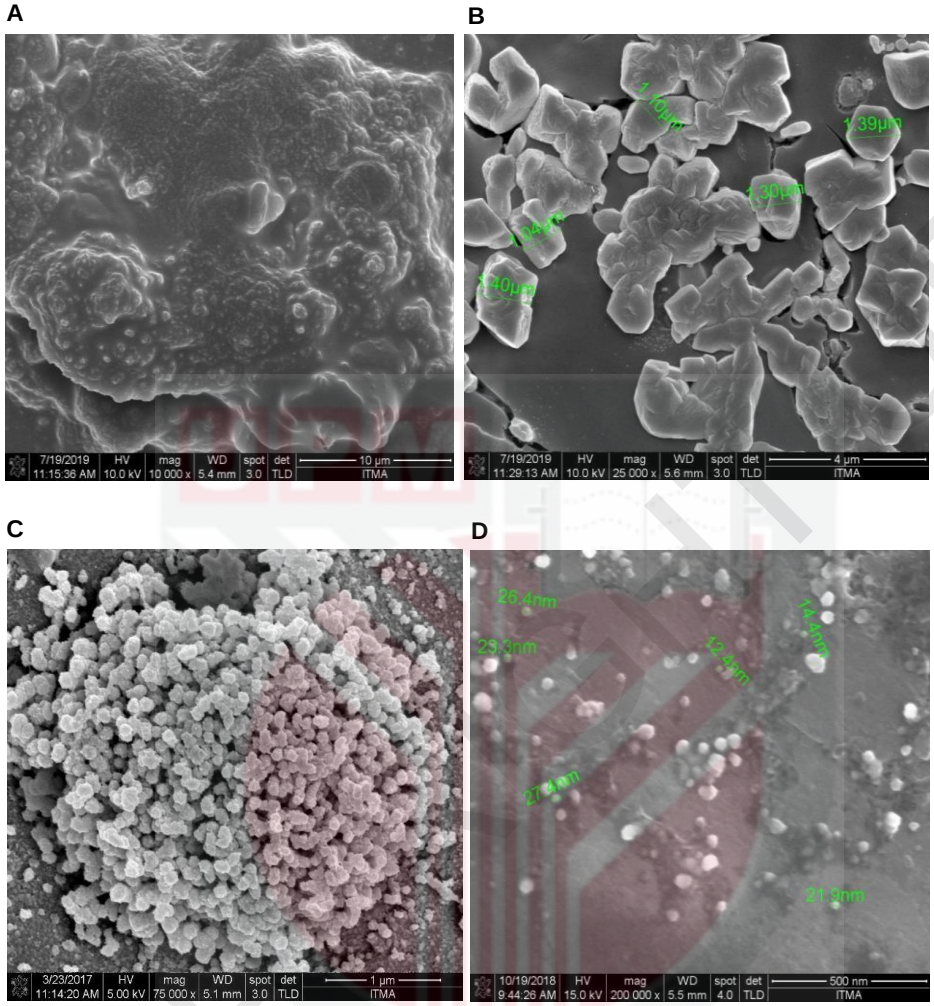
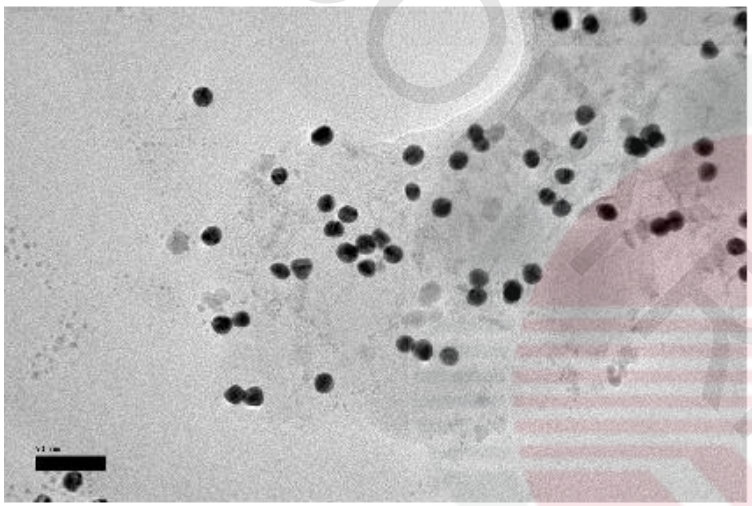


Figure 4.5.: Morphological and particle analysis via FESEM images. (A) demonstrated a clump and irregular shape of cell-free supernatant of silver nanoparticles-producing bacteria (B) is the lattice shape of $AgNO_3$ precursor at 10 mM concentration with the size about 1 µm (C) is the aggregation of bacterial-synthesised silver nanoparticles with uniform spherical shapes (D) are the higher magnification of bacterial-synthesised silver nanoparticles as each nanoparticle has a diameter of less than 25 nm.

A



B

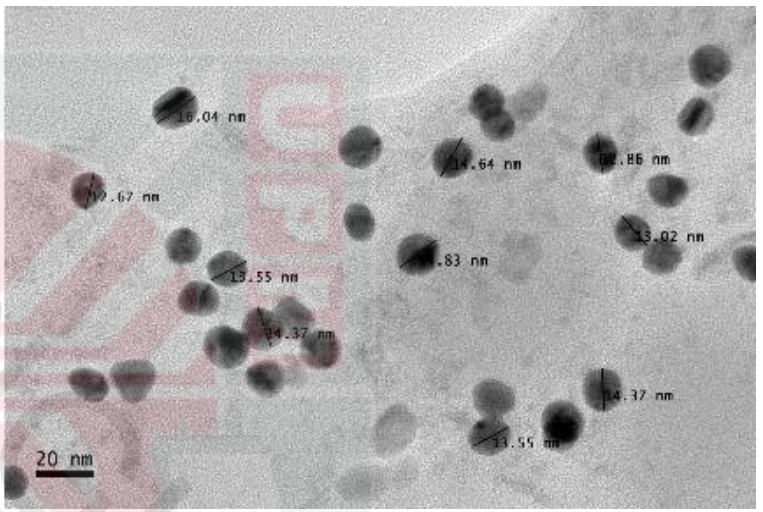


Figure 4.6: Further morphological characterisation of bacterial–synthesised silver nanoparticles using TEM images. (A) showed uniform and monodisperse spherical shape bacterial-synthesised silver nanoparticles surrounded by membrane-like structure produced by silver nanoparticles-producing bacteria and (B) depicting the size of bacterial-synthesised silver nanoparticles with less than 20 nm, comparable with FESEM images.

Ideally, the size of nanoparticles shown in FESEM should be similar with observations from TEM images. However, the average particle size of bacterial-synthesised silver nanoparticles depicted in FESEM images were slightly greater than the sizes measured in TEM images was due to the signals detected in TEM for particle size measurement originate from the core of the metallic nanoparticles and penetrates through the surface organic layer while in FESEM, the organic residues on the surface of the nanoparticles surface cannot be ignored but is taken into account when measuring nanoparticle size at the surface (Eaton et al., 2017). Another interesting observation in TEM micrographs at high magnification was the existence of layers surrounding the bacterial-synthesised silver nanoparticles. The aggregated nanoparticles depicted in the figure do not have direct contact with one another due to the fact that nanoparticles are stabilised by capping proteins secreted from bacteria. It is also postulated that Cytochrome C is the enzyme that may play an important role as capping agent which will affect the stability of silver nanoparticles formed by this process as the formation is quite stable for a period of five months at room ambient temperature (Abdeen et al., 2014).

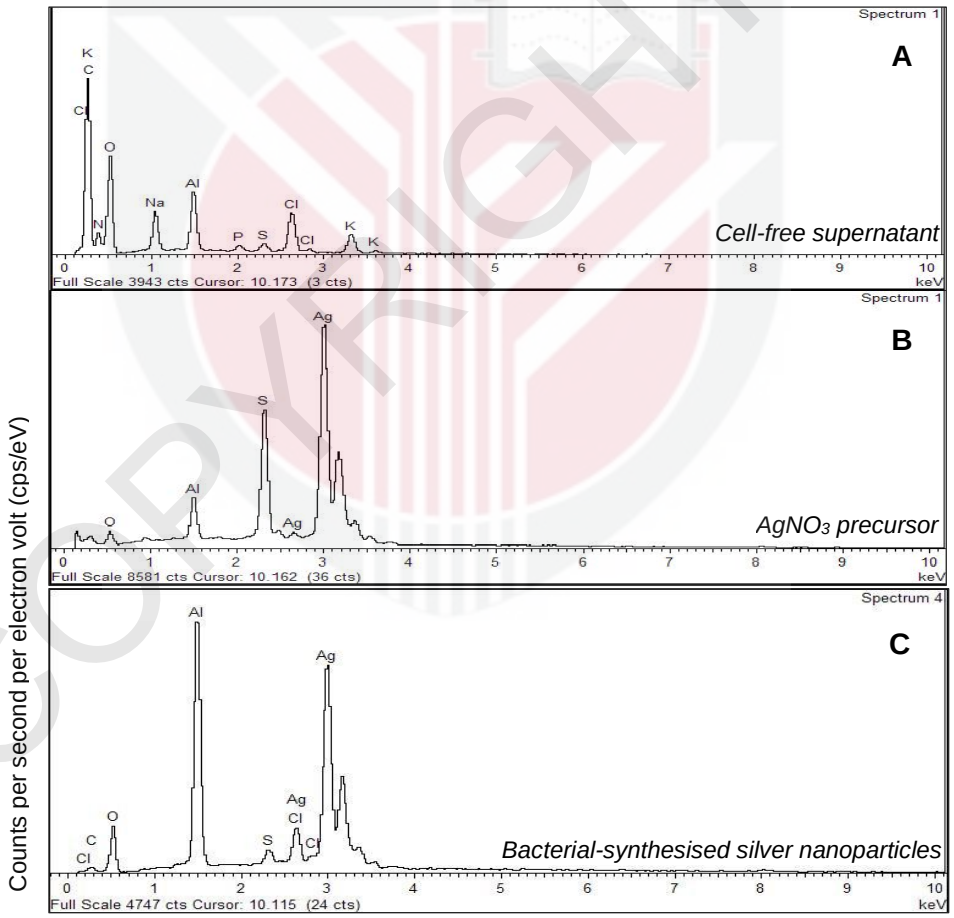
4.2.4 Elemental composition assessment of bacterial-synthesised silver nanoparticles using EDX

The EDX microanalysis is an elemental analysis technique corresponded with electron microscopy based on the generation of characteristic X-rays that reveals the presence of elements present in the specimens (Scimeca et al.,2018). Figure 4.7 shows the EDX spectrum representing the elemental peak comparison of (A) cell-free free supernatant, (B) $AgNO_3$ precursor, and (C) bacterial-synthesised silver nanoparticles. Figure 4.7 (D) tabulates the elemental breakdown of cell-free supernatant, $AgNO_3$ precursor and bacterial-synthesised silver nanoparticles respectively.

The EDX analysis provides both qualitative and quantitative information as the position of a peak in the spectrum and its energy identifies the element as depicted in Figure 4.7 (A), (B) and (C). Meanwhile, the area under the peak is proportional to the number of atoms of the element in the irradiated area. X- rays are formed as the transitions of electrons from higher shells to lower shells by atoms of the specimen interact with electron beams (Scimeca et al., 2018).

Figure 4.7 (D) illustrates the EDX elemental breakdown of bacterial-synthesised silver nanoparticles comparable to cell-free supernatant and $AgNO_3$ precursor as controls in atomic percentages. The composition of cell-free supernatant mainly consists of polysaccharides and proteins as elements like C (45.03%), O (31.29%), N (15.17%), S (0.36%), and P (0.29%) with salt residues such as Na (2.35%), Cl (2.05%) and K (1.11%) present in the analysis. Chemical elements present in $AgNO_3$ precursor solutions primarily involve Ag (42.06%), followed by O (23.81%) and S (26.02%) elements. Multiple peaks such as C (3.99%), O

(36.74%), S (2.57%) and Cl (4.23%) elements were present besides Ag (27.41%) in (iii) postulates the bacterial-synthesised silver nanoparticles were surrounded by organic substances originating from cellular and media components from cell-free supernatant as the same elements but differs in atomic weight percentage were also present in (i). This observation corresponded with the presence of layer covering bacterial-synthesised silver nanoparticles as shown in TEM micrograph (Figure 4.6) as the biofilm layers are the byproducts of bacterial synthesised silver nanoparticles. This discovery comparable with another study using Spirulinamethanolic extract (SME) as the TEM analysis revealed the presence of organic layer surrounding the silver nanoparticles and overall analysis results indicated that the bioactive molecules from SME would play a major role in the synthesis, capping and stabilisation of Ag^{+} (LewisOscar et al., 2021). On the other hand, the presence of component C (3.99%) is from the cell-free supernatant, suggests fabrication of bacterial-synthesised silver nanoparticles. High atomic percentage of O (36.74%) are partially originated from air atmosphere (El-Saadony et al., 2019).



D

Elements	Atomic weight percentage (%)		
	(i) Cell-free supernatant	(ii) $AgNO_3$ precursor	(iii) bacterial-synthesised silver nanoparticles
Carbon (C)	45.03	0.00	3.99
Nitrogen (N)	15.17	0.00	0.00
Oxygen (O)	31.29	23.81	36.74
Sodium (Na)	2.35	0.00	0.00
Aluminum (Al)	2.36	8.11	27.72
Phosphorus (P)	0.29	0.00	0.00
Sulphur (S)	0.36	26.02	2.57
Chlorine (Cl)	2.05	0.00	4.23
Potassium (K)	1.11	0.00	0.00
Silver (Ag)	0.00	42.06	27.41

Figure 4.7: The elemental composition of targeted samples using EDX. The elemental peaks were showed in (A) cell-free supernatant, (B) $AgNO_3$ precursor, and (C) bacterial-synthesised silver nanoparticles while (D) shows quantitative elemental breakdown comparison of bacterial-synthesised silver nanoparticles alongside with cell-free supernatant and $AgNO_3$ precursor as controls. The EDX result showed the Ag element was present together with organic residues derived from cell-free supernatant prior to generation of bacterial-synthesised silver nanoparticles.

Elements such as S (2.57%) and Cl (4.23%) were reported as the presence of organic residues generated by bacteria as supported by another study which shows similar EDX analysis as S and Cl in silver nanoparticles generated using endophytic bacterium *Bacillus siamensis* strain C1, which was isolated from the medicinal plant *Coriandrum sativum* (Ibrahim et al., 2019). Therefore, the purity of bacterial synthesised silver nanoparticles should be improved by removing the biofilm layer surrounding nanoparticles. Al element is present in all samples as the samples prepared on aluminum stubs prior to analysis.

In this analysis, the amount of Ag in atomic percentage produced in bacterial-synthesised silver nanoparticles (iii) were 27.41%. Comparatively, the atomic percentage amount of Ag in another studies varies among microorganisms. For instance, biosynthesis of silver nanoparticles using strains such as *E. coli*, *Bacillus megaterium*, *Acinetobacter sp*. and *Stenotrophomonas maltophilia* resulted less than 10% of atomic percentage in Ag element (Zaki et al., 2011). Meanwhile, another study reported high value in atomic percentage of Ag element from endophytic bacterium *B. siamensis* strain C1, resulted in 91.80% (Ibrahim et al., 2019). In addition, the reduction of % atomic weight Ag in bacterial-synthesised silver nanoparticles from 42.06% in (ii) to 27.41% (iii). Therefore, a conversion rate of Ag elements was calculated with some modifications. The degree of conversion of silver was determined as the difference between initial concentration and the final concentration of silver (Dlugosz and Banach, 2019). Therefore, the conversion rate of silver amount in bacterial-synthesised silver nanoparticles was 34.83%. Referring to the conversion rate value, low yield of bacterial-synthesised silver nanoparticles is influenced by a combination of factors. Concentration of $AgNO_3$ as a precursor should be considered to increase as there are more silver ions for reduction process. However, optimal concentration range need to be determined for this specific bacterial strain as excessive substrate concentration can lead to deformed nanoparticles formation. Other than that, higher concentration of catalyst such as nitrate reductase enzymes can facilitate in reduction process more efficiently to achieve higher nanoparticles yield. This can be achieved by determining the optimal growth rate of silver- nanoparticles producing bacteria to harvest more catalyst (Ibrahim et al., 2021). However, the low rate of conversion might be attributed to bacterial species or strain that are being used to synthesise silver nanoparticles as different bacterial species or strains have varying abilities to produce silver nanoparticles. Some bacteria are naturally better suited for this process due to their specific metabolic pathways or the presence of unique enzymes. Since this silver nanoparticles-producing bacteria was naturally evolved and not-well-adapted to have nanoparticle synthesis ability may result in lower yields.

4.3 Antimicrobial potential of bacterial-synthesised silver nanoparticles

The generation of bacterial-synthesised silver nanoparticles as it occurs at absorbance peak of 420 nm in UV-vis spectrometry; z-average of 139.73±13.63 nm with PDI value of 0.2±0.05 in colloidal solution; round and well-defined nanoparticles with average of less than 25 nm as photographed by electron microscopy; 27.41% atomic weight with 34.83% degree of conversion of silver amount. Therefore, the antimicrobial potential of bacterial-synthesised silver nanoparticles against gram-negative *E. coli* DH5α using disk diffusion assay comparable with conventional antibiotics. The same bacteria were tested in bacterial solution to determine the rate of growth inhibition by bacterial-synthesised silver nanoparticles using bacterial growth inhibition assay. Therefore, the data obtained from these assay works will be an avenue for enhancing the antimicrobial properties of bacterial-synthesised silver nanoparticles in future, widening the range of multiple bacteria and other antimicrobial agents.

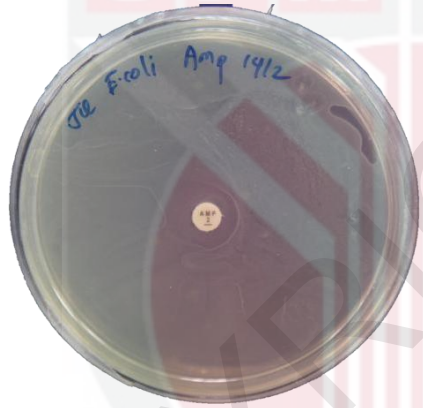
4.3.1 Growth inhibition zones of *E. coli* treated with bacterial-synthesised silver nanoparticles

Figure 4.8 (A) and (B) show the growth inhibition zones results of the disk diffusion assay to perform preliminary evaluation of the antimicrobial potential of bacterial-synthesised silver nanoparticles against gram-negative *E. coli* DH5α strain. Blank disk and distilled water treated disk served as negative controls in this experiment. The growth of the bacteria was also tested with disks treated with sterile distilled water as negative control meanwhile antibiotics such as ampicillin (2 ug/mL) and kanamycin (30 ug/mL) were used as positive controls in this experiment. Lastly, $AgNO_3$ precursor with final concentration of 10 mM was used to compare antimicrobial activity towards *E. coli* DH5α strain with bacterial-synthesised silver nanoparticles solution. Figure 4.8 (C) is the secondary data generated from (B) to relatively compare the rate of killing efficiency of silver between $AgNO_3$ precursor and bacterial-synthesised silver nanoparticles, with normalised concentration. The secondary data from Figure 4.8 (C) was generated deriving from the elemental composition analysis as described in subsection 4.25. The amount of Ag in atomic weight percentage for $AgNO_3$ were not similar with the bacterial-synthesised silver nanoparticles as described in Figure 4.7. In addition, the conversion rate of 34.83% was defined as 100% of Ag in $AgNO_3$ was not totally converted into bacterial-synthesised silver nanoparticles. Therefore, the secondary data from Figure 4.8 (C) was generated to provide a fair comparison of the antimicrobial efficiency between bacterial-synthesised silver nanoparticles and $AgNO_3$.

A

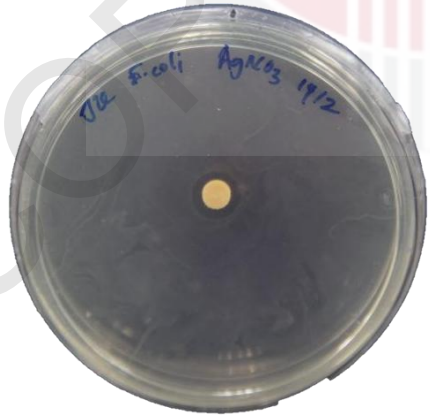
(i) Blank disk

(ii) Distilled water

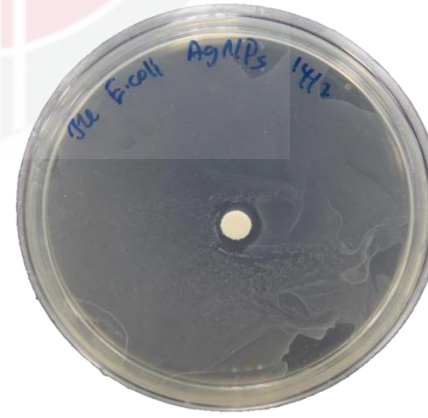


(iii) Ampicillin (2 ug/mL)

(iv) Kanamycin (30 ug/mL)



(v) $AgNO_3$ precursor (10 mM)



(vi) Bacterial-synthesised silver nanoparticles

1 cm

B

Testing agents	Inhibition zone (cm)				
	E. coli DH5α				
	R1	R2	R3	Mean	SD
Blank disk	0.00	0.00	0.00	0.00	0.00
Distilled water	0.00	0.00	0.00	0.00	0.00
Ampicillin (2 ug/mL)	0.00	0.00	0.00	0.00	0.00
Kanamycin (30 ug/mL)	1.80	2.00	2.00	1.93	0.12
$AgNO_3$ precursor (10 mM)	1.00	1.00	1.10	1.03	**** 0.06
AgNPs (500µL)	0.80	0.80	1.00	0.87	**** 0.12

Clinical and Laboratory Standards Institute (CLSI) guidelines

Antibiotic (ug)	Test Cultures (zone diameters in mm)		
	Resistant	Intermediate	Susceptible
Ampicillin (10 ug)	≤13	14-16	≥17
Kanamycin (30 ug)	≤13	14-17	≥18

***Adapted in part from CLSI document M100-S23 (M02-A11): Performance standards for antimicrobial susceptibility testing**

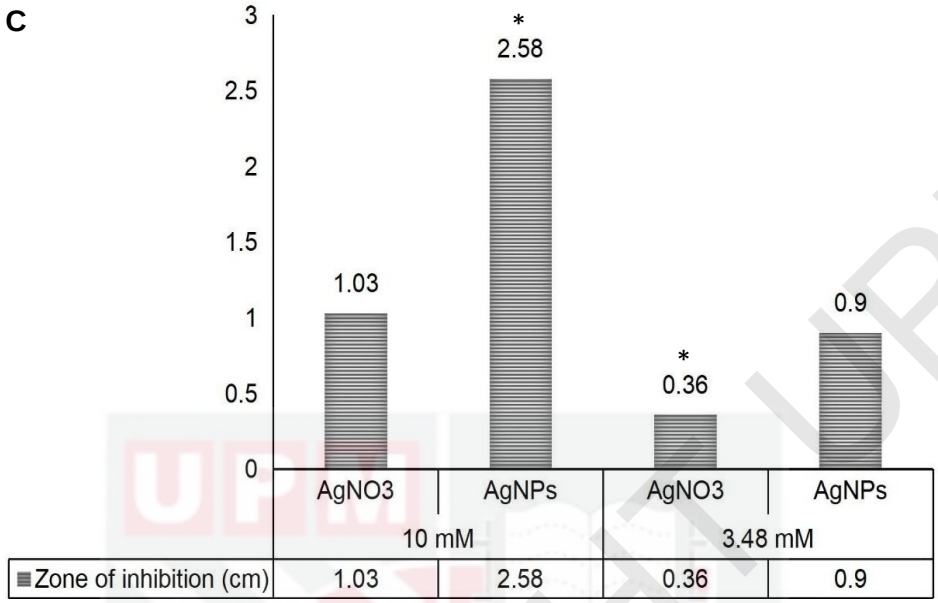


Figure 4.8: Antibacterial evaluation of bacterial–synthesised silver nanoparticles comparable to the other antimicrobial agents. (A) Visual observation and (B) quantitative data of the disk diffusion assay in triplicate against *E. coli* DH5α strain as there was no growth inhibition in *E. coli* DH5α when tested with negative controls such as blank disk and distilled water together with ampicillin (2 ug/mL) as this bacterial strain has been transformed to confer resistance towards ampicillin. Kanamycin (30 ug/mL) possessed the largest inhibition zone 1.93±0.12 cm followed by $AgNO_3$ precursor (10 mM) and bacterial-synthesised silver nanoparticles. Statistical analysis was conducted to compare the effects of treatment with silver nanoparticles to those of distilled water (Negative control). "*" indicates significance with $p < 0.05$; "": $p < 0.01$; "***": $p < 0.001$; $p < 0.0001$ and ns: not significant. (C) is the comparison of killing efficiency rate between $AgNO_3$ ($AgNO_3$ precursor) and AgNPs (bacterial-synthesised silver nanoparticles), given the normalised concentration. It is postulated that the killing efficiency of silver contained in bacterial-synthesised silver nanoparticles is higher than in $AgNO_3$ precursor because of the distinct nano dimensions of bacterial-synthesised silver nanoparticles which contributes to higher efficiency rate of killing.**

Based on Figure 4.8 (A) and (B), there were no inhibition ring exhibited on *E. coli* DH5α strain culture when treated with blank disk and distilled water. Similarly, there was no growth inhibition in the *E. coli* DH5α bacteria treated with 2 ug/mL ampicillin, indicates resistance to ampicillin based on CLSI guidelines. The ampicillin antibiotic was served as control and the result was expected as the bacteria strain used was resistant to ampicillin. This resistance ability was facilitated through the presence of an endogenous plasmid that carried an ampicillin resistance gene, encodes for beta-lactamase enzyme. The beta-lactamase is capable of degrading ampicillin, causing the ampicillin ineffective against the bacteria (Joji et al., 2021, Saeed, 2014). This example of resistance development of *E. coli* DH5α to ampicillin will raise public health concern as if someone becomes infected with ampicillin-resistant bacteria, the commonly used ampicillin antibiotic will be no longer useful. The range of available treatment options becomes narrower, harder to control widespread infections and hospital treatment will be costly.

However, the bacteria were found to be susceptible towards the other class of antibiotic used such as kanamycin at the concentration of 30 ug/mL as the kanamycin has the largest inhibition zone of 1.93±0.12 cm. Even though Kanamycin, the aminoglycoside antibiotic has the highest antimicrobial activity, there are a few *E. coli* strains that are proven to confer resistance towards Kanamycin. For instance, the same *E. coli* DH5α bacteria can resist towards kanamycin as it is engineered to carry plasmids with kanamycin resistance genes from *P. aeruginosa* isolates (Hosseini et al, 2008). Other than that, *E. coli* BL21 (DE3) has the plasmids carrying both ampicillin and kanamycin resistance genes for recombinant protein production (Yang et al., 2001). There are also pathogenic strains of *E. coli* that can naturally exhibit resistance to kanamycin and other classes of antibiotics over time and these strains can possess significant public health concerns as limited treatment options in clinical settings. For example, some strains of Shiga toxin-producing *E. coli* exhibit resistance to kanamycin and have been associated with outbreaks of foodborne illness (Galarce et al., 2020). Extraintestinal pathogenic *E. coli* strains have been reported to be resistant to kanamycin and cause urinary tract infections, bloodstream infections, and other infections related to gastrointestinal tract (Ukah et al., 2018).

Therefore, it is crucial to find an alternative solution to overcome antimicrobial resistance. Silver-based antimicrobial agents have been used in various applications, such as wound dressings, medical devices, water purification, and topical creams due to their effectiveness against the bacteria. The presence of silver can cause disruption of cell membrane, interference with DNA replication, enzymes inhibition, and generation of reactive oxygen species (ROS) of the bacteria, which are distinct pathways of killing than antibiotics. This test is to compare the comparative mechanistic antimicrobial action of bacterial-synthesised silver nanoparticles and Ag^+ from $AgNO_3$ precursor. The results shown in Figure 4.8 (B) as the inhibition ring is present in $AgNO_3$ precursor with 1.03±0.06 cm and bacterial-synthesised silver nanoparticles with 0.87±0.12 cm respectively. From these results, it seems the Ag^+ ions from $AgNO_3$ precursor have higher antimicrobial activity than nano dimension of silver in bacterial-

synthesised silver nanoparticles. However, referring to Figure 4.8 (C), the antimicrobial activity efficiency of bacterial- synthesised silver nanoparticles are superior than $AgNO_3$ precursor, given normalised concentration in mM. The initial concentration (mM) was derived from the conversion rate as described in subsection of 4.2.5 and the signs of asterisks (*) are the calculated data. In 10 mM concentration of $AgNO_3$ precursor and bacterial-synthesised silver nanoparticles, the bacterial- synthesised silver nanoparticles confer a higher zone of inhibition of 2.58 cm (calculated data) than $AgNO_3$ precursor at 1.03 cm zone of inhibition. The same trend was observed in 3.48 mM concentration, as the larger inhibition zone of 0.9 cm in bacterial-synthesised silver nanoparticles than $AgNO_3$ precursor, which only 0.36 cm (calculated data).

Subsequently, the rate of killing efficiency of bacterial-synthesised silver nanoparticles was 150.5% (refer calculation at subsection 3.2.3.1) which also means 1.5 times higher than non-nano silver in $AgNO_3$ precursor. This is due to the extremely small size in nano-dimension of silver, with larger surface area per volume ratio, so more atoms can interact with bacteria and subsequently enhance the superior antimicrobial properties of silver comparative to different mechanism of action as the presence of Ag^+ ions induced by $AgNO_3$. A comparison study of mechanism of actions between chemically-synthesised silver nanoparticles (8 and 29 nm) and $AgNO_3$ with the same concentrations of 10µg/mL was also conducted. However, there are no inhibition zones observed in different *E. coli* (Trans 1-T1) strain when treated with 8 and 29 nm of chemically-synthesised silver nanoparticles while 10 mm of inhibition zone was recorded when the bacteria treated with $AgNO_3$ (Mosselhy et al., 2015). This project can be advantageous and high potential as the bacterial-synthesised silver nanoparticles still has the antimicrobial effect towards gram-negative *E. coli*, but need to establish the test by normalising and widening the concentration range, together with antimicrobial assessment with wide-range of bacteria.

Afterward, susceptibility profile of *E. coli* DH5α was assessed using CLSI M100 guideline, which allowed determining whether the organisms were susceptible, intermediate or resistant to each antimicrobial agent tested, according to the different susceptibility interpretative criteria.

4.3.2 Bacterial growth inhibition assay

Further assessment of any antimicrobial effects of bacterial-synthesised silver nanoparticles towards the growth of the same *E. coli* DH5α strain in a suspension was also conducted. This evaluation was performed by treating 1% and 10% (v/v) concentrations of bacterial-synthesised silver nanoparticles to the bacterial culture during the lag phase (0-30 minutes) and log phase (30 minutes) respectively. In the lag phase, the bacterial-synthesised silver nanoparticles were added to the bacterial culture when it was just inoculated to determine the capability of bacterial-synthesised silver nanoparticles in inhibiting the bacterial cells doubling time can be determined. In the log phase, the capability of bacterial-synthesised silver nanoparticles in slowing down or inhibiting the fast-bacterial growth rate can be studied by adding the bacterial- synthesised silver nanoparticles during the rapid growth period of *E. coli* DH5α strain. In both evaluations, three bacterial cultures of *E. coli* DH5α strain in suspension were cultured in different conditions: a bacterial culture without the treatment (control), a bacterial culture with treatment of 1% (v/v) concentration of bacterial-synthesised silver nanoparticles, and a bacterial culture treated with 10% (v/v) concentration of bacterial-synthesised silver nanoparticles. The results were shown in Figure 4.9 (A).

Referring Figure 4.9 (A), the bacterial culture that was treated with 1% (v/v) of bacterial-synthesised silver nanoparticles in both lag and log phases were found to induce the bacterial growth as the OD reading were higher than non- treated bacterial culture (control). This might due to the negligible 1% (v/v) concentration which has no effect on the bacterial growth. However, when the concentration of bacterial-synthesised silver nanoparticles was increased to 10% (v/v), bacterial growth was suppressed by more than 50% compared to the normal bacterial growth curve. This indicates that 10% (v/v) concentration is enough to inhibit the growth of bacteria in suspension as OD increased is significantly less than 1% treatment in both lag and log phases as shown in Figure 4.9 (B).

This observation correlates with a study to evaluate the microbial growth of *E. coli* and *B. subtilis* using chemically-synthesised citrate-capped silver nanoparticles at different concentrations of 10, 30, and 50 mg/mL respectively. In *E. coli*, the results showed that higher concentrations reduced the growth of the bacteria, comparable to non-treated bacterial culture. However, the effect of chemically-synthesised citrate-capped silver nanoparticles was lesser in *B. subtilis* due to thick peptidoglycan layer (Kim et al., 2011). From this study, the low concentration of 1% (v/v) did not affect the growth of the bacteria. This might due to the low amount of atomic weight percentage and conversion rate of silver in bacterial-synthesised silver nanoparticles comparable to silver in $AgNO_3$ as discussed in subsection 4.2.5. Comparatively, the growth of *Cupriavidus necator* (*C. necator*) H16 was evaluated when treated with spherical silver nanoparticles.

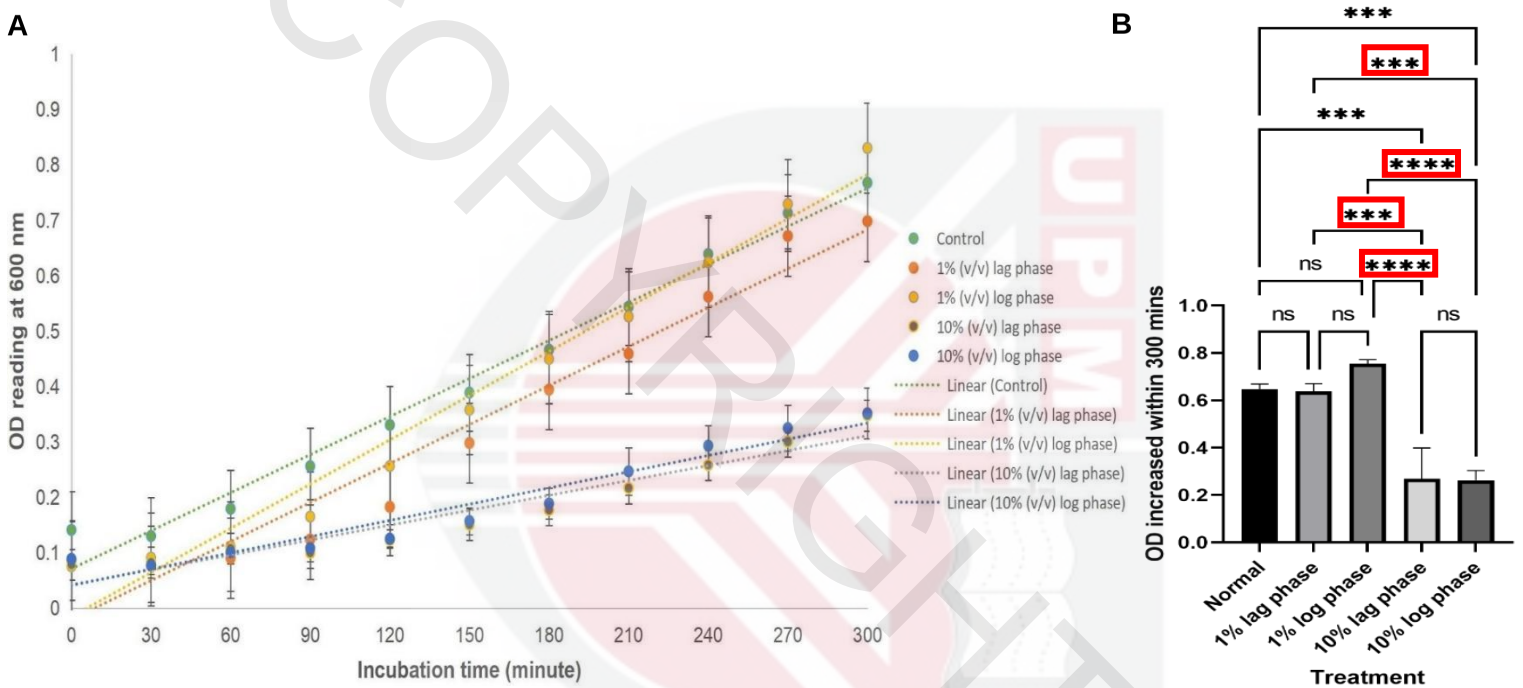


Figure 4.9: Growth inhibition of bacterial-synthesised silver nanoparticles towards *E. coli* DH5α strain suspension culture (A) The evaluation of growth curve of *E. coli* DH5α strain suspension culture incubated with different treatments added at lag and log phases until 300 minutes incubation time. (B) Histogram graph of OD increased within 300 minutes after treatment. The 10% (v/v) concentration of bacterial-synthesised silver nanoparticles has a significant antimicrobial effect in suspension culture (red box), comparable to non-treated and 1% (v/v) treatment of bacterial cultures, respectively. The symbol of "*" indicates significance with $p < 0.05$; "": $p < 0.01$; "***": $p < 0.001$; $p < 0.0001$ and ns: not significant**

Low amount of 20 µg/mL of silver nanoparticles did not have any effect on the growth of *C. necator*. However, when the concentration of silver nanoparticles was increased, the growth in *C. necator* cultures was significantly inhibited as low as in the presence of 60 µg/mL (Schacht et al., 2013). Similar observation was reported by Bao et al. (2015), where high concentration of bacterial-synthesised silver nanoparticles significantly decreased the growth rate of *E. coli* as compared to low concentration of bacterial-synthesised silver nanoparticles, which correlated with the result found in this assay.

Possible inhibition mechanism of bacterial-synthesised silver nanoparticles in suspension culture is due to the aggregation surrounding bacteria cells followed by nanoparticles uptake and dissolution of Ag^+ to disrupt the cell membrane comparing to the effect on direct Ag^+ in silver alone. This was proven by the electron microscopy micrograph picturing the accumulation of silver nanoparticles covering the membrane of the bacteria (Kim et al., 2011). Predictably, higher content of Ag in atomic weight percentage in bacterial-synthesised silver nanoparticles to inhibit the growth of targeted bacteria cells. However, it is important to determine the optimal concentration used, especially in biomedical applications as toxicity results should be conducted carefully in nanotoxicological studies to be suitable alternative to other therapeutics.

The antimicrobial effect of bacterial-synthesised silver nanoparticles was also dependent on the microbial tests employed. Comparing the performance of bacterial-synthesised silver nanoparticles in both assays, bacterial-synthesised silver nanoparticles had more antimicrobial efficiency. This is because the physicochemical properties of silver nanoparticles have been changed in the presence of electrolytes contained in LB media, comparing with distilled water. The hydrodynamic diameter of silver nanoparticles in LB medium was recorded in DLS to be two to three times greater than the hydrodynamic diameter of silver nanoparticles in water (Kim et al., 2011). Previous study also reported that the presence of salts such as potassium chloride and sodium chloride in growth media induced the aggregation of nanoparticles immediately (Meyer et al., 2010). Generally, the antimicrobial effect of bacterial-synthesised silver nanoparticles using the bacterial growth inhibition assay was a meaningful indicator to be employed in suspension environment such as a potential candidate of antimicrobial agent in wastewater treatment.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Conclusions

This thesis describes the isolation of a locally produced silver nanoparticles-producing bacteria from the abundant tropical mangrove ecosystem in Malaysia showed aerobic extracellular synthesis of silver nanoparticles in environmental conditions. The bacteria have undergone evolutionary changes to thrive in highly toxic settings saturated with metallic compounds, wherein they efficiently absorb these metals and generate less harmful metallic nanoparticles. In this study, the silver nanoparticles-producing bacteria has proven to synthesise silver nanoparticles by inducing $AgNO_3$ as a precursor in the cell-free supernatant with the final concentration of 10 mM. The reaction color was changed from yellow to dark brown was an early indicator of production of bacterial-synthesised silver nanoparticles. Subsequently, the colony morphological and bacterial growth characterization analyses of the silver nanoparticles-producing bacteria have been discussed while the growth and doubling time of the silver nanoparticles-producing bacteria have been determined as the calculated doubling time was 53 minutes. Therefore, phylogenetic identification was performed using 16S rRNA sequencing as the silver nanoparticles-producing bacteria are highly homologous to *Bacillus* strains, specifically *B. cereus* strain KTSMBNL, *B. thuringiensis* strain BTJ-S-1, *B. cereus* strain UITSAI, and *B. subtilis* strain Mans5.

The bacterial-synthesised silver nanoparticles that have been formed were first detected by peak absorbance at 420 nm via UV-vis spectrometry and the OD reading was decreased over time. The average nanoparticle size in a solution was 139.73 nm with a low polydispersity value of 0.20 using DLS analysis depicted this particular silver nanoparticles-producing bacteria can synthesise narrow size distribution of silver nanoparticles which are favorable for efficient industrial production. Morphological and particle characterisations of bacterial-synthesised silver nanoparticles have been validated using SEM and TEM analyses which both displayed every single nanoparticle produced was less than 25 nm with minor aggregation. It was also observed that an extracellular biofilm layer surrounded the bacterial-synthesised silver nanoparticles in the TEM micrograph postulating the capping agent to maintain the integrity and stability of bacterial-synthesised silver nanoparticles. On top of that, the elemental composition of the bacterial-synthesised silver nanoparticles has been carried out as the amount of atomic weight percentage of silver was 27.41% with the conversion rate of 34.83% comparative with silver amount in $AgNO_3$ precursor. In addition, the presence of organic substances alongside with bacterial-synthesised silver nanoparticles such as polysaccharides, proteins and salt

residues were originating from cell-free supernatant of silver nanoparticles-producing bacteria which was also corresponded with the presence of biofilm surrounding the bacterial-synthesised silver nanoparticles observed from TEM morphological analysis.

The antimicrobial potential of bacterial-synthesised silver nanoparticles to be developed as an alternative for antimicrobial agent against gram-negative *E. coli* DH5α strain was evaluated using disk diffusion. The disk diffusion assay results shown there is no antimicrobial activity when the *E. coli* DH5α bacteria treated with ampicillin as this particular bacterial strain has developed a resistance to ampicillin due to the presence of a plasmid that carries an ampicillin resistance gene, encodes for beta-lactamase enzyme to degrade ampicillin. However, the tested bacteria were shown to be susceptible to kanamycin as the kanamycin has the largest inhibition zone of 1.93+0.12 cm, followed by $AgNO_3$ precursor with 1.03+0.06 cm and bacterial-synthesised silver nanoparticles with 0.87+0.12 cm respectively. Therefore, bacterial-synthesised silver nanoparticles have been a potential candidate to overcome or complement the role of antibiotics as it was compared to have 1.5 times higher killing efficiency than non-nano dimension of silver presence in $AgNO_3$ with excellent efficiency rate of 150.5%. The bacterial-synthesised silver nanoparticles also inhibited the growth of the *E. coli* DH5α bacterial suspension culture in lag and log phases as the growth reduced by 50% via bacterial growth inhibition assay with the concentration with 10% (v/v) concentration. These antimicrobial evaluation results postulate the bacterial-synthesised silver nanoparticles are viable to be an alternative and work synergistically with antibiotics to restrain antimicrobial resistance.

5.2 Recommendations for future research

Despite of knowing the genus of the bacteria, the identity of the silver nanoparticles-producing bacteria can be further extended by conducting whole sequencing. The obtained genomic data will be compared with reference databases by analysing the entire genome to determine the species of the organism being sequenced. The whole genome sequencing is practically a useful tool for taxonomy and understand the genetic diversity within and between species.

The discovery of generating monodispersed silver nanoparticles with low cost and environmentally friendly method using a reliable bacterial system and minimal usage of media and reagents is a good platform to be industrialised to scale-up the production of bacterial-synthesised silver nanoparticles. However, the bacterial-synthesised silver nanoparticles generation needs to be optimised with the goal of high yield synthesis. Other than that, if there is any leftover or unused $AgNO_3$ precursor in the supernatant after producing silver nanoparticles from bacteria, it may raise concerns about potential toxicity as the residual $AgNO_3$ precursor in the supernatant could lead to the presence of free Ag^+, which may exhibit antimicrobial properties but can also be toxic to cells, including human cells. Therefore, further study of the parameters influencing the optimal

production of bacterial-synthesised silver nanoparticles with maximum conversion rate is essential. Statistical optimisation using Response surface methodology (RSM) is suggested to carry out this study to determine the optimal parameters to synthesise the most desired nanoparticles which can be the formulation to be employed for industrial runs. Additionally, thorough purification steps should be employed to remove any unconverted silver nitrate or by-products to ensure the safety of the synthesised silver nanoparticles for intended applications, such as antimicrobial agents or other biomedical uses.

Nanoparticle characterisation should be further extended to study the chemical composition, surface functional groups, and structural properties using Fourier-transform Infrared spectroscopy (FTIR) to obtain a comprehensive understanding of bacterial-synthesised silver nanoparticles. FTIR data helps in analysing surface functionalisation, monitoring chemical reactions, and identifying the presence of specific functional groups on the nanoparticle surface. X-ray diffraction (XRD) method could be added in extended nanoparticle characterisation as it provides detailed information about the crystal structure and phase composition of nanomaterial. This information is important to elucidate the properties of bacterial-synthesised silver nanoparticles in various applications.

The wide range study of antimicrobial potential of bacterial-synthesised silver nanoparticles should be carried out to determine the killing efficacy of bacterial-synthesised silver nanoparticles towards targeted pathogens. This includes testing with different types of strains such as gram-positive bacteria, and also the bacteria associated with hospital-acquired infections such as *S. aureus*, *Klebsiella* spp., *Enterobacter* spp., and *Streptococcus* spp.. Substantial study associated with minimal inhibitory concentration (MIC) of bacterial-synthesised silver nanoparticles also need to be carried out to accurately determine the effectiveness of antimicrobial agents of bacterial- synthesised silver nanoparticles. Other than that, toxicity test is suggested to assess the biocompatibility of bacterial-synthesised silver nanoparticles as compared to non-biologically synthesised silver nanoparticles to be applied for biomedical applications. Once the establishment of optimal generation of bacterial-synthesised silver nanoparticles with high efficacy has been finalised, this bacterial system will be a great avenue for industrial efficient production of this alternative antimicrobial agent.

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APPENDICES

Appendix 1: Bacterial growth curve and doubling time determination of silver-nanoparticles-producing bacteria

Time	Absorbance			
Hour	**B1**	**B2**	**Absorbance**	**Standard Deviation**
0	0.018	0.052	0.035	0.024
1	0.038	0.124	0.081	0.06
2	0.029	0.222	0.126	0.136
3	0.021	0.548	0.285	0.373
4	0.019	0.850	0.435	0.588
5	0.023	1.026	0.525	0.709
6	0.052	1.100	0.576	0.741
7	0.124	1.158	0.641	0.731
8	0.222	1.264	0.743	0.737
9	0.548	1.347	0.948	0.565
10	0.850	1.410	1.130	0.396
11	1.026	1.461	1.2435	0.308
12	1.100	1.505	1.3025	0.286
13	1.158	1.540	1.349	0.270
14	1.264	1.590	1.427	0.230
15	1.347	1.613	1.48	0.188
16	1.410	1.631	1.5205	0.156
17	1.461	1.657	1.559	0.139
18	1.505	1.630	1.5675	0.088

Doubling time formulation:
Chosen time frame is in gray box:

$$\frac{A\ (after)\ /\ A\ (before)}{T\ (after) - T\ (before)}$$

Therefore, in 60 minutes, the growth rate is 2.266932271.
Doubling time of silver-nanoparticles-producing bacteria is 53 min

Appendix 2: Level of SPR absorbance peak at 420 nm against incubation time

Time (Hour)	0	24	48	72
B1	2.054	1.700	1.829	1.917
B2	1.896	1.650	1.924	1.629
B3	1.457	1.322	1.481	1.443
Mean	1.802	1.557	1.745	1.663

Appendix 3: The raw data obtained to measure the size and polydispersity analysis of bacterial-synthesised silver nanoparticles

y Intensity

Malvern

Sample Details

Sample Name:	AgNPs 10mM Precipitate Light 2 3		
SOP Name:	JIE SOP		
General Notes:			
File Name:	Jeff.dts	**Dispersant Name:**	Water
Record Number:	5819	**Dispersant RI:**	1.330
Material RI:	0.54	**Viscosity (mPa.s):**	1.0032
Material Absorbtion:	0.00	**Measurement Date and Time:**	Tuesday, December 12, 2017 ...

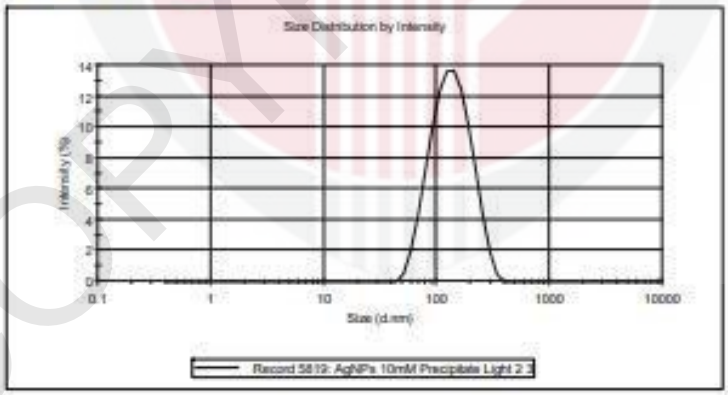
System

Temperature (°C):	20.0	**Duration Used (s):**	70
Count Rate (kcps):	190.1	**Measurement Position (mm):**	0.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	3

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	119.2	**Peak 1:**	142.0	100.0	55.74
PdI:	0.151	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.932	**Peak 3:**	0.000	0.0	0.000

Result quality : Good



Appendix 4: Bacterial growth inhibition assays at average of OD reading 600 nm at lag and log phases

Mean

Time (minute)	0	30	60	90	120	150	180	210	240	270	300
Control	0.151	0.142	0.191	0.275	0.360	0.423	0.508	0.581	0.682	0.747	0.798
1% (v/v) lag	0.077	0.068	0.087	0.126	0.182	0.299	0.393	0.465	0.569	0.684	0.715
1% (v/v) log	0.076	0.091	0.112	0.165	0.257	0.358	0.450	0.526	0.624	0.729	0.83
10% (v/v) lag	0.079	0.082	0.108	0.100	0.123	0.151	0.177	0.217	0.258	0.301	0.348
10% (v/v) log	0.089	0.077	0.101	0.108	0.125	0.157	0.189	0.247	0.293	0.325	0.352

Standard deviation

Time (minute)	0	30	60	90	120	150	180	210	240	270	300
Control	0.006	0.008	0.011	0.033	0.067	0.107	0.144	0.163	0.196	0.233	0.249
1% (v/v) lag	0.011	0.011	0.005	0.007	0.005	0.013	0.003	0.007	0.014	0.009	0.010
1% (v/v) log	0.003	0.005	0.012	0.015	0.021	0.013	0.022	0.019	0.027	0.020	0.020
10% (v/v) lag	0.011	0.010	0.003	0.019	0.029	0.053	0.078	0.097	0.106	0.115	0.128
10% (v/v) log	0.005	0.006	0.006	0.015	0.016	0.024	0.028	0.043	0.036	0.041	0.046

BIODATA OF STUDENT

Farah Najwa Nabila Binti Mohd Hatta was born in Mentakab, Pahang and later raised in Kluang, Johor. Her educational journey includes a current Master's degree in Nanobiotechnology (Research Mode) from Universiti Putra Malaysia (UPM) where she strides in developing her specialisation in nanotechnology, all while playing a role in mentoring undergraduate students and assisting in research projects. Her earlier academic accomplishments include a Bachelor's degree in Cell and Molecular Biology and a Foundation in Agricultural Sciences, both from UPM.

In the professional realm, her experience extends to the Malaysia Automotive, Robotics and IoT Institute (MARii), where she served as a Biomedical Management Trainee. Here, she spearheaded a vital project involving COVID-19 antibody diagnostic tests, leveraging Smart Automation Line (SAL) Technology. Previously she commited to education when she worked as a Part-time Lecturer cum Facilitator at UPM's Co-curriculum and Student Development Centre from September 2019 to February 2020. As a Research Assistant under UPM's Faculty of Biotechnology and Biomolecular Sciences from October 2017 to January 2018, she was ventured into nanotechnology research. In autumn 2023, she was appointed as one of the visiting researchers as part of the SAKURA Science Exchange Program at Kyushu Institute of Technology, Japan.

In summary, her academic journey and professional experiences have equipped with a strong foundation in biotechnology, nanotechnology, research, and project management. She is eager to continue making meaningful contributions to the fields of biotechnology and healthcare, driven by a passion for innovation and a commitment to environmental sustainability.