BIOSYNTHESIS AND CHARACTERISATION OF SILVER NANOPARTICLES USING *Streptomyces sp*. PBD311B WITH *IN VITRO* AND *IN SILICO* BIOLOGICAL ACTIVITY TOWARDS *Pseudomonas aeruginosa*

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by

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LIST OF ABBREVIATIONS

g	Gram
mg	milligram
µg	microgram
°C	degree Celsius
µL	Micrometer
mL	Milliliter
L	Liter
µm	Micrometre
nm	nanometre
mm	millimeter
cm	centimetre
PH	Potential of Hydrogen
w/v	Weight per volume
v/v	Volume per volume
rpm	Revolution per minute
OD	Optical density
dH2O	Distilled water
h	hour
Abs	absorbance
mA	Milliamp
kV	kilovolt

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BIOSINTESIS DAN PENCIRIAN NANOPARTIKEL PERAK MENGGUNAKAN *Streptomyces* sp. PBD311B SERTA PENILAIAN AKTIVITI BIOLOGI *IN VITRO* DAN *IN SILICO* TERHADAP *Pseudomonas aeruginosa*

ABSTRAK

Pseudomonas aeruginosa ialah patogen rintang pelbagai ubat yang menggunakan sistem penderiaan kuorum (QS) untuk mengawal atur virulens dan pembentukan biofilm, menjadikannya sukar untuk dirawat dengan antibiotik konvensional. Oleh itu, pembangunan strategi alternatif antibakteria dan anti-QS adalah penting untuk memerangi jangkitan yang disebabkan olehnya. Kajian ini bertujuan untuk mensintesis nanopartikel perak (bioAgNPs) secara biosintetik menggunakan *Streptomyces* sp. PBD-311B serta menilai sifat antibakteria dan sifat anti-QS terhadap *P. aeruginosa* sp. BioAgNPs telah disintesis menggunakan *Streptomyces* sp. PBD-311B serta dicirikan melalui spektroskopi ultralembayung–nampak (UV-Vis), mikroskop elektron transmisi (TEM), dan mikroskop elektron imbasan (SEM). Aktiviti antibakteria ditentukan melalui kaedah resapan cakera dan analisis TEM, manakala kesan anti-QS dinilai melalui ujian pergerakan renang dan penghasilan biofilm. Interaksi protein korona bioAgNPs dengan protein membrane luar bakteria dikaji melalui elektroforesis gel poliakrilamida–dodesil sulfat natrium (SDS-PAGE), kromatografi cecair–spektrometri jisim (LC-MS), dan kajian dok molekul. Pembentukan bioAgNPs berjaya ditunjukkan melalui perubahan warna daripada kuning cerah kepada coklat gelap, dengan puncak LSPR antara 413–426 nm, menunjukkan sifat polidispersiti. Analisis TEM menunjukkan bioAgNPs berbentuk sfera dengan saiz antara 2 hingga 15 nm. Kepekatan perencatan minimum (MIC) bioAgNPs terhadap *P. aeruginosa* sp. ialah 2.4 mg/mL, dengan perencatan bakteria yang signifikan pada peringkat awal (30 minit) dan lewat (24 jam). Tahap sub-MIC bioAgNPs menunjukkan pengurangan ketara dalam pergerakan renang dan pembentukan biofilm. Pembentukan protein korona disahkan pada ~63 kDa, dengan tiga protein hipotesis dikenal pasti: esterase (PDB ID: 1ESC), PikA1 (PDB ID: 8CZC), dan PikA2 (PDB ID: 3PQ9). Kajian dok

molekul menunjukkan afiniti ikatan yang kuat antara protein korona bioAgNPs dan protein membran luar *P. aeruginosa*, OprF (PDB ID: 1WP1) dan OprD (PDB ID: 3D5K). Kajian ini membuktikan bahawa bioAgNPs menunjukkan aktiviti antibakteria dan aktiviti anti-QS yang berkesan terhadap *P. aeruginosa* sp. Interaksi terpilih antara protein korona bioAgNPs dan protein membran luar bakteria menyerlahkan potensinya dalam mengganggu laluan QS, menawarkan pendekatan alternatif yang berpotensi untuk menangani jangkitan bakteria rintang antibiotik.

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ABSTRACT

Pseudomonas aeruginosa is a multidrug-resistant pathogen that utilizes quorum sensing (QS) to regulate virulence and biofilm formation, making it a challenging target for conventional antibiotics. The development of alternative antibacterial and anti-QS strategies is crucial to combat its infections. This study aims to biosynthesize silver nanoparticles (bioAgNPs) using *Streptomyces* sp. PBD-311B and evaluate their antibacterial and anti-quorum sensing properties against *P. aeruginosa* sp. BioAgNPs were synthesized using *Streptomyces* sp. PBD-311B and characterized through ultraviolet–visible spectroscopy (UV-Vis)**,** transmission electron microscopy (TEM)**,** and scanning electron microscopy (SEM). Antibacterial activity was determined using disc diffusion and TEM analysis, while the anti-QS effects were evaluated using swimming motility and biofilm production. The interaction of bioAgNPs protein corona with bacterial outer membrane protein was investigated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)**,** liquid chromatography–mass spectrometry (LC-MS)**,** and molecular docking studies. Successful bioAgNPs formation was indicated by a color change from light yellow to dark brown, with localized surface plasmon resonance (LSPR) peaks between 413–426 nm, suggesting polydispersity. TEM analysis showed that the bioAgNPs were predominantly spherical with sizes ranging from 2 to 15 nm. The minimum inhibitory concentration (MIC) of bioAgNPs against *P. aeruginosa* sp. was determined as 2.4 mg/mL, showing significant bacterial inhibition at both early (30 min) and late (24 h) stages. Sub-MIC levels of bioAgNPs significantly reduced swimming motility and biofilm formation. Protein corona formation was confirmed at ~63 kDa, with three identified hypothetical proteins: esterase (PDB ID: 1ESC),

PikA1 (PDB ID: 8CZC), and PikA2 (PDB ID: 3PQ9). Molecular docking revealed strong binding affinity between bioAgNPs proteins corona and *P. aeruginosa* outer membrane proteins, OprF (PDB ID: 1WP1) and OprD (PDB ID: 3D5K). This study demonstrates that bioAgNPs exhibit potent antibacterial and anti-QS activities against *P. aeruginosa* sp. The selective interaction between protein corona bioAgNPs and bacterial outer membrane proteins highlights their potential in disrupting QS pathways, offering a promising alternative approach for combating antibiotic-resistant bacterial infections.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium that is distributed around humans and causing a lot of acute and chronic infections (Li et al., 2022). According to Shariati's group, it is a major pathogen for hospital infection, especially burns and wounds (Shariati et al., 2018). According to a different study by Tzaneva et al. (2016), it resulted in 11.8% of chronic wounds. Furthermore, a high degree of antibiotic resistance can arise from the overuse or abuse of antibiotics, leading to endocarditis, meningitis, pneumonia, nosocomial infections, and fulminant infections of the bones and joints in patients with cystic fibrosis (Singh et al., 2015). Overall, *P.aeruginosa* is responsible for biofilm-associated infections that can reduce the sensitivity towards antibiotics and increased resistance to the attack of host immune mechanisms (Sen et al., 2021; Suwal et al., 2021). Various virulence factors and biofilm formation have been proven to be regulated by *P. aeruginosa* quorum sensing system (Bjarnsholt *et al*., 2010).

Quorum sensing (QS) is known as a shared molecular communication mechanism that probe stimulates for the production of biofilms, which are impacted by both biotic and abiotic factors, as well as numerous virulence genes (Kostylev et al., 2019). There are four QS systems identified for *P. aeruginosa*; integrated quorum signalling (IQS), RhlI/RhlR, LasI/LasR, and Pseudomonas quinolone signalling (PQS), all of which use different autoinducers (Cosa et al., 2020). To effectively combat *P. aeruginosa* infections, new strategies are needed such as

targeting the bacteria's interconnected and hierarchically regulated QS systems. These systems control the production of virulence factors and contribute to antibiotic resistance (Ueda and Wood, 2009). Potential strategies include disrupting QS pathways (quorum quenching) to prevent communication between bacterial cells, inhibiting biofilm formation, and enhancing the efficacy of antibiotics (Ueda and Wood, 2009). Interestingly, the use of nanoparticles to interfere with QS signalling and biofilm structure offers a promising avenue for reducing bacterial virulence and resistance (Rajkhowa et al., 2024).

Manufacturing nanoparticles is a rapidly growing field in various branches of science such as physics, chemistry, biology and bioengineering (Radzig et al., 2013). Moreover, nanoparticles show massive interest in the biological or biomedical field because it is capability to target specific cells or tissues promoting the effectiveness of drug delivery and diagnostic imaging (Patra et al, 2018). Among various metal nanoparticles, silver (Ag) stands out as particularly important for drug delivery due to its broad-spectrum antimicrobial properties, including antibacterial, antifungal, and antiviral activities (Bruna et al., 2021). Silver nanoparticles (AgNPs) are not only chemically stable and easy to synthesize, but also highly effective against a wide range of pathogens (Balavijayalakshmi and Ramalakshmi, 2017; Kim et al., 2023). AgNP can be synthesized using physical, chemical and biological methods. Biosynthesis of AgNPs is considered a better approach to overcome the limitations of physical and chemical methods, as it offers a simple, low-cost nanomanufacturing route that enables rapid production with high yield and relatively uniform shape distribution of nanoparticles (Das and Chakraborty, 2018). As a result, biological systems including bacteria, fungi, and plant extracts are used in green technology because of their ease of use, cheap

operating costs, and environmentally friendly (Sukanya et al., 2013; Singh et al., 2015; Zhang et al., 2016; Qais et al., 2021).

To date, the biosynthesis of silver nanoparticles (AgNPs) using bacteria (John et al., 2020), fungi (Shahzad et al., 2019), and plants (Ahmed et al., 2016) has been widely studied and reported. *Streptomyces* sp. is the largest genus of actinobacteria, and many of its species can produce secondary metabolites such as antibiotics, which also play an important role in the reduction and stabilization of AgNPs during silver salt reduction (Bakhtiari-Sardari et al., 2020). The use of *Streptomyces* sp. for producing AgNPs offers several advantages, including fast production rate due to its short cell generation time (Adiguzel et al., 2018). Extending the biosynthesis duration allows more complete nucleation and growth control, leading to smaller nanoparticles with a narrower size distribution. These characteristics are known to enhance cellular uptake, antimicrobial activity, and overall biomedical efficacy. When a nanoparticle enters a biological environment, proteins and other biomolecules quickly adsorb onto its surface, forming a protein corona. This corona may be composed of loosely bound (soft) or tightly bound (hard) layers, significantly affecting the nanoparticle's biological identity, cellular interactions, and overall behavior in vivo (Joo and Aggarwal, 2018). Consequently, this corona also influences the interaction between AgNPs with any biofilm component (Joo and Aggarwal, 2018). Nanoparticles capped with biomolecules, such as proteins, stabilize AgNPs through interactions between proteins and nanosilver (Durán et al., 2010). These interactions, involving covalent bonds and electrostatic forces, drive key biophysical and metabolic processes. AgNPs can also form complexes with proteins via electrostatic interactions, which minimally affect protein conformation

and function, as well as through binding to thiol (HS–, from cysteine) or amine (H_2N–) groups on the proteins (Chaloupka et al., 2010).

In this work, *Streptomyces* sp. PDB-311B was used to synthesize silver nanoparticles (AgNPs), and their antibacterial and anti-QS properties against *Pseudomonas aeruginosa* were assessed. The biosynthesis of bioAgNPs was performed by reducing $AgNO_3$ using cell pellets of *Streptomyces* sp. PDB-311B. The synthesized bioAgNPs was characterized using UV-Vis spectroscopy and Transmission Electron Microscopy (TEM) analysis. Then, the antibacterial activity of bioAgNPs were assessed using disc diffusion and tetrazolium microplate assay (TEMA) method. Additionally, the observation of swimming motility, biofilm formation, TEM and SEM analysis were evaluated for the assessment of anti-QS activity. SDS-PAGE analysis was employed to observe the protein corona of the bioAgNPs, followed by the identification of the purified protein using LC-MS. Finally, *in silico* analysis was conducted to identify the interactions between the protein corona of bioAgNPs and bacterial outer membrane proteins, enabling a deeper understanding of the anti-QS system in *Pseudomonas aeruginosa* sp.

1.2 Research Problem

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen responsible for a wide range of acute and chronic infections, contributing significantly to morbidity and mortality rates worldwide (Tumbarello et al., 2011). Its intrinsic and acquired multidrug resistance, along with its capacity to form biofilms, poses a substantial challenge in clinical treatment and infection control (Cao et al., 2004). Within biofilm structures, bacterial cells are embedded in a self-produced matrix that protects them from antibiotics and host immune responses

(Sharma et al., 2019). As a result, conventional antibiotics often fail to eradicate these infections effectively.

One promising strategy to combat such resistant infections is through anti-quorum sensing (QS) therapy, which targets the bacterial communication systems that regulate biofilm formation and virulence factor production. Disrupting QS pathways can attenuate bacterial pathogenicity without exerting selective pressure for resistance development. In this context, silver nanoparticles (AgNPs) have emerged as potent antimicrobial agents due to their broad-spectrum activity and distinct mechanisms of action, such as disruption of metabolic processes and inactivation of vital enzymes (Dakal et al., 2016).

Among various biological synthesis approaches, utilizing *Streptomyces* sp. for the biosynthesis of AgNPs offers distinct advantages. *Streptomyces* species are well known for their ability to produce a wide array of secondary metabolites with antibacterial, antifungal, and anticancer properties. These metabolites play a crucial role in the reduction of Ag^{+} ions and stabilization of the resulting nanoparticles. Moreover, the *Streptomyces*-derived protein corona capping the AgNPs may significantly influence their interaction with bacterial outer membrane proteins, potentially enhancing their ability to interfere with quorum sensing pathways. However, despite the growing interest in biologically synthesized AgNPs, there is still limited understanding of how the biochemical composition of AgNPs—especially the protein corona derived from different Streptomyces strains—affects their antimicrobial and anti-QS efficacy. In particular, the interactions between protein-capped AgNPs and outer membrane proteins of *P. aeruginosa* remain poorly characterized.

Therefore, this study aims to investigate and compare the biological synthesis of AgNPs using *Streptomyces* sp. PBD-311B with other reported *Streptomyces* species, focusing on the role of the protein corona in modulating interactions with *P. aeruginosa* outer membrane proteins. By integrating biosynthesis, characterization, and in silico docking studies, this research seeks to elucidate the mechanism by which *Streptomyces*-derived AgNPs disrupt quorum sensing and biofilm formation, ultimately contributing to the development of novel and effective antibacterial therapies.

1.3 Research objectives

This study focuses on the biosynthesis of silver nanoparticles (AgNPs) and the elucidation of its antibacterial and anti-quorum sensing property. The specific objectives of this study is as follows:

1. To biosynthesize and characterize biologically synthesized silver nanoparticles (bioAgNPs) produced using the cell lysate of *Streptomyces* sp. PBD-311B.

2. To evaluate the antibacterial and anti-quorum sensing effects of the bioAgNPs against *Pseudomonas aeruginosa* sp.

3. To predict the molecular interactions between the protein corona of bioAgNPs and the outer membrane proteins of *Pseudomonas aeruginosa* sp. using HADDOCK.

CHAPTER 2

LITERATURE REVIEW

2.1 Silver nanoparticles (AgNPs)

Since ancient times, burns and chronic wounds have been treated with silver (Ag). Silver nitrate ($AgNO_3$) is frequently used to treat different abscesses, salivary gland fistulae, and venereal infections (Klasen, 2000). Because Ag exhibits toxicity to a variety of microbes (Liau et al., 1997), Ag-based combinations have been widely utilized for antibacterial applications (Nomiya et al., 2004). Research by Al-Ogaidi et al., (2017) reported a antibacterial effects of AgNPs against *S.aureus* and *E.coli* with no significant cytotoxicity activity. In brief, silver's historical applications in wound care have paved the way for the development of silver-based antibacterial solutions (Dhiman et al., 2019).

2.1.1 Size and shape of AgNPs

The size of AgNPs is defined in the 1-100 nm (Bruna et al., 2021). Many researchers have reported that the antibiotic activity of AgNPs depends on their size and shape, as these physical characteristics influence the surface area-to-volume ratio, reactivity, and ability to interact with bacterial membranes (Sotiriou and Pratsinis, 2011; Van et al., 2012). Smaller and more spherical nanoparticles, for example, tend to exhibit higher antibacterial activity due to better penetration and larger surface contact with microbial cells (Sirelkhatim et al., 2015). AgNPs has great capacity and higher surface (high surface-to-volume ratios) compared to silver in its bulk form, resulted in chemical and physical differences such as unique electrical, optical, and catalytic properties, leading to the investigation and fabrication of products for targeted drug delivery, diagnosis, detection and imaging (Silva et al., 2017; Yaqoob et al., 2020).

The size and shape of AgNPs are the primary determinants of their toxicity (Shang et al., 2014). Several studies reported that nanoparticles with a smaller size exhibited greater antibacterial activity（El-Nour et al., 2010; Agnihotri et al., 2014). Morone's group demonstrated that the smaller size of AgNPs attached to the cell membrane of the target bacteria and disrupted cell functions (Morones et al., 2005). Compared to larger silver nanoparticles, smaller ones have a higher surface-area-to-volume ratio, which facilitates the faster release of Ag^+ ions into the surrounding medium. This enhanced ion release contributes to stronger antibacterial activity (Sotiriou and Pratsinis, 2011).

In addition, many studies also focused on the shape of nanoparticles which effect the antibacterial activity significantly. Pal and co-workers found that nanosilver antibacterial activity varies depending on its shape (Pal et al., 2007). The shape of AgNPs such as spherical, triangular shapes and rods plays an important role in inhibiting cell growth as in **Figure 2.1**. Van et al., (2012) speculated that silver nanoparticles with sharp edges, such as triangular or cubic shapes, are more likely to penetrate bacterial cell walls compared to smooth, spherical particles. However, a study by Actis et al. (2015) reported that AgNPs in the form of spheres, cubes, and triangles showed no significant antibacterial activity against *Staphylococcus aureus*, suggesting that shape alone may not determine antimicrobial efficacy. Interestingly, it has also been reported that the (100) crystal facet, which is dominant in nanocubes, exhibits higher surface energy than the (111) facet typically found in nanospheres. This difference in surface energy may influence interactions with bacterial membranes, but further investigation is needed to clarify the relationship between nanoparticle morphology and antibacterial activity. This indicates the differences in activity between the two forms and their

respective crystal faces and helps explain why nanocubes are more reactive than nanospheres (Agarwal et al., 2013). In addition, researchers compared triangular (prismatic)/hexagonal nanoparticles of the same size but with different edges (cube or round) and observed that the sharps nanoparticles have higher antimicrobial activity because the sharp edges have higher charge density than round edges (Noguez et al., 2007). Thus, different shapes could lead to different antimicrobial properties.

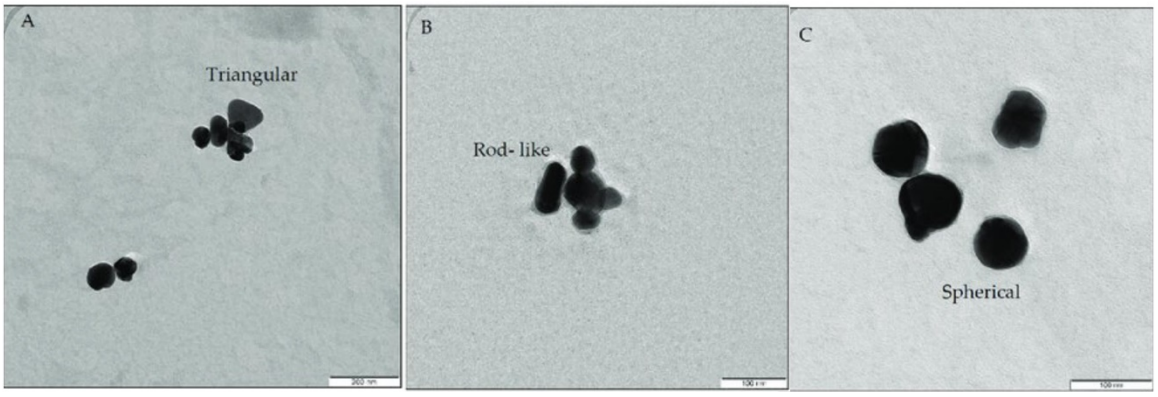

Figure 2.1 Different shapes of AgNPs as reported in Murugaiah et al., (2021).

2.1.2 Biological synthesis of AgNPs

Methods to synthesis AgNPs include physical, chemical and biological process. For physical synthesis method, it is a simple and low-cost nanomanufacturing since it can produce AgNPs rapidly with high production yield and nanoparticles of uniform sizes. But several challenges related to this method involving space requirements and energy consumption for thermal stability in tubular furnaces (Das and Chakraborty, 2018). The chemical synthesis of nanoparticles offers several advantages, including the ability to produce smaller agglomerates and achieve high yields (Awad et al., 2016). However, this method is harmful for human that cause ecological and biological hazards (Mallick, 2004; Lee et al., 2014). Therefore, physical and chemical method for development of AgNPs has several limitations, such as high cost, environmental damage, and usage of harmful chemical (Zhang et al., 2016). There is a growing need for green, cost-effective, and environmentally friendly biosynthesis methods for AgNPs. The "green synthesis" or biological method of AgNPs synthesis is considered a low-cost, safe, simple, and effective alternative particularly for its antibacterial activity（Huq et al., 2022).

The biological synthesis of AgNPs is widely available and has been used in many studies. Researchers have synthesized AgNPs using a variety of natural sources, including, yeast, fungi, algae, microorganisms, and plant extracts (Ahmad et al., 2020). Overall, green synthesis can be divided into several categories such as synthesis using microorganisms; fungi, yeast (eukaryotes), bacteria and actinomyces (prokaryotes), vegetation and plant compounds and templates; membranes viral DNA and diatoms (Rafique et al., 2017).

2.1.2(a) Biosynthesis of AgNPs using *Streptomyces* sp.

The ability of bacteria to survive in environments with high concentrations of silver ions or silver nanoparticles may facilitate the accumulation of AgNPs (Klaus et al., 1999). Despite of its challenges such as culture contamination and limited control over nanoparticle size, bacterial synthesis holds promise due to the unique ability of microorganisms to reduce heavy metal ions (Kapoor et al., 2021; Mustapha et al., 2022). Various bacterial strains have been reported to biosynthesize silver nanoparticles (AgNPs), including *Lactobacillus bulgaricus* (Naseer et al., 2021), *Rhodococcus* sp., *Pseudomonas* sp., *Bacillus* sp. (John et al., 2020), *Staphylococcus aureus* (Nanda and Saravanan, 2009), and *Escherichia coli* (Yuan et al., 2017). While each of these bacteria has shown the ability to reduce silver ions into nanoparticles, their efficiency varies in terms of nanoparticle size distribution, stability, and synthesis yield. Among these, *Bacillus* and *Pseudomonas* are often cited for their relatively rapid synthesis, but challenges such as endotoxin production (in Gram-negative strains) and limited nanoparticle uniformity remain. In contrast, *Streptomyces*, a genus of Gram-positive actinobacteria, has emerged as a superior candidate due to its strong metabolic potential, high secretion capacity of reductive enzymes and secondary metabolites, and its well-established role in producing bioactive compounds. These characteristics make *Streptomyces* not only an efficient but also a biologically safer producer of highly stable and functional AgNPs (Manivasagan et al., 2016).

Figure 2.2 shows the research trend in biosynthesis of AgNPs using *Streptomyces* over the past decade as compiled from Google scholar search. *Streptomyces* sp. is the largest genus of actinomycetes and is a Gram-positive bacterium (Shepherd et al., 2010). It constitutes about 50% of the total

actinomycetes in soil and some species that exist in nature are known as antibiotic-producer. In fact, *Streptomyces sp.* can produce antiviral, antiparasitic, antitumor, and immunosuppressant secondary bioactive metabolites (Borodina et al., 2005). Additionally, several studies have demonstrated that *Streptomyces hygrospicous* (Sadhasivam et al., 2010) and *Streptomyces* sp. VITSTK7 (Thenmozhi et al., 2013) can biosynthesize AgNPs that exhibit potent antibacterial activity, indicating their potential as effective biological producers of functional silver nanoparticles. Kalaba and co-workers (Kalaba et al., 2021) studied the antimicrobial properties of AgNPs synthesized using *Streptomyces plicatus*. In another study, Loqman et al., (2022) used *Streptomyces thinghirensis* sp. *nov*. as a green synthesis of AgNPs to eradicate multidrug-resistant bacteria. Juling and co-workers reported that an important factor in determining the cytotoxic effects of AgNPs is the protein crown (Juling et al., 2017). Pederzoli et al., (2017) demonstrated that due to their relatively large surface-area-to-volume ratio, nanoparticles facilitate mass adsorption of protein molecules on their surfaces. As highlighted by Walczyk et al. (2010), the nanoparticle-protein corona forms immediately upon exposure to biological fluids, creating a new biological identity. This corona, rather than the nanoparticle itself, primarily mediates the interactions with the cell and can potentially interfere with cellular uptake and response. The cytotoxicity, endocytosis, distribution, and

pharmacological behavior of nanoparticles are all influenced by this quickly produced crown (Jain et al., 2017).

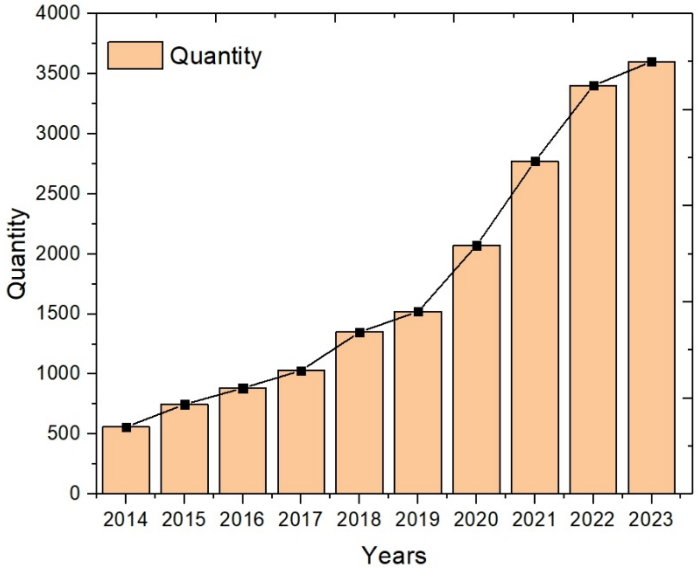

Figure 2.2 Research trend of the biosynthesis of AgNPs using *Streptomyces* sp. within last 10 years.

In addition to *Streptomyces* sp., various other bacterial species have been extensively explored for the biosynthesis of silver nanoparticles (AgNPs), owing to their ability to secrete a wide range of bioactive molecules that act as reducing and capping agents. These microorganisms can facilitate both intracellular and extracellular synthesis routes, depending on the nature of the bacterial metabolites and the interaction with silver ions (Ag^{+}).For example *Bacillus subtilis*, *Bacillus licheniformis*, and *Lactobacillus* species have been reported to successfully mediate the biosynthesis of AgNPs（Ali et al., 2019）. The mechanism typically involves the secretion of enzymes like nitrate reductase, along with peptides and proteins that not only reduce Ag^{+} into Ag^{0} but also stabilize the formed nanoparticles. For example, *Bacillus subtilis* was shown to produce spherical and stable AgNPs through extracellular synthesis, where proteins present in the supernatant played a significant role in particle capping and stabilization (Kalimuthu et al., 2008). Such as *Escherichia coli*, *Pseudomonas fluorescens*, and *Acinetobacter calcoaceticus*

have also demonstrated potential in green synthesis of AgNPs. For instance, *E. coli* has been used for intracellular synthesis of AgNPs, where particles accumulate inside the periplasmic space and are later extracted using sonication or mechanical disruption (Shahverdi et al., 2007).

The choice of bacterial species significantly influences the physicochemical characteristics of the resulting AgNPs, including their size, shape, surface charge, and antimicrobial efficacy. Overall, exploring different bacterial strains beyond *Streptomyces* expands the diversity of AgNP synthesis and provides insight into strain-specific biosynthetic pathways.

2.1.3 Mechanism of Silver Ion (Ag^+) Reduction in Biological Systems

The biosynthesis of silver nanoparticles (AgNPs) in biological systems primarily involves the reduction of silver ions (Ag^+) into elemental silver (Ag^0), followed by nucleation and growth of nanoparticles. This process is mediated by microbial metabolites and enzymes, offering a green, cost-effective, and environmentally friendly alternative to conventional chemical and physical synthesis methods（Garg et al., 2020）. Al-Rshim(2021) reported that the reduction of Ag^+ to Ag^0 in microbial systems occurs through two principal pathways: enzymatic and non-enzymatic mechanisms. Certain microorganisms, such as *Streptomyces* sp., secrete reductase enzymes—most notably nitrate reductase—that utilize intracellular cofactors such as NADH or NADPH as electron donors. These enzymes facilitate the transfer of electrons to Ag^+, reducing it to Ag^0. The simplified reaction can be represented as:

$$Ag^{+}+e^{-}\rightarrow Ag^{0}$$

This enzymatic activity can occur either intracellularly or extracellularly, depending on the nature and location of the enzymes involved. In addition to enzymatic pathways, various biomolecules produced during microbial metabolism can act as natural reducing agents. These include polyphenols, flavonoids, proteins, peptides, polysaccharides, amino acids, and vitamins such as ascorbic acid. These molecules donate electrons to Ag^{+} and initiate the reduction process without the involvement of specific enzymes (Jena et al., 2016). They are commonly found both inside the cell and in extracellular secretions.

2.2 The case study of *Pseudomonas aeruginosa* sp.

Pseudomonas aeruginosa, a Gram-negative bacterium is widely distributed in nature, and poses a significant risk for developing multidrug-resistant variants with prolonged antibiotic use (Peng et al., 2023). The highly conserved gene pool of *P.aeruginosa* contains a significant proportion of regulatory genes and networks, which are among the highest observed in known bacterial genomes. This gene pool plays a crucial role in the bacterium's ability to respond to and adapt to various environments (Stover et al., 2000). *P. aeruginosa* contain large genome (~5-7 Mbp) and diverse metabolic capacities. Thus, its prevalence in healthcare settings and resistance to antimicrobial agents make it a challenging pathogen (Singh et al., 2015). *P. aeruginosa* frequently causes endocarditis, meningitis, pneumonia in people with cystic fibrosis, burn wounds, nosocomial infections, and fulminant infections of the bones and joints. (Russotto et al., 2015).

The International Alliance for Hospital Infection Control reported that *P. aeruginosa* nosocomial infection has become a global health care problem (Rosenthal et al., 2016). It is common in healthcare settings because it is a common

companion of patients receiving medical care, and it can survive on abiotic and biological surfaces, such as medical devices resistant to disinfection methods and it can be transmitted between patients (Russotto et al., 2015). The prevalence of ventilator-associated pneumonia, central catheter-associated bloodstream infections, catheter-associated infections, and surgical/transplant highlighting the need for effective antimicrobial strategies (System et al., 2003; Nathwani et al., 2014; Trubiano and Padiglione, 2015). Therefore, science has moved towards developing effective and alternative antimicrobial strategies to prevent biofilms production of *P.aeruginosa* (Mwangi et al., 2019; Ibaraki et al., 2020). Therefore, studying a strategy to combat *Pseudomonas aeruginosa* infection is critical.

2.2.1 Quorum sensing (QS) system of *P. aeruginosa* sp.

Due to the rapid spread of multidrug resistance bacteria, researchers are currently developing strategies to inhibit bacterial communication in order to combat bacterial diseases. The pathogenicity profile of *P. aeruginosa* is associated with multiple virulence factors including protein secretion system, biofilm formation, cell surface composition, quorum sensing system (QS) and exoenzymes, which play an important role in infection severity (Jurado-Martín et al., 2021).

A bacterial cell-to-cell communication process called quorum sensing (QS) is used to assess bacterial physiology, including local population density and adaptation to a harsh and dynamic environment, including innate immune responses and antibacterial drugs (Skandamis and Nychas, 2012; Ahmad et al., 2015). The QS controls bacterial social behaviour through multiple interconnected signalling pathways (LaSarre and Federle, 2013). **Figure 2.3** illustrated that *Pseudomonas aeruginosa* has three prominent QS pathways, which consist of two LuxI/R-type systems (LasI/R and RhlI/R) and a Pseudomonas quinolone signalling system

(PQS) (Guzzo et al., (2020). By producing a diffused signalling molecule called a self-inducer (AI) in Gram-positive (oligopeptide) and Gram-negative (N-acylhomoserine lactone (AHL)) bacteria, these microorganisms are able to modulate the QS mechanism (Skandamis and Nychas, 2012). Research shows that within a community, cell responses to QS signals and the associated gene expression profile vary, leading to greater adaptability and survival chances (Grote et al., 2015). O'Loughlin and co-workers reported that the synthetic compound metathiolactone (mBTL) has an inhibitory effect on the QS receptors LasR and RhlR of *P. aeruginosa* (2013). By coordinating phenotypic alterations early in infection, QS also plays a crucial role in *P. aeruginosa* survival and colonization (González and Keshavan, 2006), and primarily engaged in the regulation of stress response metabolic pathways, biofilm formation, motion-asexual transition, motility, and antibiotic resistance mechanisms in addition to the synthesis of virulence components (Venturi, 2006; Williams and Camara, 2009; Barr et al., 2015).

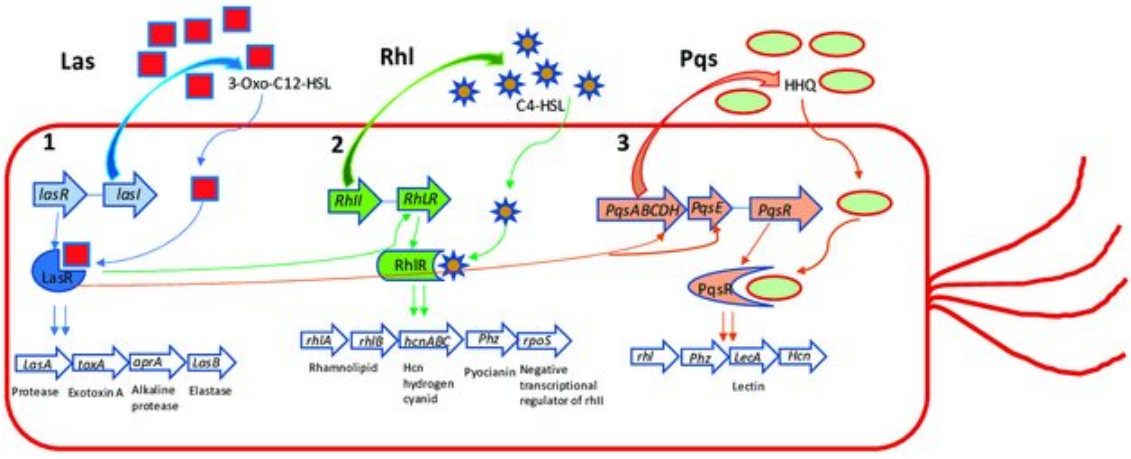

Figure 2.3 Activity of QS in *Pseudomonas aeruginosa* as adapted in Guzzo et al., (2020). The three major Pseudomonas aeruginosa QS systems (1) The LasI system synthesizes 3-oxo-C12-HSL, which interacts with the LasR receptor. This interaction results in the activation of several genes, including aprA, lasA, lasB, and toxA, along with other virulence-associated genes that fall under its regulatory control. (2) The RhlI system generates C4-HSL, which binds to the RhlR receptor, subsequently inducing the expression of genes such as phz, lasA,

rpoS, lasB, rhlAB, and hcnABC. (3) The PqsABCDH system produces HHQ, which engages with the PqsR receptor, thereby regulating the expression of genes including LecA, Phz, Hcn, and rhl.

2.2.2 Contribution to virulence and pathogenicity

Surface binding cell aggregates build up to form biofilms controlled by the QS system (Sharma et al., 2023). The QS system of *Pseudomonas aeruginosa* has been reported to be highly adaptable in response to external biological stress by producing virulence factors such as pyocyanin, clustering, and biofilm formation (Balasubramanian et al., 2013). *P. aeruginosa* utilizes various virulence mechanisms and host tissue invasion to establish infection. Biofilm production, motility (pili, flagella), pigment (pyocyanin), cytotoxin, phospholipase, elastase, and protease are among key virulence factors of *P. aeruginosa* (Liao et al., 2019). Its huge genome and key genes are responsible for adaptability of *P. aeruginosa* which includes both resistance and pathogenicity features (Poulsen et al., 2019; Stover et al., 2000).

Biofilms refer to the communities of microorganisms and usually associated with the chronic infections and antimicrobial resistance. Microorganisms developing in biofilms are significantly more resistant to antimicrobial treatments than their planktonic counterparts, and are associated with a variety of human infections and the colonization of medical devices (Sharma et al., 2023). The production of biofilms can lead to chronic infections and provide an additional mechanism of antimicrobial resistance. It was demonstrated that biofilms formed by QS mutant strains had distinct structural differences from those formed by wild-type strains (Purevdorj et al., 2002). Interfering with bacterial communication or QS can reduce microbial pathogenicity (Finch et al., 1998), including antibiotic production (Fineran et al., 2005) and differentiation of biofilm (Coneye, 2010). The

ability to withstand difficult living conditions and to withstand antibiotics are two benefits of the biofilm-QS relationship. Thus, biofilms aided in the development of numerous bacterial illnesses (Nadell et al., 2008; Newman et al., 2017). Davier' group first described the role of QS in the formation of *P. aeruginosa* biofilms (Davies et al., 1998). Another study by Perez-Perez et al., (2017) demonstrated that Beta-lactamase, efflux pumps, horizontal gene transfer, and biofilm formation are characteristics of *P. aeruginosa* that result in resistance. In addition, QS mechanisms of *P. aeruginosa* are important for regulating virulence factors and biofilm formation. According to a study, biofilm formation and antibiotic resistance that can elude host immune responses are linked to 80% of bacterial illnesses (Chakraborty et al., 2020).

Previous studies reveal the multifaceted virulence of *Pseudomonas aeruginosa*, mostly regulated by the global virulence factor regulator Vfr, a transcription factor that is dependent on cyclic adenosine phosphate (cAMP) and regulates the expression of over 200 genes (Wolfgang et al., 2003; Coggan and Wolfgang, 2012). By triggering secretion system expression, it controls virulence (Davinic et al., 2009). By triggering the expression of secretion systems, Vfr controls virulence (Davinic et al., 2009).Vfr regulates virulence by activating the expression of secretion systems (Davinic et al., 2009). Important virulence factors that promote bacterial metal resistance and allow bacterial growth in the presence of ferrous ions are iron transporters, such as pyoverdine and pyochelin (Braud et al., 2010). Acute and chronic infections are caused by certain *P. aeruginosa* characteristics, including biofilm development, motility, pyocyanin, and released toxins (Sultan et al., 2021)

The main secretory systems of *P. aeruginosa* are T2SS, T3SS, and T6SS, secreting products that act as virulence factors (Azam and Khan, 2019). T2SS secretes a variety of proteases in extracellular mediators, including elastases LasA and LasB, protease IV, phospholipase C, and exotoxin A (Kipnis et al., 2006). The T3SS of *P. aeruginosa* produces various toxins, such as ExoU, ExoS, ExoT, and ExoY, which are the cause of ventilator-associated pneumonia (Alonso et al., 2020). A powerful strategy for controlling bacterial infections is to block virulent small molecules. ExoS and ExoT with amino terminal Rho-GAP and C- C-terminal ADP-ribosyltransferase activities, respectively, interfere the signal transduction in the host such as phagocytic NADPH (nicotinamide adenine dinucleotide phosphate) (Vareechon et al., 2017). ExoU with phospholipase activity mainly destruct cellular physiology (Sultan et al., 2021). Moverover, ExoY play role in pathogenic of *P. aeruginosa* through adenylate cyclase activity (Sultan et al., 2021).

2.3 Antibacterial property of AgNPs against *P. aeruginosa*

In the past, researchers discovered that AgNP exhibited a significant antibacterial effect against various bacteria, accumulating ample data regarding their antibiotic and cytotoxic effects (Al-Ogaidi, 2017). According to reports, AgNPs are an efficient antibacterial agent that works against both Gram-positive and Gram-negative bacteria (Zhang et al., 2016). AgNPs induce cell death in *Escherichia coli*, a Gram-negative bacterium, by accumulating on the cell surface, forming "pits" in the cell wall (Sondi and Salopek-Sondi,2004; Beyth et al., 2010). Additionally, AgNPs induce cell death through the production of reactive oxygen species (ROS) in various bacteria including *Pseudomonas aeruginosa, Shigella fowleri*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Helicobacter pylori*,

Helicobacter felis, *Escherichia coli*, *Klebsiella pneumoniae* and *Bacillus subtilis* (Anthony et al., 2014; Han et al., 2014; Gurunathan et al., 2015).

Many researchers studied the antibacterial activity of AgNPs, but the antibacterial mechanisms and potential hazards are not very clear (Velusamy et al., 2016). Castiglioni and co-workers (Castiglioni et al., 2017) suggested that AgNPs cause cytotoxicity that is dose dependent. AgNPs release Ag^{+} and interact with bacterial cell membrane and wall components, exerting their antibacterial effect (Xiu et al., 2012). The primary mechanisms involve the penetration of bacterial cell walls and membranes by Ag ions, leading to increased membrane permeability and leakage of cellular components, disrupting cell function (Shrivastava et al., 2007). Abbaszadegan's group reported that electrostatic attraction between positively charged AgNPs and negatively charged microbial cell membranes facilitates nanoparticle attachment to the bacterial surface (Abbaszadegan et al., 2015). However, in biological environments, AgNPs are often surrounded by a protein corona, which can alter their surface charge, hydrophobicity, and interaction patterns with microbial membranes (Wang et al., 2016). Therefore, the presence of a protein corona may influence or modulate this electrostatic interaction, and should be considered when evaluating nanoparticle–cell membrane interactions. Additionally, Ag ions also react with proteins through sulfhydryl group, resulting in damage of DNA and proteins that cause death (Liau et al., 1997).

In brief, three main hypotheses have been proposed regarding the antimicrobial mechanism of AgNPs. The first hypothesis suggests that AgNPs exert their effects at the membrane level by penetrating the outer membrane and accumulating in the inner membrane. This accumulation disrupts membrane integrity, leading to leakage of cellular contents and loss of membrane potential, which ultimately

results in cell death (Seil and Webster, 2012). The second theory proposes that AgNPs not only damage and pass through the cell membrane, but also enter the cytoplasm, where they interfere with intracellular components such as proteins and DNA. This interference can impair essential cellular functions, including enzyme activity, replication, and metabolic pathways, further contributing to bacterial death. AgNP can alter the structure and activities of intracellular materials including proteins and DNA by interacting with sulfur or phosphorus groups (Khalandi, et al.,2017). By reacting with thiol (mercaptan) groups in enzymes, it modifies the respiratory chain in the inner mitochondrial membrane, resulting in the generation of free radicals and reactive oxygen species (ROS). This sets off apoptotic pathways and damages intracellular machinery (Seil and Webster, 2012). The third theory states that because of their size and charge, Ag ions are released from nanoparticles. These ions have the ability to interact with many parts of cells, which may change metabolic processes, damage cell membranes, and have an impact on genetic material. Accordingly, a number of variables, such as the size, shape, and surface charge of silver nanoparticles (AgNPs), affect their bactericidal action (Abbaszadegan et al., 2015).

2.4 Mechanism of interaction between nanoparticles and membrane cells of bacteria

Using *in silico* techniques to observe interactions offers the chance to find substances that prevent disease processes and advances knowledge of the mechanism of action (Yi et al., 2018). Computer simulations offer advantages over *in vitro* experiment such as saving time and resources when predicting compound efficacy prior to *in vitro* and *in vivo* testing (Ferrero et al., 2017). In other way, efficient data from *in vitro* testing can also be verified using computer studies

(Ahmad et al., 2020). For instance, inhibitory enzymes like ß-lactamase have been shown to be important in the antibacterial action of nanomaterials by *in silico* molecular docking studies (Mureed, et al., 2021).

Rather than existing alone, proteins are a component of intricate networks or pathways. The quantity of proteins (genes) in an organism, their combinatorial connections, and the selective linking, chemical, and structural alterations in the proteome all contribute to the complexity of biological systems at various levels (Lander et al., 2001). Computational techniques aid in detecting and predicting protein interactions, contributing to the recognition of the importance of describing all protein interactions in cells. Computer models help identify new therapeutic targets and build drugs by encoding and testing theories about cell function, illness etiology, and processes (Barh et al., 2014). Phylogenetic profiling, intracellular localization, post-translational changes, structural patterns, and homologous pairs are some methods for studying protein-protein interaction predictions (Rao et al., 2014).

Majumdar and colleagues used computational modeling to demonstrate the presence of multiple non-covalent interactions—including hydrogen bonding, hydrophobic forces, Van der Waals forces, and metal–receptor coordination—between active phosphorylation sites of bacterial biofilm proteins and AgNPs or the phytochemicals adsorbed on their surfaces. These interactions lead to conformational changes in the protein that inactivate its biological function (Majumdar et al., 2020).

The scientific investigations have provided evidence for the inhibitory capacity of nanoparticles on quorum sensing (QS)-mediated virulence factors, as well as

their efficacy in preventing biofilm formation. Prateeksha et al., (2017) and Gómez-Gómez et al., (2019) focused on a mechanistic approach and computational analysis of SeNPs to discover their potential as anti-QS agents. Vyshnava et al. (2016) demonstrated the anti-biofilm properties of silver nanoparticles (AgNPs) through an *in-silico* methodology. According to the *in silico* findings, Ali et al. (2017) suggested several potential mechanisms through which silver nanoparticles (AgNPs) may interfere with quorum sensing (QS) circuits. These mechanisms include (1) competitive binding of AgNPs to quorum sensing receptor proteins**,** which blocks the interaction with their natural signaling molecules (autoinducers); (2) distortion of QS signal molecule structure due to oxidative stress generated by AgNPs; and (3) disruption of the expression of QS-regulated genes**,** thereby inhibiting the production of virulence factors and biofilm formation.