

**Biogenic Synthesis of Silver Nanoparticles using Soil Fungi
against Carbapenem-Resistant *Escherichia coli* and *Klebsiella
pneumoniae***

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

**in
Microbiology
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DECLARATION

I, hereby declared that the presented work in the thesis entitled “**Biogenic Synthesis of Silver Nanoparticles using Soil Fungi against Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae***” in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the supervision of Dr. Anuprita Ray, working as Assistant Professor in Department of Molecular Biology & Genetic Engineering, School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India and Co-supervision of Prof. Gaurav Kumar, working as Professor in Amity Institute of Microbial Technology, Amity University Rajasthan. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled “**Biogenic Synthesis of Silver Nanoparticles using Soil Fungi against Carbapenem-Resistant *Escherichia coli* and *Klebsiella pneumoniae***” submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Microbiology, School of Bioengineering and Biosciences, is a research work carried out by Himanshu Jangid, Registration Number: 12116762 is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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Abstract

The rapid emergence and global dissemination of carbapenem-resistant Enterobacteriaceae (CRE), particularly carbapenem-resistant strains of *Escherichia coli* and *Klebsiella pneumoniae*, represent one of the most formidable challenges in contemporary clinical microbiology. These pathogens display remarkable resistance to multiple classes of antibiotics, including carbapenems, which are frequently relied upon as critical therapeutic options in severe multidrug-resistant Gram-negative infections. Their resistance mechanisms, primarily mediated through carbapenemase production, porin loss, and efflux pumps, severely compromise the efficacy of conventional therapies and contribute to increased morbidity, prolonged hospitalization, and elevated mortality rates among affected patients. Recognizing the severity of this public health threat, the World Health Organization (WHO) has categorized CRE under the “Critical Priority Pathogen Group” in its global priority list of antibiotic-resistant bacteria. This top-tier designation underscores the urgent need for novel antimicrobial agents and alternative strategies to combat these resilient organisms. The designation aligns with mounting evidence that CRE-related infections are associated with significantly worse clinical outcomes, with treatment failure often being the norm rather than the exception. In developing countries, including India, the burden of CRE is further exacerbated by factors such as antibiotic misuse, lack of regulatory oversight, and limited availability of advanced diagnostic tools. Clinical isolates exhibiting co-resistance to carbapenems, cephalosporins, aminoglycosides, and even colistin have been increasingly reported from tertiary care settings, fueling an antimicrobial resistance (AMR) crisis of unprecedented scale. Surveillance data also highlight that *K. pneumoniae* and *E. coli* remain the most prevalent CRE pathogens implicated in life-threatening infections, including bloodstream infections, urinary tract infections, and ventilator-associated pneumonia. Given this alarming scenario, there is an imperative global consensus on the necessity to explore innovative, eco-friendly, and sustainable therapeutic avenues to tackle CRE. Strategies that employ biologically synthesized nanomaterials, particularly silver nanoparticles (AgNPs), have demonstrated promising antimicrobial efficacy and offer a potentially transformative solution against resistant strains.

Therefore, this study was design to produce silver nanoparticle using soil fungi with antimicrobial properties against carbapenem resistant *K. pneumoniae* and *E. coli*.

Soil samples were collected from agricultural land in and around Phagwara, Punjab, India. A total of six soil samples were processed for fungal isolation, yielding 25 distinct fungal isolates. Both intracellular and extracellular extracts were prepared from each isolate and employed for the biosynthesis of silver nanoparticles (AgNPs), giving a total of 50 biosynthesis reactions. The reaction mixtures were monitored for evidence of nanoparticle synthesis through color change, followed by confirmatory spectroscopic analysis. The synthesized AgNPs were screened for antimicrobial activity using agar well diffusion assays against clinically isolated carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae*. The antibacterial activity of AgNPs were quantified by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) relative to meropenem. To explore the mechanism of action, bacterial growth kinetics, membrane integrity (via nucleotide leakage assays), DNA degradation, and ultrastructural changes observed through Field Emission Scanning Electron Microscopy (FE-SEM) were investigated. Toxicity of AgNPs was evaluated by MTT assays on 3T3 mouse fibroblast cell lines to determine biosafety. Morphological characterization of the most potent fungal isolate was performed based on macroscopic and microscopic features, including colony color, texture, and conidial morphology. Molecular identification was subsequently carried out by ITS (Internal Transcribed Spacer) region sequencing using ITS1 and ITS4 primers, and the resulting sequence was deposited in the GenBank database for accession number assignment and phylogenetic analysis. Finally, detailed physicochemical characterization of the potential biosynthesized nanoparticles was carried out using UV–Vis spectroscopy, Dynamic Light Scattering (DLS), Zeta potential analysis, Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM), and Scanning Electron Microscopy with Energy-Dispersive X-ray Spectroscopy (SEM-EDX).

Twenty-five morphologically distinct fungal isolates were obtained from six agricultural soils around Phagwara, Punjab. Both extracellular and intracellular extracts

were evaluated for silver ion reduction, generating fifty biosynthesis reactions. Among 50 reactions, 8 showed a distinct color transition from pale yellow to brown, indicating silver nanoparticle formation and they also exhibiting surface plasmon resonance between 391–450 nm during UV–visible spectroscopy analysis. These samples were subjected to antibacterial activity against test bacteria. During preliminary antibacterial activity among these samples, S1I4 (extracellular extract) exhibited highest antibacterial activity against both test bacteria including carbapenem resistant *E. coli* (24.33 ± 1.52 mm) and *K. pneumoniae* (25.33 ± 2.08 mm). Subsequent assays demonstrated MIC/MBC values of $5/50 \mu\text{g mL}^{-1}$ for *E. coli* and $25/75 \mu\text{g mL}^{-1}$ for *K. pneumoniae*, outperforming meropenem controls. These findings confirm the strong bactericidal potential of S1I4-derived AgNPs against carbapenem-resistant strains. Mechanistic evaluations showed that AgNP exposure delayed bacterial growth kinetics, extended the lag phase, and reduced biomass accumulation. Nucleotide-leakage assays recorded a time-dependent rise in 260 nm absorbance, indicating compromised membrane integrity. Agarose-gel electrophoresis revealed smeared, fragmented DNA in *E. coli*, while *K. pneumoniae* displayed distinct banding linked to plasmid co-isolation rather than typical fragmentation. FE-SEM imaging displayed severe ultrastructural damage, including membrane blebbing, irregular surfaces, and collapsed cell envelopes, validating physical disruption as a major antibacterial mechanism. MTT-based cytotoxicity profiling on 3T3 mouse fibroblasts showed dose-dependent viability loss with an IC_{50} of $100.06 \mu\text{g mL}^{-1}$. Notably, the effective antibacterial concentrations ($5\text{--}25 \mu\text{g mL}^{-1}$) were well below this threshold, confirming a favorable therapeutic index and limited mammalian cytotoxicity. The potent fungal isolate (S1I4) displayed rapid growth on PDA, forming dark brown-black, cottony colonies with radiating folds and heavy sporulation. Microscopic examination revealed septate hyphae, biserial phialides, and rough-walled globose conidia typical of the *Aspergillus niger* complex. ITS sequencing confirmed > 99 % identity with reference *A. niger* strains; phylogenetic analysis (bootstrap 81 %) placed S1I4 within the *A. niger* clade. The sequence was deposited in GenBank under the name *Aspergillus niger* HG3 and Accession No. PV203216.1. Physicochemical characterization of S1I4-derived AgNPs demonstrated its physiochemical properties. DLS analysis established an average hydrodynamic diameter of 24.55 nm ($\text{PDI} < 0.3$) and zeta potential of -23.66 mV,

confirming monodispersity and colloidal stability. FTIR spectra revealed O–H, amide, amine, and C–O vibrations signifying biomolecule-mediated reduction and capping. XRD peaks at 38.1°, 44.3°, 64.4°, and 77.4° corresponded to the (111), (200), (220), and (311) planes of FCC metallic silver with an average crystallite size (~12.8 nm). TEM and SEM images showed predominantly spherical, well-dispersed nanoparticles (29–34 nm), while EDX analysis displayed a strong Ag signal at ~3 keV with accompanying C and O peaks, verifying purity and organic capping. Collectively, these results confirm the efficient biogenic synthesis of small, spherical, stable, and crystalline silver nanoparticles by *Aspergillus niger* S1I4, exhibiting potent antibacterial activity against carbapenem-resistant pathogens with minimal cytotoxicity. In conclusion, this study demonstrates that *Aspergillus niger*-mediated silver nanoparticles (AgNPs) represent a potent, eco-friendly antimicrobial strategy against carbapenem-resistant *E. coli* and *K. pneumoniae*. The biosynthesized AgNPs exhibited strong bactericidal activity, multi-target mechanisms involving membrane disruption and genotoxic effects, and minimal toxicity toward normal cells. Comprehensive characterization confirmed their nanoscale stability, crystallinity, and biofunctional surface chemistry, supporting their biomedical applicability. Future research should focus on *in vivo* efficacy validation, long-term biosafety assessments, and formulation of AgNP-based therapeutics. Scaling up fungal-mediated synthesis could further advance sustainable nanotechnology solutions against multidrug-resistant pathogens.

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List of Abbreviations

Abbreviation	Full Form
AgNPs	Silver Nanoparticles
AMR	Antimicrobial Resistance
CRE	Carbapenem-Resistant Enterobacteriaceae
MDR	Multidrug-Resistant
WHO	World Health Organization
CDC	Centers for Disease Control and Prevention
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
DLS	Dynamic Light Scattering
PDI	Polydispersity Index
FTIR	Fourier Transform Infrared Spectroscopy
XRD	X-ray Diffraction
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
EDX	Energy-Dispersive X-ray Spectroscopy
FE-SEM	Field Emission Scanning Electron Microscopy
UV–Vis	Ultraviolet–Visible Spectrophotometry
SPR	Surface Plasmon Resonance
ROS	Reactive Oxygen Species
ITS	Internal Transcribed Spacer
PCR	Polymerase Chain Reaction
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetics Analysis
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth

MHA	Mueller Hinton Agar
TSB	Tryptic Soy Broth
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
DMSO	Dimethyl Sulfoxide
FCC	Face-Centered Cubic
KBr	Potassium Bromide
rpm	Revolutions Per Minute
nm	Nanometer
µg/mL	Micrograms per Milliliter
°C	Degree Celsius
CFU/mL	Colony Forming Units per Milliliter
IC ₅₀	Half Maximal Inhibitory Concentration
3T3	Mouse Embryonic Fibroblast Cell Line
PIMS	Punjab Institute of Medical Sciences
AM	Absorbance Measurement
LCBP	Lactophenol Cotton Blue Preparation
LB	Luria–Bertani Medium
SD	Standard Deviation
UV	Ultraviolet
OD	Optical Density

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LIST OF APPENDICES

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(A. Chemicals, B. Media & Buffers, C. Antibiotics, and D. Instruments)

Appendix – II: Buffers and reagents prepared

Appendix – III: Publications and conference presentation

Chapter 1

1. Introduction

Bacterial infections continue to represent one of the most significant global health challenges, accounting for a substantial portion of disease burden across all regions and age groups. Despite advancements in antimicrobial therapy, diagnostics, and public health infrastructure, bacterial diseases remain a leading cause of morbidity and mortality worldwide. In 2019, over 7.7 million deaths were directly caused by bacterial infections, making them the second leading cause of death globally, following only ischemic heart disease (Ikuta et al., 2022). Lower respiratory tract infections, bloodstream infections, and peritoneal infections were identified as the top contributors to this mortality burden (Naghavi et al., 2024). The impact of bacterial infections is not confined to low-resource settings. While low- and middle-income countries (LMICs) face a disproportionate burden due to inadequate access to healthcare, diagnostics, and sanitation, high-income countries also experience substantial hospitalization rates and clinical complications from bacterial infections. For instance, in the United States alone, sepsis a severe manifestation of bacterial infection leads to over 1.7 million hospitalizations and substantial healthcare utilization annually (Maki & Zervos, 2021). Infection-related morbidity disproportionately affects vulnerable populations, including neonates, the elderly, immunocompromised individuals, and patients undergoing major surgeries or intensive care. In many cases, the absence of rapid and reliable diagnostic methods delays treatment initiation, resulting in worsened clinical outcomes and extended hospital stays (Garvey, 2024). Furthermore, in LMICs, bacterial infections remain a leading cause of neonatal and maternal mortality, particularly in areas where healthcare access and early intervention capabilities are limited (Dramowski et al., 2025). Beyond their clinical consequences, bacterial infections impose considerable economic costs on health systems due to prolonged hospitalization, repeated interventions, and intensive care requirements. These costs are especially burdensome in under-resourced health settings, where the financial implications often result in delayed or incomplete care (Costabella et al., 2023). Accurate assessment of the global burden of bacterial diseases is therefore essential to

inform policy, allocate resources effectively, and prioritize innovations in prevention, diagnosis, and treatment.

The global crisis of antimicrobial resistance (AMR) has escalated rapidly in recent decades, with bacterial infections increasingly demonstrating reduced susceptibility to standard antibiotic regimens. Once considered isolated clinical occurrences, resistant infections are now systematically categorized into clinically defined stages: antimicrobial resistance (AMR), multidrug resistance (MDR), extensively drug resistance (XDR), and pan-drug resistance (PDR). These classifications reflect the progressive erosion of treatment options available to healthcare providers (Ahmed et al., 2024; Nwobodo et al., 2022). In clinical terms, AMR refers to resistance against one or more antimicrobial agents. MDR is defined as non-susceptibility to at least one drug in three or more antimicrobial categories, while XDR isolates are susceptible to only one or two remaining classes. PDR organisms exhibit resistance to all available antimicrobial classes, leaving clinicians with no effective therapeutic choices (Basak et al., 2016; Magiorakos et al., 2012). These resistance phenotypes are no longer rare or theoretical. Multiple surveillance studies have confirmed the growing incidence of MDR and XDR infections in both hospital and community settings globally (van Duin & Paterson, 2016). Healthcare environments such as intensive care units, surgical wards, and neonatal care facilities remain particularly vulnerable, where infection control challenges and antibiotic pressure accelerate the spread of resistant strains (Strich & Palmore, 2017). Factors such as inappropriate prescribing, over-the-counter availability of antibiotics, and insufficient global regulation have contributed to a surge in resistance even in routine infections (Salam et al., 2023). In response to this escalating antimicrobial resistance (AMR) crisis, international organizations such as the World Health Organization (WHO) have recognized AMR as one of the top ten global public health threats of the 21st century. Without urgent and coordinated intervention, the continued rise in resistance could make routine medical procedures including surgeries, cancer treatments, and childbirth dangerously unpredictable due to the risk of infections that no longer respond to available antibiotics (Coque et al., 2023). What was once an issue

of localized treatment failure has become a global threat to medical security, warranting urgent surveillance, stewardship, and innovation on a global scale.

Beyond its devastating clinical implications, antimicrobial resistance (AMR) has emerged as a major economic threat to global health systems and development. According to projections by the World Bank, a high-AMR scenario could reduce global GDP by as much as 3.8% by 2050, resulting in an estimated cumulative loss of up to USD 85 trillion. This economic fallout is expected to disproportionately impact low- and middle-income countries (LMICs), where healthcare infrastructure is already fragile, and the ability to absorb the economic shock of resistant infections is limited (Ahmed et al., 2017; Haque, 2021). In terms of direct costs, AMR imposes a substantial financial burden on healthcare systems. In the United States alone, resistant infections contribute an additional USD 20 billion in direct healthcare costs annually, along with over USD 35 billion in lost productivity due to extended illness and premature deaths (Haruna et al., 2024). In Europe, countries like Spain have reported that AMR is responsible for more than 6,000 preventable deaths each year and an estimated annual economic cost of €338.6 million, amounting to roughly 0.03% of the national GDP (Siaba & Casal, 2025). India, which bears one of the highest burdens of AMR globally, faces a uniquely acute crisis. A study from a tertiary care centre reported that resistant infections increase ICU stays by an average of 5–10 days per patient, leading to significantly higher treatment costs and resource consumption (Kumar et al., 2013). Moreover, the absence of effective second-line treatments forces clinicians to rely on expensive, often fewer effective alternatives, thereby straining both public and private healthcare budgets (Laxminarayan et al., 2016). The economic impact also extends beyond hospitals into broader public health systems. Resistant infections reduce labour productivity by prolonging recovery times, increase out-of-pocket expenditure for patients, and drive up national pharmaceutical spending due to overuse of broad-spectrum or last-resort antibiotics (Maurmo et al., 2025). When multiplied across tens of millions of annual cases, this translates into a compounding economic loss for developing economies. A key concern is the lack of cost-effective interventions for AMR in these regions. Most LMICs do not have sustainable antibiotic stewardship programs, comprehensive surveillance systems, or accessible diagnostic tools gaps that

further worsen both the clinical and economic burden. Without innovative, scalable, and eco-friendly solutions to control resistant pathogens, AMR threatens to reverse decades of global progress in infectious disease control and economic development. Thus, addressing AMR is not merely a public health imperative but an economic necessity. By offering a multi-targeted and sustainable solution, such approaches could substantially reduce the economic costs associated with resistant infections while also preserving the efficacy of existing antibiotics (Snair, 2024).

In 2017, the World Health Organization (WHO) introduced its first Priority Pathogens List (PPL) to guide global research and development efforts aimed at addressing the growing threat of antimicrobial resistance (AMR). The list classifies antibiotic-resistant bacteria into three tiers Critical, High, and Medium Priority based on factors such as clinical severity, resistance prevalence, transmissibility, treatment limitations, and overall public health impact (Tacconelli et al., 2018). The Critical Priority Tier includes pathogens posing the most urgent threat to human health, particularly Gram-negative bacteria with extensive resistance and few effective treatment options. This tier comprises carbapenem-resistant and third-generation cephalosporin-resistant Enterobacterales, *Acinetobacter baumannii* resistant to carbapenems, and *Mycobacterium tuberculosis* resistant to rifampicin. The High Priority Tier includes organisms such as fluoroquinolone-resistant *Salmonella Typhi*, *Shigella* species, and non-typhoidal *Salmonella*, vancomycin-resistant *Enterococcus faecium*, carbapenem-resistant *Pseudomonas aeruginosa*, third-generation cephalosporin- and/or fluoroquinolone-resistant *Neisseria gonorrhoeae*, and methicillin-resistant *Staphylococcus aureus*. The Medium Priority Tier includes macrolide-resistant Group A *Streptococci* and *Streptococcus pneumoniae*, ampicillin-resistant *Haemophilus influenzae*, and penicillin-resistant Group B *Streptococci* (Vaithinathan & Vanitha, 2018; Mancuso et al., 2021).

Within the Critical Tier, members of the Enterobacterales family particularly *Klebsiella pneumoniae* and *Escherichia coli* are of special concern due to their broad presence, increasing reports of resistance and widespread association with hospital-acquired infections. These organisms are major contributors to bloodstream, urinary tract, and

respiratory infections, especially in intensive care units and other high-dependency clinical environments. Their ability to rapidly acquire and disseminate resistance mechanisms has resulted in rising clinical failure rates globally (Chang et al., 2021; Saparamadu & Ratnayake, 2022). In light of these threats, the WHO has called for urgent investment in the discovery of new antimicrobials targeting Critical Tier pathogens. This prioritization underscores the pressing need for sustainable alternatives to conventional antibiotics, especially against bacteria that are becoming increasingly unresponsive to existing therapeutic options (Mancuso et al., 2021).

Carbapenem-resistant Enterobacteriaceae (CRE), particularly *Escherichia coli* and *Klebsiella pneumoniae*, represent one of the most pressing threats in modern infectious diseases. These microorganisms are among the most common Gram-negative pathogens causing bloodstream, urinary tract, and respiratory infections in both hospital and community settings. When resistance to carbapenems arises the class of antibiotics long regarded as the last line of defense treatment options become extremely limited, and therapeutic failures are frequent. The clinical burden of CRE infections is reflected in their exceptionally high mortality rates. Bloodstream infections caused by carbapenem-resistant *K. pneumoniae* and *E. coli* are associated with case fatality rates exceeding 50%, especially among critically ill or immunocompromised patients (PEREZ & VAN DUIN, 2013; Suay-García & Pérez-Gracia, 2019a). Studies across diverse healthcare systems have confirmed that carbapenem resistance is increasingly common, with *K. pneumoniae* showing prevalence rates as high as 46–53% in clinical isolates, while carbapenem-resistant *E. coli* is steadily rising worldwide (Mohammadpour et al., 2025). The situation is particularly acute in low- and middle-income countries, including India, where hospital-based surveillance has reported alarming levels of carbapenem resistance among *K. pneumoniae*, frequently exceeding 50% in intensive care units and surgical wards (Lathakumari et al., 2025). The consequences of this resistance extend beyond individual treatment failure. Patients with CRE infections face prolonged hospital stays, increased healthcare costs, and markedly worse prognoses compared to those infected with carbapenem-susceptible strains (Tangsawad et al., 2022). In addition, the ability of *K. pneumoniae* and *E. coli* to share resistance traits via plasmid-mediated horizontal gene transfer accelerates the

spread of carbapenem resistance across bacterial populations, turning previously treatable infections into lethal conditions in both hospital and community settings (Jean et al., 2022). Recognizing the magnitude of this crisis, the World Health Organization has designated carbapenem-resistant *K. pneumoniae* and *E. coli* as part of the “Priority 1: Critical” group in its Global Priority Pathogens List, underscoring the urgent need for novel antimicrobials and innovative therapeutic strategies. Without immediate intervention, the global rise of CRE threatens to dismantle decades of medical progress, leaving even routine infections without reliable treatment options (Sati et al., 2025; Tacconelli et al., 2018).

Despite the discovery of antibiotics revolutionizing modern medicine, these compounds are now facing serious limitations. Most notably, the widespread emergence of antibiotic resistance has rendered many traditional treatments ineffective. Compounding this issue is the alarming slowdown in the discovery of novel antibiotics, leaving clinicians with limited options against drug-resistant infections. A key concern is that conventional antibiotics often exhibit high toxicity, particularly in critically ill patients. Agents such as colistin and aminoglycosides, once abandoned due to nephrotoxicity and cytotoxicity, are being reintroduced out of necessity a desperate attempt to combat resistant strains despite their known dangers (Oliveira et al., 2024). Moreover, most antibiotics in current use have predictable mechanisms of action, making them vulnerable to bacterial resistance. This has led to scenarios where even first-line and reserve antibiotics are ineffective. Alarming, new antibiotic approvals have stagnated, especially for Gram-negative pathogens like *Klebsiella pneumoniae* and *E. coli*. The antibiotic pipeline has not kept pace with resistance trends, leaving gaps in our therapeutic arsenal. According to the WHO, nearly all newly developed antibiotics over the past 30 years are variations of existing classes, offering minimal advantage against emerging superbugs. The lack of innovative scaffolds and underinvestment in antibiotic research reflect both scientific and economic hurdles in drug development. Collectively, these limitations underscore a critical need for alternative antimicrobial strategies that go beyond the conventional antibiotic framework (Muteeb et al., 2023).

In response to the mounting failure of conventional antibiotics, nanotechnology has emerged as a promising and innovative approach for combating infectious diseases. Nanoparticles, due to their ultra-small size (1–100 nm), high surface area-to-volume ratio, and tunable physicochemical properties, have shown potential to interact with bacterial cells in ways that traditional antibiotics cannot (Hetta et al., 2023). In recent years, a variety of nanomaterials have been explored as potential antimicrobial agents beyond silver nanoparticles. These include gold nanoparticles (AuNPs), zinc oxide nanoparticles (ZnO NPs), titanium dioxide nanoparticles (TiO₂ NPs), and carbon-based nanomaterials such as graphene oxide and carbon nanotubes. Each of these nanomaterials exhibits distinct antimicrobial mechanisms. For instance, ZnO and TiO₂ nanoparticles are known for their strong photocatalytic activity and reactive oxygen species (ROS) generation, while gold nanoparticles are valued for their biocompatibility and ease of functionalization for targeted applications. Carbon-based nanomaterials demonstrate physical disruption of bacterial membranes and interference with microbial metabolism. However, despite these advancements, many alternative nanomaterials face limitations such as higher production costs, stability challenges, or reduced broad-spectrum efficacy compared to silver nanoparticles. As a result, silver nanoparticles continue to be widely recognized as one of the most effective and versatile nanomaterials for antimicrobial applications (Srivastava et al., 2021; Hosny et al., 2025). Silver has been recognized for its antimicrobial properties since antiquity, but its nanoscale form significantly amplifies its bioactivity. AgNPs operate through multiple, synergistic mechanisms that compromise bacterial viability. One of the principal mechanisms involves the generation of reactive oxygen species (ROS) such as superoxide anions and hydrogen peroxide which induce oxidative stress in microbial cells. This leads to lipid peroxidation, protein denaturation, and ultimately, fragmentation of bacterial DNA, causing irreversible cellular damage (Yadav & Tare, 2024). Additionally, AgNPs are known to disrupt bacterial membranes, increasing permeability and leading to leakage of intracellular contents. They bind to sulfur- and phosphorus-containing compounds in the cell membrane and enzymes, interfering with energy metabolism and structural integrity. This interaction is enhanced by the nanoparticles' shape, size, and surface charge, which can be optimized depending on the synthesis method. A significant advantage of AgNPs lies in their ability to penetrate

and dismantle mature biofilms, which are typically impervious to most antibiotics. Biofilms are dense microbial communities encased in extracellular polymeric substances that act as a diffusion barrier. AgNPs overcome this barrier by targeting quorum sensing, bacterial motility, and iron metabolism thereby preventing biofilm maturation and enhancing bacterial clearance (Zhang et al., 2020). Another notable mechanism involves the release of Ag⁺ ions from the nanoparticle surface. These ions interfere with intracellular processes such as DNA replication and protein synthesis. When combined with photoactivation or functionalization using plant or fungal biomolecules, the bactericidal potential of AgNPs is significantly enhanced. Some formulations can even act against persister cells dormant phenotypes resistant to most antibiotics by triggering apoptosis-like pathways within bacteria (Godakhindi et al., 2025). AgNPs also demonstrate broad-spectrum activity, effectively targeting both Gram-positive and Gram-negative bacteria, including multidrug-resistant species such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*. Studies have reported that these nanoparticles compromise bacterial respiration, disrupt membrane potential, and induce leakage of nucleotides and ions, often leading to rapid bacteriolysis (Lee et al., 2014; Mateo & Jiménez, 2022). Beyond their immediate antimicrobial properties, AgNPs are being explored in drug delivery, wound healing, implant coatings, and water purification underscoring their translational relevance.

In this study, silver nanoparticles synthesized via biological (fungal) methods are proposed as a novel, biocompatible, and effective strategy to target high-priority antibiotic-resistant bacteria, especially those resistant to carbapenems. Their multi-targeted mechanisms and adaptability offer significant promise for integration into next-generation antimicrobial platforms. The method used to synthesize silver nanoparticles (AgNPs) significantly influences their biological activity, toxicity profile, cost-efficiency, and scalability. Conventional physical and chemical synthesis techniques often rely on toxic solvents, high energy inputs, and produce non-biocompatible byproducts, limiting their applicability in clinical or environmental settings (Abbas et al., 2024; Fahim et al., 2024). In contrast, biological synthesis methods offer an eco-friendly alternative and among these, fungal-mediated synthesis stands out as particularly efficient and robust. Fungi surpass bacterial and plant systems

in nanoparticle biosynthesis due to their high tolerance to metals, the ability to secrete large quantities of extracellular enzymes, and their superior protein-mediated reduction systems. These metabolic and enzymatic features allow for rapid, extracellular nanoparticle production with better shape control, stability, and reproducibility. Fungal AgNPs are also capped naturally by proteins and secondary metabolites, which not only stabilize the particles but also enhance their antimicrobial efficacy (Guilger-Casagrande & de Lima, 2019; Rami et al., 2024; Sidhu et al., 2025). Compared to plant-based methods, fungal systems such as *Aspergillus niger* and *Penicillium* spp. provide more consistent nanoparticle morphology, narrower size distributions, and higher synthesis yields, due to more controlled, enzyme-mediated redox reactions (Bawskar et al., 2015; Gade et al., 2008; Mathur & Pillai, 2025). These physical attributes play a critical role in influencing the antimicrobial behavior of the nanoparticles, especially against resistant bacterial strains. Beyond synthesis efficiency, fungal AgNPs exhibit strong antimicrobial potential due to their multi-targeted mechanisms. These include disruption of bacterial membranes, oxidative stress through reactive oxygen species (ROS), and interference with intracellular biomolecules such as DNA and proteins. Fungal AgNPs also often display synergistic effects when combined with conventional antibiotics, enhancing bactericidal activity and lowering resistance potential. Their ability to penetrate dense biofilms and maintain long-term colloidal stability further extends their therapeutic value (Gupta et al., 2022). In summary, fungal-mediated AgNP synthesis is not only cleaner and more scalable, but biologically superior making it a powerful platform for developing next-generation antimicrobials to combat critical priority pathogens.

The antimicrobial efficacy of silver nanoparticles (AgNPs), particularly those synthesized through fungal pathways, stems from their ability to engage multiple, simultaneous targets within bacterial cells. This multi-modal mechanism of action is especially relevant in the fight against multidrug-resistant organisms, which often evade conventional antibiotics due to their single-site specificity (Barua & Buragohain, 2024; Vanlalveni et al., 2024). A primary mechanism involves disruption of the bacterial cell membrane and cell wall, where AgNPs adhere to the cell wall, compromise its structural integrity, and increase membrane permeability. This leads to

the uncontrolled leakage of intracellular components and eventual cell lysis (Mikhailova, 2020; More et al., 2023). Simultaneously, AgNPs promote the generation of reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide. These ROS induce oxidative stress that damages cellular macromolecules including lipids, proteins, and nucleic acids ultimately leading to cellular dysfunction and death. Further, AgNPs interact with bacterial DNA, causing strand breakage, interference with replication machinery, and inhibition of transcriptional processes. These genotoxic effects are central to bacterial inactivation and have been experimentally validated in this study through nucleotide leakage assays, growth kinetics analysis, and DNA damage evaluations. Disruption of membrane function was also confirmed via morphological assessment and leakage tests, reinforcing the hypothesis of AgNP-induced cell envelope breakdown. This offers a preliminary safety profile for future clinical translation and supports the therapeutic viability of the biosynthesized nanoparticles. By targeting bacterial survival at multiple levels membrane integrity, redox homeostasis, and genomic stability these nanoparticles represent a promising strategy to overcome current limitations in antimicrobial therapy, particularly for carbapenem-resistant pathogens (Adeyemi et al., 2020; Kaweeteerawat et al., 2017).

This study aims to explore the biosynthesis of silver nanoparticles using soil-derived fungal isolates and to evaluate their antimicrobial efficacy against carbapenem-resistant strains of *Escherichia coli* and *Klebsiella pneumoniae*. Given the urgent threat posed by multidrug-resistant Gram-negative bacteria and the critical limitations of conventional antibiotics, this research focuses on developing eco-friendly, biologically synthesized nanoparticles as potential alternatives. Fungal culture was isolated from agricultural soil samples and screened for their ability to synthesize silver nanoparticles. The resulting AgNPs were then evaluated for antibacterial activity against carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae*, representing WHO-designated critical priority pathogens. Among the various biosynthesized nanoparticles, the most effective formulation was selected for mechanistic investigations to understand its mode of antibacterial action. Mechanism studies involved growth kinetics analysis, nucleotide leakage assays, DNA damage evaluation, and scanning electron microscopy to assess cell wall and membrane integrity. In

parallel, the toxicity of the selected AgNP formulation was assessed using 3T3-L1 murine fibroblast cells via MTT assay to ensure preliminary biocompatibility. Furthermore, the fungal strain responsible for producing the most potent nanoparticles was subjected to detailed morphological, physiological, and molecular characterization to confirm its identity and potential for further biofabrication work. This research contributes meaningfully to the field of antimicrobial nanotechnology by addressing two major gaps: the lack of novel, eco-sustainable synthesis methods and the need for effective therapeutics against carbapenem-resistant Enterobacteriaceae. Through the integration of microbiology, nanoscience, and toxicology, the study proposes a viable, biologically-based strategy to counteract antimicrobial resistance in high-priority bacterial pathogens.

Chapter 2

2.1 Review of Literature

2.1.1 Microbial Infections, Antibiotic Resistance, and Impact on Human Health

Microbial infections are pathogenic infections by pathogenic microorganisms such as bacteria, viruses, fungi, and parasites that enter host tissues and interfere with normal physiological processes. Such infections have been the determinants of human history, including population health, societal designs, and economic growth from ancient pandemics to the present-day outbreaks. Microbial infections continue to be a significant cause of disease burden across the globe and account for significant morbidity, mortality, and disability-adjusted life years (DALYs) lost worldwide, despite the medical innovations and breakthroughs. The most recent estimates suggest that bacterial infections alone claimed 7.7 million lives in 2019 roughly a quarter of all deaths worldwide making them the second leading cause of death worldwide (Murray et al., 2022). Viral agents like influenza viruses, HIV, and SARS-CoV-2 still lead to massive epidemics and pandemics, whereas fungal infections, caused by *Candida* spp. and *Aspergillus* spp., are responsible for high morbidity in immunocompromised patients (Roudbary et al., 2021). Malaria and schistosomiasis are endemic in most low- and middle-income countries, and malaria alone has an estimated death toll of 619,000 in 2021 (WHO, 2022). The persistent and evolving threat of microbial infections underscores their profound impact on human health and the necessity for ongoing global surveillance, prevention, and treatment strategies.

Microbial infections arise when pathogenic microorganisms successfully enter a susceptible host, evade immune defenses, and disrupt normal physiological processes. The onset of infection is influenced by the virulence of the microbe, its ability to survive in the environment, and the host's immune status (Zachary, 2017). Transmission pathways are diverse and depend on the pathogen's characteristics. Common routes include direct contact with infected individuals or contaminated surfaces, airborne spread via respiratory droplets or aerosols, ingestion of contaminated food or water, and, less frequently, vector-borne transmission by insects or animals (Tien & Earn,

2010). Certain environments, such as healthcare facilities, intensify transmission risk due to high pathogen loads and the presence of vulnerable hosts; here, contact, airborne, and fomite-mediated routes are especially significant (Husain & Meshram, 2017). The risk of acquiring infection is shaped by multiple host and environmental factors. Immunocompromised individuals, such as those undergoing chemotherapy or with chronic illnesses, are more susceptible to opportunistic pathogens. Poor sanitation, inadequate infection control measures, overcrowding, and global travel also contribute to the spread of infectious agents (Tien & Earn, 2010). The resilience of pathogens outside the host sometimes persisting for days or weeks further facilitates transmission in community and hospital settings. Collectively, these causes, routes, and risk factors underscore the need for robust surveillance systems, stringent hygiene practices, and targeted interventions to reduce the global burden of microbial diseases. Bacterial infections remain a leading cause of morbidity and mortality globally, imposing a substantial public health and economic burden. A global estimate of the burden of bacteria estimated that in 2019, bacterial pathogens were linked to 7.7 million deaths, which is almost one out of every eight of all global deaths, with them being the second most common cause of death worldwide (Murray et al., 2022). *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* represent Gram-negative organisms that play a significant role in the development of bloodstream infections, urinary tract infections, and pneumonia both in and outside a healthcare setting (Shi & Xie, 2023). Even today, gram-positive pathogens such as *S. aureus* and *S. pneumoniae* are still leading causes of skin and soft tissue infections, respiratory diseases, and sepsis, with methicillin-resistant *S. aureus* (MRSA) alone causing more than 100,000 deaths yearly (Murray et al., 2022). This burden is more disproportionately in low- and middle-income countries (LMICs) where the healthcare infrastructure is weak, and insufficient infection control measures promote the spread of the pathogen. As an example, in sub-Saharan Africa and South Asia, neonatal sepsis related to bacterial pathogens represents a large share of avoidable child deaths (“The Burden of Bacterial Antimicrobial Resistance in the WHO African Region in 2019,” 2023). The effects are further compounded by infections caused by drug-resistant strains, which result in longer hospital stays, greater intensity of care, and mortality rates (Chen et al., 2021). In addition to their direct health

effects, bacterial infections are a major cause of disability-adjusted life years (DALYs) lost, a measure of both premature mortality and long-term morbidity.

Antimicrobial resistance costs humanity in both human and economic sense, and the price is astronomical, globally. In 2019, AMR directly caused an estimated 1.27 million deaths globally and was connected to almost 4.95 million deaths worldwide, which makes it one of the causes of death, ranking higher than HIV/AIDS and malaria (Murray et al., 2022). Sub-Saharan Africa and South Asia have the highest mortality cases because they lack effective antibiotics and a prevalence of resistant pathogens. Much of this burden is linked to drug-resistant *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, and *A. baumannii* (Murray et al., 2022). The economic consequences are widespread. According to an analysis by the World Bank, the global GDP may decrease by as much as 3.8 percent by 2050, with the annual cost of healthcare increasing drastically because of longer stays in hospitals, the requirement of intensive care, and higher expenditure on second or third-line medications. Resistant infections are already causing billions of unnecessary medical expenditures in higher-income countries, and they worsen poverty cycles in low- and middle-income countries by impacting the poorest and the most economically vulnerable groups (National Academies of Sciences et al., 2021). Besides direct costs, society suffers in terms of low productivity, loss of income, and higher health disparities. The scale of this crisis has caused the World Health Organization to list AMR among the ten most important global public health threats that must be addressed through a joint effort to curb its rates (Coque et al., 2023). The implications of antimicrobial resistance (AMR) on the economy and society are much broader than the direct medical expense of treatment. Resistant infections require more costly and toxic medications, extended hospital stays, and greater dependence on intensive care, and the extra cost per patient can be between thousands of dollars and tens of thousands in high-income health care systems (Maragakis et al., 2008). On a macroeconomic scale, the cumulative loss of global output to AMR is expected to reach up to US\$100 trillion by 2050 without any action, mainly because of the decreased labor productivity, increased mortality, and higher spending on healthcare (Jasovský et al., 2016). This would compromise the security of medical procedures, including surgery, chemotherapy, and organ transplantations, which took decades to achieve

(Smith & Coast, 2012). Social impact of AMR, too, is not evenly spread, as countries of low and middle income are affected disproportionately due to the unavailability of second-line drugs and advanced medical care. This also increases inequality and poverty traps, and since chronic morbidity and early mortality decrease household income and increase health spending on catastrophic disease (Keenan et al., 2025). In addition, AMR undermines social resilience, which results in the inability to trust healthcare systems and an increased social burden on families and communities that take care of chronically ill patients (Dadgostar, 2019). In order to address this multi-dimensional crisis, there would be a need to integrate the economic modeling and social science knowledge so that the treatment is not merely medically effective, but also socially equitable and affordable (Kruk et al., 2018). Carbapenem resistance has become one of the most dangerous and acute threats to the health of the population in the context of the larger antimicrobial resistance crisis. The rise of carbapenem-producing organisms, particularly *K. pneumoniae*, *E. coli*, *A. baumannii*, and *P. aeruginosa*, has progressively undermined carbapenems, which were once viewed as last-line antibiotics to treat multidrug-resistant Gram-negative infections (Meletis, 2016). The quick spread of carbapenemase genes- many through mobile genetic elements- has seen their distribution across continents, with the highest levels in Asia, Europe, and some parts of South America (Codjoe & Donkor, 2017). Carbapenem-resistant pathogens cause infections that are most clinically relevant, as they are related to a high level of morbidity, mortality, and prolonged length of stay in the hospital, as well as limited treatment options, which makes them one of the unifying themes in AMR discussion (Elshamy & Aboshanab, 2019). Taking into account their disastrous clinical outcomes and the high rate of developing resistance, carbapenem-resistant bacteria should be addressed with special focus, which will be discussed in more detail in the subsequent section.

2.1.2. Carbapenem Antibiotics and Their Resistance

Carbapenems are a subgroup of antibiotics called β -lactam, which have an extremely wide range of antibacterial activity against Gram-positive, Gram-negative, and anaerobic pathogens (Papp-Wallace et al., 2011). They are structurally related to

penicillins and, by binding to crucial penicillin-binding proteins (PBPs), they cause the inhibition of bacterial cell wall synthesis and the subsequent death of the bacteria. They are invaluable in the management of multidrug resistance as they are unique in the fact that they are resistant to most beta-lactamases, including extended-spectrum beta-lactamases (ESBLs) (El-Gamal et al., 2017). Carbapenems evolved with the late 1970s discovery of thienamycin of *Streptomyces cattleya*, which was later developed into semi-synthetic derivatives to address the problem of instability. The first carbapenem to be available clinically was imipenem in the 1980s in combination with cilastatin to prevent inactivation by dehydropeptidase-1 in the kidney (Kahan et al., 1983; Papp-Wallace et al., 2011). Since then, other agents of meropenem, ertapenem, and doripenem have been established, but they have different pharmacokinetics and antimicrobial spectra (El-Gamal et al., 2017). In clinical practice, carbapenems are now restricted to severe, life-threatening infections, especially those caused by multidrug-resistant Gram-negative organisms. Imipenem's activity against Gram-positive organisms is slightly increased than meropenem and doripenem, which are more active against Gram-negative pathogens, including *P. aeruginosa*. Ertapenem possesses a longer half-life and is mostly administered in situations of community-acquired infections and once-daily doses; however, it is less effective against *P. aeruginosa* and *Acinetobacter* spp. (Ayub & Islam, 2015). Because of their efficiency and status as a last resort, carbapenems have been an important part of contemporary antimicrobial management, but emerging resistance currently jeopardizes their clinical potential (Nepal & Paudel, 2020).

Carbapenems are regarded as cornerstone agents in the treatment of severe, life-threatening infections caused by multidrug-resistant (MDR) bacteria. They are frequently indicated for septicemia, hospital-acquired and ventilator-associated pneumonia, complicated urinary tract infections (cUTIs), intra-abdominal infections, and febrile neutropenia in immunocompromised patients (Paterson & Depestel, 2009). In surgical and trauma patients, they serve as empirical therapy where there is a high risk of infection with extended-spectrum β -lactamase (ESBL)-producing Enterobacterales or other resistant Gram-negative pathogens (Ayub & Islam, 2015). The World Health Organization (WHO) has classified carbapenems on its Model List

of Essential Medicines as “Reserve” group agents under the AWaRe (Access, Watch, Reserve) classification, designating them for use only in confirmed or strongly suspected cases of infection with MDR organisms to preserve their effectiveness (WHO, 2023b). Their designation as “last resort” antibiotics is due to their activity against pathogens resistant to nearly all other available β -lactams, making them critical for preventing treatment failures in high-risk infections (Nepal & Paudel, 2020). However, this very reliance has contributed to escalating resistance rates, necessitating judicious use within antimicrobial stewardship frameworks to prolong their clinical utility. In the last 20 years, there has been a significant increase in the use of carbapenem antibiotics in the global setting, as the number of infections due to extended-spectrum β -lactamase (ESBL)-producing and multidrug-resistant Gram-negative bacteria has also grown. This trend has been mainly facilitated by hospital environments, where carbapenems have been used empirically in intensive care units to treat severely ill patients, where there is a risk of multi-resistant pathogens (Hays et al., 2022). Nevertheless, such an increase in utilization has been closely linked with the occurrence and spread of carbapenem-resistant organisms. Researchers have established that there is a strong relationship between the amount of carbapenem used and the resistance rates, especially in *K. pneumonia* and *A. baumannii*, highlighting the importance of overuse (Perez et al., 2010). Patterns of use also vary greatly between high-income countries (HICs) and low- and middle-income countries (LMICs). Effective antimicrobial stewardship programs moderate carbapenem consumption in a few HICs, although resistance remains an issue. By contrast, LMICs are more likely to have access to non-regulated antibiotics, diagnostic failure, and inadequate infection control measures, which leads to both inappropriate antibiotic prescribing and more likelihood of acquiring resistance (Sartelli et al., 2020). These variations indicate the criticality of globally coordinated policies on stewardship, considering local health services and pathogen epidemiology.

Carbapenem resistance is reduced susceptibility or complete inability of one or more carbapenem antibiotics to bacterial pathogens. The same can be found in critical Gram-negative organisms, especially *A. baumannii*, *P. aeruginosa*, and Enterobacterales species, including *K. pneumoniae* and *E. coli* (Codjoe & Donkor, 2017). Evidence on

worldwide surveillance indicates that the resistance to carbapenems in *A. baumannii* is usually above 50 percent in most regions, with *P. aeruginosa* having a variable resistance of 10-50 percent based on geography and the location of the hospital (Li et al., 2025). Resistance is less prevalent in Enterobacterales overall, but with alarming signs of increasing resistance, especially in *K. pneumoniae*, where the prevalence can exceed 40 per cent in some low- and middle-income countries (Asokan et al., 2025). In clinical practice, carbapenem-resistant infections have a much greater morbidity and mortality than carbapenem-sensitive infections. In severe forms, including blood infections, mortality rates may be above 50%, and the outcomes are aggravated by a delay in the start of an effective treatment (So-ngern et al., 2023). Patients usually need a long stay in the hospital and complicated multidrug therapy, which predisposes them to adverse drug reactions and secondary infection. The economic impacts are also severe. In high-income economies, long hospital stays and increased care requirements associated with carbapenem-resistant infections are expensive, increasing the cost of care by tens of thousands of dollars per patient. Further complicating health disparities, the need to resort to expensive second-line drugs further contributes to the economic pressure that is already present, as these second-line drugs are more unavailable in low- and middle-income settings (Sendekie et al., 2023). Carbapenem resistance is one of the most challenging issues of antimicrobial resistance, yet, among them, carbapenem-resistant Enterobacterales (CRE) deserve a special place because of their fast-global spread, high fatality rates, and astonishing horizontal transfer of the resistance genes to other bacterial species. This effective dispersion is mostly facilitated by plasmids that bear the genes encoding carbapenemases, including *blaKPC*, *blaNDM*, and *blaOXA-48*, which are capable of transferring in different hosts and retaining in clinical as well as environmental conditions devoid of antibiotics (Smith et al., 2025). This combination of clonal growth and horizontal transfer of genes via plasmids has allowed CRE to create profound reservoirs in hospitals and communities, spawning outbreaks that are now hard to control (Caliskan-Aydogan & Alocilja, 2023).

2.1.3 Carbapenem-Resistant Enterobacterales (CRE)

Carbapenem-resistant Enterobacterales (CRE) have been described as Gram-negative bacteria, ordered as Enterobacterales, with *E. coli*, *K. pneumoniae*, and Enterobacter species being resistant to at least one carbapenem antibiotic. CRE refers to Enterobacterales that are resistant to at least one carbapenem (imipenem, meropenem, doripenem, or ertapenem) or produce an enzyme known as carbapenemase, which can hydrolyze carbapenems (Ma et al., 2023). U.S. Centers for Disease Control and Prevention (CDC) also lists CRE as Enterobacterales that were resistant to at least 1 carbapenem or reported to produce carbapenemases, or detected via standardized antimicrobial susceptibility testing. Both agencies consider these organisms to be on the list of the so-called urgent threats because of their low treatment opportunities, mortality rates, and the possibility of quick transmission in healthcare and community contexts (CDC, 2024).

Carbapenem-resistant Enterobacterales (CRE) have increased in prevalence by up to 100-fold in the last 20 years, and there is substantial regional variation, especially between continents. According to surveillance data, the rate of CRE in *K. pneumoniae* is higher than 40 percent in the regions of Southern and Eastern Europe, but in some countries of Northern Europe, where antibiotic stewardship programs are strict, the rates are less than 5 percent. The prevalence of CRE in bloodstream infections caused by *K. pneumoniae* is between 2 to 10 percent, and this may be higher in long-term acute care hospitals and intensive care units in the United States. Unregulated antibiotic use, poor diagnostics, and insufficient infection control often contribute to a higher CRE prevalence in low- and middle-income countries (LMICs). As an illustration, in some South and Southeast Asia regions, the *K. pneumoniae* carbapenem resistance rates are above 50%, and *E. coli* rates are above 15%, and the hotspots are in India, Pakistan, and Vietnam (Jean et al., 2022). CRE prevalence in *K. pneumoniae* includes between 20 and 45 percent in Latin America, especially in Brazil and Argentina (de Araujo et al., 2025). The transmission settings are usually healthcare-related, and outbreak occurs mainly in the intensive care units, long-term care facilities, and the wards attending immunocompromised patients. Contaminated surfaces, invasive devices (central lines, ventilators, urinary catheters), and transfer of colonized patients across healthcare institutions facilitate CRE dissemination (Collins, 2008). Although community-

associated CRE is not yet very common, there have been some occasional cases, which have aroused fears of spreading it to a wider community outside the hospital settings. Carbapenem-resistant Enterobacterales (CRE) are a series of Gram-negative bacteria that have high clinical effects. The most common and worrying species among them are *K. pneumoniae*, *E. coli*, and *Enterobacter cloacae* complex, both in hospitals and in the community. *K. pneumoniae* is also constantly the most reported CRE species worldwide, most often associated with healthcare-associated infections (HAIs) like bloodstream infections, ventilator-associated pneumonia, and complicated UTIs. In U.S. surveillance, *K. pneumoniae* caused 63 percent of CRE isolates, with ST 258 as the most common and carbapenem production, especially KPC, as the major factor of resistance (Iovleva & Doi, 2017). This dominance has also been documented in China, where hypervirulent KPC-ST11 lineages have contributed to outbreaks in ICUs. *E. coli* is the second most common CRE in most regions and has both community-acquired and hospital-acquired cases. It is important to note that *E. coli* ST410 and ST167 were linked to carbapenemase, including NDM-5 and KPC-2, which indicates the high risk of spreading them worldwide (Lian et al., 2025). CRE *E. coli* are less prevalent than *K. pneumoniae* but can provide silent reservoirs of resistance genes within communities. Another critical CRE pathogen is the *E. cloacae* complex, especially in ICUs and outbreaks due to invasive devices. It accounted for 18 percent of CRE isolates in U.S. data and was often a KPC producer and sometimes an NDM producer. These three species (*K. pneumoniae*, *E. coli*, and *E. cloacae* complex) stand out in terms of their clinical and epidemiological importance within the CRE landscape as they are the dominant ones. They not only spur morbidity and mortality but also become sources of major horizontal transfer of carbapenemase genes, further increasing the AMR threat. CRE is the cause of a wide range of severe infections that are severe such as bloodstream infections (BSIs), ventilator-associated and hospital-acquired pneumonia, complicated urinary tract infections (cUTIs), and intra-abdominal infections. CRE-induced BSIs are especially hazardous, and mortality rates among hospitalized patients are frequently as high as 40-50 percent, compared to those of carbapenem-susceptible equivalents (Logan & Weinstein, 2017). CRE pneumonia, particularly among ventilated patients in the ICU, has been associated with increased adverse clinical outcomes and more extended mechanical ventilation needs. CRE-related cUTIs may

progress to pyelonephritis or bacteremia, especially in catheterized patients, and intra-abdominal infections commonly arise after surgery or following gastrointestinal perforation and are difficult to manage. The most vulnerable groups are immunocompromised, organ transplant recipients, oncology patients on chemotherapy, and neonates in intensive care. CRE infections in such groups are associated with increased mortality, reduced therapeutic possibilities, and more frequent recurrences, caused by long-term colonization. The results are further complicated by the high rate of toxic or less efficient salvage regimens, including colistin or tigecycline-based combinations, which impose further safety risks (Gao et al., 2019; Sharma et al., 2021).

Carbapenem-resistant Enterobacterales (CRE) pose a significant financial and populational burden on a global scale. The treatment of CRE infection is also much more expensive than carbapenem-susceptible infections due to the need to extend the hospital stay, expand ICU stays, and the need to use costly salvage drugs, such as polymyxins, ceftazidime-avibactam, or combination therapy (Stewart et al., 2019). CRE infections are estimated to drive more than 66,000 dollars per patient in United States healthcare costs, primarily because of extended inpatient hospitalization and ICU bed occupancy. The direct cost of treatment is not the only burden on the healthcare systems. Outbreaks in hospitals and long-term care facilities may require the shutdown of wards, the use of isolation precautions, and intense environmental cleaning at the cost of other services (Larsson et al., 2025). In LMICs, there is inadequate access to newer antibiotics, necessitating the use of older, more toxic agents, further worsening patient outcomes and furthering resistance. They have been noted as a top priority by both the World Health Organization (WHO) and the U.S. Centers for Disease Control and Prevention (CDC), and they are in great need of increased surveillance, robust infection control measures, and the quick discovery of drugs to curb the CRE issue (Nammi et al., 2025; WHO, 2023). The clinical and financial burden of CRE is likely to grow without concerted effort, and it could overwhelm health infrastructure, especially in high-prevalence areas. Lastly Table 2.1.1 have shown economic and healthcare burden of cre infections across different regions/countries.

Table 2.1.1: Economic and Healthcare Burden of CRE Infections Across Different Regions/Countries

Region/Country	Average Cost per CRE Infection (USD)	Annual Economic Burden (USD)	Mortality Rate (%)	Addition al Healthcare Costs per Infection (USD)	Societal Cost (USD)	Source
United States	\$22,484 - \$66,031	\$4.6 billion	40-50%	\$10,000 - \$20,000	\$100,000 per productivity loss	(Bartsch et al., 2017)
European Union (EU)	€40,000 - €50,000	€1.5 billion	40-50%	€15,000 - €25,000	€1.1 billion	(Y. Li et al., 2018)
United Kingdom	£40,000 - £60,000	£1.1 billion	35-45%	£15,000 - £30,000	£1 billion	(Decraene et al., 2018)
China	\$14,252 - \$30,000	\$2.5 billion	30-40%	\$7,000 - \$15,000	High economic impact	(Zhen et al., 2020)
Australia	AUD\$155,784	AUD\$500 million	20-30%	AUD\$12,000 - AUD\$18,000	Significant healthcare burden	(Rodríguez-Acevedo et al., 2020)
India	\$5,000 - \$10,000	\$1 billion (projected)	50%	50% of annual income	High economic impact	(K. Sharma et al., 2023)

Southeast Asia	Varies (High in Indonesia and the Philippines)	\$300 million (projected)	30-50%	Significant healthcare costs	Severe financial distress	(Malchio et al., 2019)
Brazil	\$8,000 - \$15,000	\$500 million (projected)	40%	Significant costs in coastal areas	Significant public health burden	(Andrade et al., 2020)

The current worldwide rise in carbapenem-resistant Enterobacterales (CRE) is a major turning point in antimicrobial resistance, as they can spread rapidly due to mobile genetic elements and plasmid-encoded carbapenemase production, which often occurs across a variety of health care and community settings. The combination of high morbidity, few treatment alternatives, and the development of strains that co-carry multiple resistance determinants, including those against last-line agents, such as colistin, further compounds the need to conduct specific research. The molecular and biochemical mechanisms that support carbapenem resistance, such as enzymatic degradation, porin alterations, and efflux systems, are vital to the development of effective diagnostics, novel therapeutics, and control measures (Bulens et al., 2023). The following part focuses on the analysis of the molecular and biochemical processes that fuel carbapenem resistance in detail and form the basis of novel counterstrategies.

2.1.4 Mechanisms of Carbapenem Resistance

Carbapenem resistance has become one of the most daunting challenges of contemporary infectious disease control that challenging the effectiveness of β -lactam antibiotics previously regarded as a backbone of the treatment of multidrug-resistant Gram-negative infections. The issue is especially severe in carbapenem-resistant Enterobacterales (CRE), including *K. pneumoniae* and *E. coli*, along with opportunistic

pathogens, including *A. baumannii* and *P. aeruginosa*. Resistance is caused by a wide range of strategies, which can be roughly classified into enzyme and non-enzymatic mechanisms, and they frequently interact to affect drug efficacy synergistically. The horizontal spread of carbapenemase genes by mobile genetic elements, especially plasmids, transposons, and integrons, is a leading cause of dissemination around the world and creates an avenue of high rates of dissemination across species and environments. In addition to classical conjugation, the gene transfer through outer membrane vesicles (OMVs) has been reported to be a highly effective mechanism of spreading of *bla*OXA and *bla*KPC carbapenemase-producing genes among clinical isolates (Aurilio et al., 2022). This allows resistance to propagate not only in healthcare facilities but also in community and environmental reservoirs, which is a complicated problem in preventing infections. With the interplay between various resistance pathways and the mobility of resistance determinants, effective containment will necessitate molecular level knowledge, as well as genomic surveillance, as will be discussed in the following subsections describing enzyme based, non-enzymatic, and horizontal gene transfer-mediated mechanisms. Mechanism of Carbapenem resistance in Enterobacteriaceae illustrate in Figure 2.1.1. Table show trends in molecular mechanism of carbapenem resistance in Enterobacteriaceae.

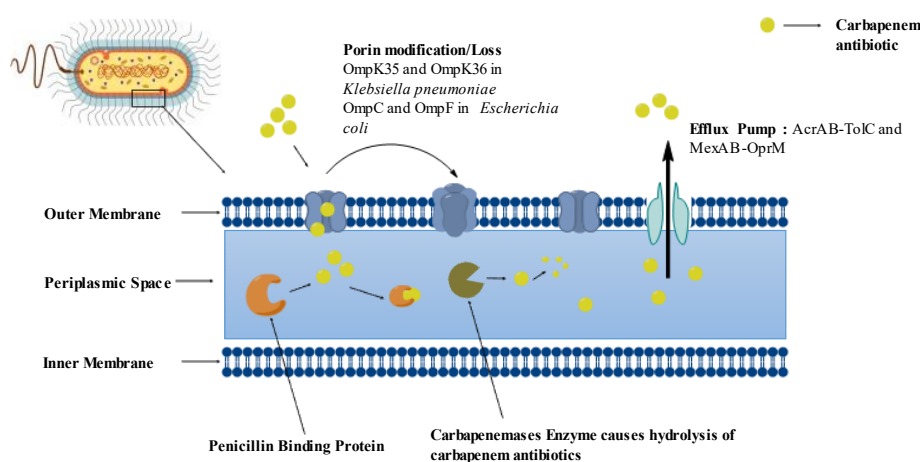


Figure 2.1.1: Mechanism of Carbapenem resistance in Enterobacteriaceae (Adapted and modified from (Aurilio et al., 2022; Ye et al., 2018) .

Table 2.1.2: Trends in Molecular Mechanism of Carbapenem Resistance in Enterobacteriaceae

Molecular Mechanism	Gene	Prevalence	Geographical Distribution	Associated Bacterial Strains	Clinical Impact	Detection Methods	Reference
Metallo- β -lactamases (MBLs)	bla _{NDM}	High in South Asia, rising globally	Widespread, with hotspots in India, Pakistan, and the Balkans	<i>E. coli</i> , <i>K. pneumoniae</i> , and other <i>Enterobacteriaceae</i>	High morbidity and mortality due to limited treatment options	PCR, sequencing, phenotypic assays	(Firoozeh et al., 2017)
Serine β -lactamases	bla _{KPC}	High in North America, parts of Europe, and Asia	Global, with prevalence in the United States, Greece, and China	<i>K. pneumoniae</i> , <i>E. coli</i>	Treatment challenges associated with hospital outbreaks	PCR, Modified Hodge test, Carbapenem inactivation test	(Cui et al., 2019)
Oxacillinases	bla _{OXA-48}	Moderate, increasing in the Middle East	Prevalent in Turkey, the Middle East, and	<i>K. pneumoniae</i> , <i>E. coli</i>	Reduced efficacy of carbapenems, complex	PCR, sequencing, MALDI-TOF MS	(Boyd et al., 2022)

		and Europe	North Africa		therapeutic management		
AmpC β -lactamas	Various bla _{AMP} C	Variable, often plasmid-mediated	Global, with variable distribution	<i>Enterobacter</i> spp., <i>Citrobacter freundii</i>	Complicated infections often require combination therapy	PCR, phenotypic assays	(Aryal et al., 2020)
ESBLs with carbapenemase activity	bla _{CTX-M} , bla _{TEM} , bla _{SHV}	Widespread, endemic in certain regions	Global, high in Asia and South America	<i>K. pneumoniae</i> , <i>E. coli</i> , and other <i>Enterobacteriaceae</i>	Increased use of last-resort antibiotics, higher healthcare costs	PCR, confirmatory phenotypic tests	(Castanheira et al., 2021)
Carbapenem-hydrolyzing Class D β -lactamas	bla _{OXA-23} , bla _{OXA-24/40}	Emerging in various regions	Sporadic global cases, clusters in Europe	<i>Acinetobacter baumannii</i> , <i>Pseudomonas aeruginosa</i>	Complicates CRE management, increasing treatment failures	PCR, sequencing	(Vranci et al., 2020)

Guiana Extended Spectrum	bla GES	Low to moderate	Latin America , Europe	<i>K. pneumoniae</i> , <i>E. coli</i>	Can confer resistance to a wide range of β - lactams	PCR, phenotypic confirmation	(Caliskan- Aydogan & Alocilja , 2023)
Imipenemase Metallo- β - lactamases	bla IMP	Moderate	Asia- Pacific, South America	Various <i>Enterobacteriaceae</i>	Broad resistance profile, challenging clinical management	PCR, phenotypic tests	(Pongchaikul & Mongkolsuk, 2022)
Verona Integron- encoded Metallo- β - lactamase	bla VIM	Moderate, increasing	Europe, Americas	<i>K. pneumoniae</i> , <i>Pseudomonas aeruginosa</i>	Requires alternative therapeutic strategies, often polymicrobial infections	PCR, sequencing	(Pelaez Bejarano et al., 2022)

Chicago β - lactamas e	bla CM _y	Low	Focused on certain urban areas	<i>E. coli</i> , <i>Salmonella</i> <i>spp.</i>	Variable impact, often associat ed with commu nity- acquired infectio ns	PCR, isoelect ric focusin g	(Bush & Bradfor d, 2020; Husna et al., 2023)
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2.1.4.1 Carbapenemase enzymes

The most prevalent and dangerous resistance mechanism is the production of carbapenemase enzymes, because these β -lactamases cleave carbapenems by destroying the β -lactam ring necessary to exert their antimicrobial properties. According to the Ambler classification, carbapenemases are divided into three broad classes. Plasmid-mediated serine carbapenemases, including *K. pneumoniae* carbapenemase (KPC), have caused endemic outbreaks in the United States, Greece, and Italy with high morbidity and mortality. Class B metallo-beta-lactamases (MBLs), such as NDM, VIM, and IMP, are zinc-dependent and do not respond to almost any other beta-lactam except monobactams. NDM-1 has spread very fast in India, Pakistan, and Sri Lanka, and the rest of the world has detected it in Europe, North America, and Africa. Class D Oxacillinase-like carbapenemases (OXA-48-like enzymes) show low-level carbapenemase activity and frequently are associated with porin mutations and efflux systems, resulting in high-level resistance. Such enzymes are especially common in Turkey, North Africa, and the Middle East, which facilitates local outbreaks. Infections with carbapenem-resistant Enterobacteriaceae (CRE) are increasing globally, with geographic differences in the prevalent resistance mechanisms. *K. pneumoniae* that produces KPC is the leading cause of hospital outbreaks in the United States and South America. The epidemic outbreaks of OXA-48-producing *K.*

pneumoniae have occurred in France, Germany, and Greece in Europe. NDM-1-producing CRE is common in Asia and the Middle East, where it is rapidly spread by medical tourism and international travel. In the meantime, the new research in Africa shows that the prevalence is on the rise especially in Nigeria and South Africa where not only OXA-48 but also NDM-1 strain has been identified (Aurilio et al., 2022; Hammoudi Halat & Ayoub Moubareck, 2020; Meletis, 2016).

2.4.1.2 Non-Enzymatic Mechanisms of Carbapenem Resistance

Although production of carbapenemase is the most predominant mechanism of resistance in Enterobacteriaceae, non-enzymatic mechanisms are also important in imparting carbapenem resistance (Figure 2.1.2). These mechanisms are over expression of efflux pumps and also porin mutations, which are known to reduce intracellular levels of the antibiotics and enhance carbapenem resistance. In many cases, these mechanisms synergise with carbapenemases and make treatment strategies even more complicated (Suay-García & Pérez-Gracia, 2019). Efflux pumps are transport systems embedded in the bacterial membranes that expel antibiotic molecules out of the bacterial cell, decreasing the concentration of these molecules and preventing the efficacy of carbapenems. They are resistance-nodulation-cell division (RND) family pumps and have been linked to multidrug resistance (MDR) phenotypes in *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*. The AcrAB-TolC efflux pump system is the most characterized in Enterobacteriaceae, and it contributes significantly to carbapenem resistance. AcrAB-TolC overexpression contributes to reduced carbapenem, cephalosporin, and fluoroquinolone susceptibility, which is one of the most important non-enzymatic resistance mechanisms. *P. aeruginosa* possesses MexAB-OprM and MexXY-OprM efflux systems, which add to extensive drug resistance (XDR) phenotypes and further complicate the available treatment options. The latest genomic analysis has supported that mutations in efflux pump regulatory genes play a role in conferring high-level carbapenem resistance in *K. pneumoniae*. Decreased susceptibility to imipenem and meropenem has been associated with a frameshift mutation in the CusF efflux system and an insertion mutation in the nickel/cobalt efflux protein RcnA (Huang et al., 2022).

In addition to carbapenem resistance regulated by porin mutations, the permeability of the membrane becomes lower, and carbapenems cannot enter the bacteria effectively. The loss or mutation of outer membrane porins OmpK35 and OmpK36 is highly linked with carbapenem resistance at high levels, especially in strains that co-express OXA-48 carbapenemase in *K. pneumoniae*. A report on the carbapenem-resistant *P. aeruginosa* isolates in the ICU identified that the loss of porin in OprD and overexpression of MexXY efflux pumps were the most common mechanisms of imipenem and meropenem resistance. In *A. baumannii*, reduced expression of carO, omp33-36 and oprD porins were seen in 75%, 66% and 72.3% of carbapenem-resistant isolates, respectively. Porin mutation is frequently observed with carbapenemase production in clinical settings, increasing resistance phenotype. Lack of OmpK36 in carbapenem-resistant *K. pneumoniae* isolates has been associated with high levels of resistance to imipenem (MIC 16 mg/L and meropenem (MIC 4 mg/L) (Kong et al., 2018; Wong et al., 2019).

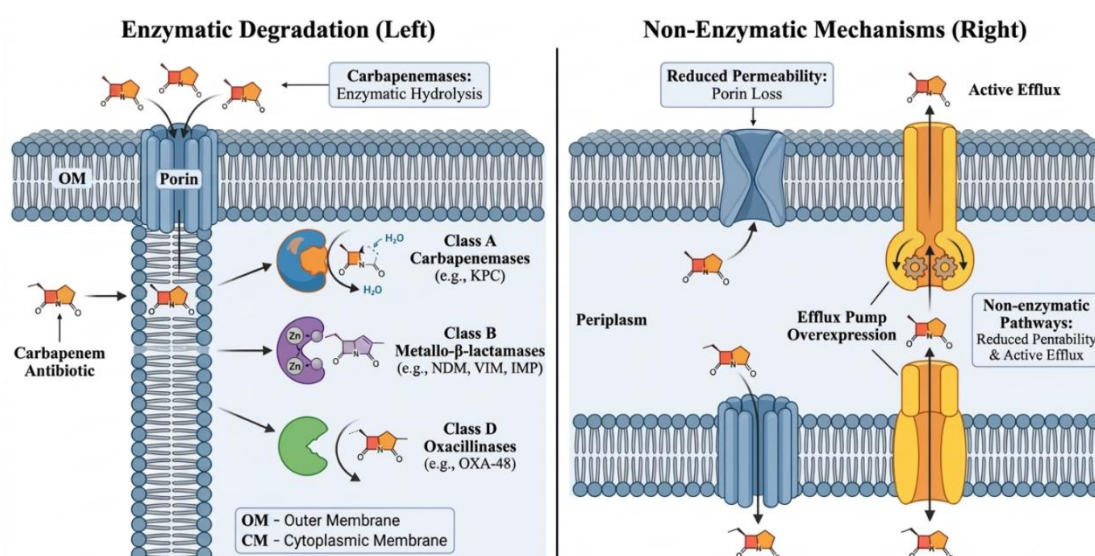


Figure 2.1.2: Mechanisms of carbapenem resistance in Gram-negative bacteria. Resistance may occur via enzymatic hydrolysis of carbapenems by carbapenemases or through non-enzymatic pathways involving reduced permeability and active efflux. *Left:* Enzymatic degradation by periplasmic carbapenemases Class A (KPC), Class B (NDM, VIM, IMP metallo- β -lactamases), and Class D (OXA-48 oxacillinases). *Right:* Non-enzymatic mechanisms include porin loss reducing drug entry and efflux pump

overexpression expelling antibiotics. (OM – outer membrane; CM – cytoplasmic membrane.)

2.4.1.3. Horizontal Gene Transfer (HGT) and Global Dissemination of Carbapenem Resistance

Horizontal gene transfer (HGT) is the major mechanism that promotes the spread of carbapenem resistance worldwide, as the resistance genes can quickly spread across bacterial populations. The most common carbapenemase genes, including KPC, NDM, OXA-48, IMP, and VIM, are typically plasmid-encoded, transposon-encoded, or integron-encoded, which allows the bacteria to become resistant due to conjugation, transformation, or transduction (P 019 triz). One of the main challenges is the fact that the carbapenem-resistant *E. coli* and *K. pneumoniae* can remain intact in the hospital setting, as they can be found on equipment, surfaces, and even in the wastewater. Such environmental resistance has led to the worldwide spread of NDM-1-producing CRE, especially in India, Pakistan, and the Middle East, where the spread of resistance through travel in healthcare has been a significant factor (Hardiman et al., 2016). In a genomic study of carbapenem-resistant *P. aeruginosa*, including a large number of isolates, investigators discovered that 95.6 percent of strains possessed chromosomal mutations implicating carbapenem resistance, whereas only <1 percent of strains possessed carbapenemase genes. The study emphasized the reduced OprD expression and overexpression of MexAB-OprM efflux pumps as the most significant mechanisms of resistance, which further highlighted the significance of the non-enzymatic resistance pathway (Esposito et al., 2021). The emergence of carbapenem resistance in *E. coli*, *K. pneumoniae*, and other Gram-negative pathogens has become one of the most pressing global health concerns and greatly undermines the efficacy of last-resort antibiotics. The mechanisms of resistance include carbapenemase production, overproduction of efflux pumps, mutations in porin channels, and horizontal gene transfer (HGT), which are the ways of rapid spread of carbapenem-resistant Enterobacteriaceae (CRE). These multi-resistant mechanisms have made most clinical infections incurable, with high morbidity and mortality rates across the globe. Although production of carbapenemases is still considered the most predominant resistance

mechanism, non-enzyme-based mechanisms (efflux pumps overexpression and mutations in porins) are also significant in increasing resistance phenotypes (Arnold et al., 2011; Mancuso et al., 2023).

Research has shown that most strains of CRE possess several resistance mechanisms at the same time, hence leaving them almost incurable by the use of traditional antibiotics. Moreover, horizontal gene transfer (HGT) has contributed to the worldwide spread of resistance genes, and it is because of this that these bacteria are multiplying in healthcare facilities and within the community at an alarming rate. Because of the few treatment alternatives available and the emergence of carbapenem resistance, new approaches to antimicrobial therapy are urgently required. The development of nanotechnology-based treatments, especially silver nanoparticles (AgNPs), has demonstrated positive outcomes in the fight against multidrug-resistant (MDR) and carbapenem-resistant bacteria. As opposed to conventional antibiotics, AgNPs have broad-spectrum antibacterial properties, which they achieve by disrupting bacterial membranes, producing reactive oxygen species (ROS), and targeting other important enzymatic pathways. These peculiar modes of action render AgNPs a very promising prospect in the solution of the rising problem of antibiotic resistance.

2.1.5. Silver and the Evolution of Silver Nanoparticles

Silver has a long history of prominence in medicine due to its wide-spectrum antimicrobial activity. Societies of ancient times, such as the Greeks, Romans, and Egyptians, stored drinking water in silver vessels, put silver coins into milk to avoid spoilage, and put silver plates on wounds to minimize the possibilities of infection (Sim et al., 2018). By the 19th century, silver nitrate was already applied in ophthalmology, where it was used to prevent neonatal conjunctivitis, whereas silver preparations in colloidal form were used in the treatment of wounds and burns. The advent of silver sulfadiazine in the middle of the 20th century offered a stable topical antimicrobial burn therapy, due to its long-lasting antimicrobial effect (Nishida & Risemberg, 1975). Even though the antibiotic revolution during the 20th century created a shift in focus to other treatments like silver-based treatments, the emergence of antimicrobial resistance (AMR) globally has once again given the attention back to silver, especially silver

nanoparticles (AgNPs), which is more potent, stable, and bioavailable than bulk silver. With the advent of nanotechnology, silver can now be manufactured at the nanoscale (1100 nm), which has substantively enhanced its antimicrobial potential. AgNPs with small size and large surface-area-to-volume ratio have a better potential to bind and enter microbial membranes, inhibit metabolic reactions, produce reactive oxygen species, and release silver ions (Ag^+) in a controlled fashion (Casals et al., 2025). They are also further modified by adding biocompatible polymers or biomolecules that increase their stability and dispersibility and target bacterial targets. These attributes have made them find use in wound dressing, surgical instrument coating, catheters, and implant surfaces, and as a vehicle to carry antimicrobial, anticancer, and anti-inflammatory compounds (Keskin et al., 2021; Liang et al., 2022).

AgNPs have shown exceptional activity against enterobacterales with carbapenem resistance (CRE) and other multidrug-resistant (MDR) pathogens in the context of the growing AMR. *Fusarium oxysporum*-mediated synthesis of biogenic Ag/AgCl nanoparticles has demonstrated strong inhibitory effects on KPC-producing and ESBL-positive *K. pneumoniae*, and *E. cloacae*, with synergy seen against these pathogens with imipenem (Khalifa et al., 2025; Wan et al., 2016). AgNPs synthesized by green methods have been able to increase the sensitivity of *E. coli*, *K. pneumoniae*, and *Acinetobacter baumannii* to cefixime and colistin, with fractional inhibitory concentration index values ranging between 0.25 and 0.49, which is strong synergy. Moreover, the conjugation of AgNPs with colistin or meropenem has lowered minimum inhibitory concentrations up to 1024-fold against MDR *E. coli* and *K. pneumoniae* and is highly effective in biofilm clearance. These results demonstrate the promise of AgNPs alone and in combination agents as an effective means of countering infections addressable using current antibiotics. Surface plasmon resonance (SPR) is one of the most significant physicochemical characteristics of AgNPs since it is an optical effect induced by the collective oscillation of conduction electrons when illuminated by light. This results in intense absorption and scattering at 400-450 nm, which can be measured with uv-vis spectroscopy and is directly related to the size, shape, and dispersion of nanoparticles. Their nanoscale size and the large surface-area-to-volume ratio allow close contact with bacterial cell walls, improving the cytotoxicity

and making it easier to penetrate into microbial cells (Abdelrazik et al., 2023; Das et al., 2020).

Structurally, AgNPs prefer a face-centered cubic (FCC) lattice structure, which leads to enhanced stability, conductivity, biocompatibility, and the organic or polymeric layer to avoid aggregation and to enhance target specificity. FTIR, TEM, SEM, XRD, and dynamic light scattering (DLS) are the essential characterization techniques to prove the formation of particles, determine the surface functional groups, morphology, and the crystal structure, as well as to evaluate the colloidal stability. Optimizing these physicochemical parameters is essential in converting AgNP technology into clinically safe and effective practice. Silver, used in ancient medicine to the most modern nanomedicine, is a piece of history itself as it takes a massive step in antimicrobial creativity in nanoparticulate form. AgNPs show potency against MDR and CRE pathogens, offer synergistic effects with last-line antibiotics, and have tunable properties that can improve safety and efficacy, making them an important part of future infection control solutions in the age of antimicrobial resistance (Hasan et al., 2022; P. K et al., 2023; Singh & Mijakovic, 2022).

2.1.6 Characterization of Silver Nanoparticles

Accurate characterization of silver nanoparticles (AgNPs) is critical in defining their morphology, size distribution, surface chemistry, crystallinity and stability, which directly affects biological activity and safety. The formation and functional properties of biosynthesized AgNPs, such as those produced in fungal systems, are normally assessed by a set of complementary methods to ascertain their existence. UV-Vis spectroscopy has been frequently used to identify surface plasmon resonance (SPR) effect, which is a characteristic of nanoscale silver. The absorption peaks of synthesized AgNPs are usually 410-450 nm and agree with previous studies of fungal, bacterial, and plant-mediated synthesis. Fourier-transform infrared spectroscopy (FTIR) determines biomolecules that induce reduction and stabilization. The peaks of amine, hydroxyl, and carbonyl functional groups indicate the presence of proteins, enzymes, and phenolic compounds on the surface of the nanoparticles, which cap them. Such biocompatible surfaces increase the colloidal stability and biocompatibility. Electron

microscopy techniques such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM) have the potential to provide the direct imaging of morphology and size distribution of the particles. Biosynthesized AgNPs are commonly spherical or quasi-spherical in shape with a size range normally 10-50 nm in fungal systems and even smaller in some yeast-based syntheses. X-ray diffraction (XRD) indicates crystalline structure, with Bragg reflections observed at 2 theta values indicating (111), (200), (220), and (311) layers corresponding to a face-centered cubic (FCC) lattice. This crystallinity enhances the release of silver ions which increases antimicrobial potency. The hydrodynamic size and the zeta potential that indicates the colloidal stability are measured by Dynamic Light Scattering (DLS). Values of between -20 and -30 mV indicate moderate stability, which is applicable to bio-applications. The use of this multi-technique characterization platform guarantees that not only are silver nanoparticles well-characterized in terms of structure, but that they are optimized with regard to stability, safety and functional performance in antimicrobial and biomedical applications (Dhaka et al., 2023; Siddiqui et al., 2023; Zhang et al., 2016).

2.1.7 Synthesis of Silver Nanoparticles

The approach of synthesis of silver nanoparticles (AgNPs) determines the size, shape, stability, surface chemistry and antimicrobial activity decisively. These physicochemical properties have a direct effect on important functional properties, including surface plasmon resonance activity, ion release rates, and interaction with microbial membranes, which determine the therapeutic potential and safety profile. Differing synthesis pathway, physical, chemical, or biological, influences the nucleation and growth process such that nanoparticles with different morphologies are obtained, among others, nanospheres, nanorods, nanoprisms, and decahedrons (Bamal et al., 2021; Sharma et al., 2022). Particle size distribution and aggregation behaviour can be significantly impacted by even small adjustments in precursor type, reducing agent, stabilizer, reaction temperature, or pH ultimately affecting antimicrobial efficacy. As an example, nanoparticles of smaller diameter and better-defined morphology tend to have greater bactericidal effect because of a larger surface area and a more effective production of reactive oxygen species (Ajitha et al., 2015). The

selection of capping agents (synthetic polymers to biomolecules) also influences colloidal stability, biocompatibility and shelf life. With the current urgent issue of antimicrobial resistance, synthesis control is not only an academic exercise, but a precondition to the design of AgNPs which are consistently targeted, and ideally safe clinically (Javed et al., 2022).

The methodology of synthesis of silver nanoparticles (AgNPs) is key in the aspects of their size, shape, stability and eventually their antimicrobial efficacy. The differences in the synthesis parameters that include temperature, precursor concentration, type of reducing agent, stabilizers and energy source can have a profound effect on the nucleation and growth mechanisms, thus changing the physicochemical characteristics and biological interactions of the nanoparticles (Xu et al., 2020). Generally, the production of AgNP can be divided into physical, chemical and biological methods, and all of them have their advantages and disadvantages. In physical synthesis methods, the top-down approach is used where bulk silver is physically broken down into nanoscale particles by means of mechanical, thermal, or irradiation-based techniques. Evaporation-condensation, e.g., gives high-purity nanoparticles in an inert atmosphere, but it consumes large amounts of energy and is not readily scalable. Laser ablation has the advantage of producing colloiddally stable AgNPs in liquids without contamination by chemicals, although the method is limited by the high cost of equipment and low yields. Ball milling can be scalable and solvent-free, especially when combined with green reducing agents, but it is not yet possible to control the size precisely. Microwave-assisted synthesis is characterized by fast heating and fast reaction processes that lead to even nanoparticles when the parameters are optimized. Although physical methods provide products of high purity and reproducibility, they require high energy consumption, costly instrumentation and mass production constraints (Iravani et al., 2014; Nguyen et al., 2023). Chemical synthesis follows a bottom-up approach where silver ions are reduced to metallic nanoparticles with a chemical agent in the presence of stabilizers. The most common methods include chemical reduction using reagents like sodium borohydride, trisodium citrate, or hydrazine, which enables precise control of the size and shape of nanoparticles, but results in the production of toxic by-products (Gudikandula & Maringanti, 2016).

Microemulsion synthesis involves nanoscale reactors (surfactants) to create highly similar particles, but the removal of surfactants may be difficult and this may constrain biomedical uses. A cleaner and more environmentally benign alternative, but which needs photoactive conditions to be efficient, is photochemical synthesis, which uses UV or visible light to trigger the reduction. Electrochemical synthesis is capable of generating very pure AgNPs with controllable properties, yet requires careful control of the reaction conditions, including voltage, current density, and electrolyte composition (Tojo et al., 2010). In most cases, chemical methods are appreciated due to their reproducibility and standardization, but reactions involving toxic reagents and purification requirements confer to the environment and safety concerns. The selected synthesis path, whether it is physical or chemical, determines the yield and reproducibility of AgNPs, as well as the functional properties of AgNPs in antimicrobial and biomedical applications (Pechyen et al., 2024). This renders the process of choosing a method to be a decisive process in designing nanoparticles with the aim of targeted clinical or environmental application. Green or biological synthesis of silver nanoparticles (AgNPs) has become a possible green alternative to the commonly used physical and chemical approaches to the synthesis of silver nanoparticles, utilising naturally available reducing and capping agents, derived through plants, bacteria, fungi, and algae. In contrast to conventional synthesis methods, where potentially dangerous chemicals are frequently used and energy requirements are significant, green synthesis can be conducted under gentle conditions, with no negative environmental impact, and produces biocompatible nanoparticles that can be used in biomedical products (Eker et al., 2025). Its mode of action is usually that bioactive compounds, including flavonoids, terpenoids, phenolic acids, proteins, and polysaccharides, can serve as electron donors to convert Ag^2 to elemental silver and stabilizers to ensure the silver is not aggregated. Plants are the most popular biological source based on the phytochemical diversity that allows rapid synthesis without aseptic maintenance of the culture (Stozhko et al., 2024). Although slower, bacterial and fungal systems provide more regulated particle size and shape, and fungi, especially, can secrete large amounts of extracellular enzymes that can be used to stabilize nanoparticles. Algal-based synthesis, which is less studied, is an unexplored path, considering the potential of algae to grow in a wide variety of aquatic conditions and

produce nanoparticles even in saline conditions. Green synthesis has obvious benefits: it is cheap, scalable, and results in AgNPs with improved biocompatibility and decreased cytotoxicity. Nevertheless, there are still limitations, such as a lower reaction rate than chemical methods, the size and shape of the particles are inconsistent as the biomolecule composition varies seasonally or across sources, and it is difficult to standardize production protocols in an industrial setting (Adeleke et al., 2024; Kulkarni et al., 2023). These limitations notwithstanding, the methodology is well aligned with the concepts of green chemistry and has shown broad-spectrum antimicrobial, antifungal and even anticancer activity, making it a pillar in the development of next-generation nanomedicines. Fungal-mediated synthesis is fast emerging as an outstanding green method, among the various biological alternatives, because it yields high amounts of silver nanoparticles, allows easy extracellular secretion of the nanoparticles, forming more stable and homogenous nanoparticles (Ratan et al., 2020; Thakur et al., 2025). Further complemented by the scalability of fungi to culture and their extensive set of enzymes, fungal systems are a plausible platform to produce sustainable nanomaterials, a discussion of which will be covered in the following section.

2.1.8. Fungal-Mediated Silver Nanoparticle Synthesis

Fungi have become one of the most promising biological systems to produce silver nanoparticles (AgNPs) with distinct scalability, yield and stability advantages. They are ideally located to be used in large-scale production of eco-friendly nanoparticles, due to their capacity to generate high biomass and secrete a wide range of extracellular enzymes, proteins and secondary metabolites. During fungal-mediated synthesis, silver ions (Ag^+) are reduced to elemental silver (Ag^0), mainly by enzymatic and metabolite reduction, whereas fungal proteins, fungal polysaccharides, and other biomolecules are used as capping and stabilizing agents to prevent agglomeration and increase colloidal stability (Ali et al., 2025; Guilger-Casagrande & Lima, 2019). This biosynthetic efficiency and natural stabilization impart to AgNPs desirable size homogeneity, morphological control and long-term dispersion stability. Fungal synthesis may be either extracellular or intracellular. Industrial and biomedical applications typically

prefer the extracellular route, whereby cell-free filtrates of fungal cultures are used, because it is simple, nanoparticle recovery is easily achieved, and downstream processing needs are less. Under such process, silver ions in the reaction medium come in contact with fungal-produced reductases and secondary metabolites, which trigger nucleation and growth into nanoparticles without the necessity of breaking up the fungal biomass (Hosny et al., 2025; Sarsar et al., 2016). Conversely, intracellular synthesis is done in the fungal cell wall or cytoplasm and results in high purity nanoparticles but with further processing being necessary to extract the nanoparticles. The different fungi employed in synthesis of AgNPs have been effective in different modifications of particle size, morphology, and stability (Singh et al., 2023). The popular species *Fusarium oxysporum* yields mainly spherical AgNPs with a size range of 3 to 25 nm and antifungal activity against pathogenic *Candida* and *Cryptococcus*. *Penicillium chrysogenum* and *Fusarium chlamydosporum* produce highly monodisperse AgNPs with a size of 6-26 nm, their stabilization by proteinaceous coatings leading to enhanced dispersibility and biocompatibility, and robust antimicrobial and antimycotoxin properties (Ishida et al., 2014). *Tricoderma harzianum* synthesizes extremely small nanoparticles (7.8 nm on average) with high stability and great phytopathogenic fungi inhibition, including *Fusarium oxysporum* and *Alternaria alternata* (Ahmad et al., 2024). Similarly, *Aspergillus niger* synthesizes AgNPs which are found in the size range of 80-100 nm that exhibit high antifungal activity against *Penicillium citrinum*, *Rhizopus stolonifer*, and *F. oxysporum* (Hashem et al., 2022).

These cases illustrate how fungi can be effective bio factories to produce AgNPs, but their production depends on the metabolic profile and secretion pattern of each species, with potential effects on nanoparticle size distribution, morphology, and functional surface chemistry. The ability to perform large-scale synthesis extracellularly, coupled with the inherent synthesis of capping agents creates a unique situation to make fungal-mediated AgNP synthesis a high yield sustainable process that is consistent with the principles of green nanotechnology. Not only does this biological route decrease dependence on toxic chemicals, but it also yields biocompatible nanoparticles that are more stable than commercial nanoparticles, further making them extremely appealing to clinical, agricultural, and environmental use. The fungal

mediated synthesis of silver nanoparticles (AgNPs) is quite sensitive to a number of physicochemical and biological factors and all of these factors can majorly affect the yield, morphology, stability and antimicrobial activity of the particles. Out of these, the pH is one of the most important factors, which influences not only the ionization of fungal metabolites, but also the rate of nucleation of silver atoms. It has been demonstrated that slightly alkaline conditions, usually with a pH of about 8.9, are better at encouraging the synthesis of smaller, more homogeneous AgNPs because the nitrate reductase and other reducing enzymes are more active in alkaline conditions, as opposed to acidic conditions which produce larger and less monodisperse particles (Abdel-Hadi et al., 2023). Temperature is also an important factor not only in enhancing the reduction kinetics but also in particle size control; e.g., *F. oxysporum* and *A. terreus* generate nanoparticles of optimal sizes at moderate temperatures of 28 to 40 °C, with higher temperatures occasionally resulting in aggregation or morphological defects. The amount of silver nitrate (AgNO_3) determines the number of nucleation sites directly and, thus, the AgNPs size distribution. Too low concentrations of precursors tend to leave reduction incomplete, whereas too high concentrations of precursors may exceed the capping abilities of fungal biomolecules, resulting in the clumping of particles (Nasr Azadani et al., 2024). The duration of the incubation is also significant because long incubation can lead to further nucleation and growth that could potentially yield better crystallinity or result in excessive particle enlargement unless vigilantly controlled. Another determinant is the physiological stage of fungal growth, in which early stationary phase cultures are usually found to have the most enzyme activity and metabolite secretion to allow efficient Ag⁺ reduction. Fungi are a better choice in the AgNP biosynthesis as compared to other biological sources like plants, bacteria and algae. They tend to yield more nanoparticles due to their capacity to massively synthesize biomass under low-cost culture environments, their ability to secrete a vast array of extracellular enzymes and metabolites, and inefficient metal reduction, and the production of capping agents that offer an enhanced colloidal stability. In addition, the downstream purification is easier in the case of extracellular synthesis pathway typical of fungi, which allows simpler scaling and commercialization of the process. Similarly, AgNPs produced by *T. harzianum* also demonstrated a significant reduction in carbapenem resistance in *K. pneumoniae* clinical isolates, reducing minimum inhibitory

concentrations (MICs) of meropenem and colistin, and increasing bactericidal effects (Mohamed et al., 2023). In addition to CRE, a number of fungal species have produced broad-spectrum activity AgNPs against MDR pathogens. AgNPs produced by *A. terreus* showed potent bactericidal activity against MDR *E. coli* and *K. pneumoniae* with total growth inhibition at 25 ug/mL and below (González et al., 2023). AgNPs synthesized by *P. oxalicum* have been demonstrated to have an impact on clinical MDR *E. coli* and *P. aeruginosa*, which break down biofilms and reduce bacterial density in the test tube (Liao et al., 2019). AgNPs extracted by marine microbe (*Penicillium simplicissimum*) demonstrated high stability and can be used to counter MDR *K. pneumoniae*, suggesting that it can be used to control waterborne pathogens (Shaligram et al., 2009). Together, these results demonstrate that fungal-derived AgNPs can potentially target MDR pathogens and provide a practical strategy to treat CRE infections by their direct antimicrobial effect and synergistic interaction with last-line antibiotics. Their ability to kill even the most resistant strains makes them an indispensable component of future antimicrobial plans especially in light of the growing antibiotic resistance. Lastly Table 2.1.3 show comprehensive overview of fungal-mediated silver nanoparticle (AgNPs) and their antimicrobial applications.

Table 2.1.3: Comprehensive Overview of Fungal-Mediated Silver Nanoparticle (AgNP) and their Antimicrobial Applications

Fungi Used	Size & Shape	Synthesis Condition	Target Bacteria	Reference
<i>Aspergillus niger</i> & <i>A. terreus</i>	Spherical nanoparticles were (20-50 nm), Rod Shaped (5-10 nm)	Reduction of AgNO ₃	<i>E. coli</i> , <i>Proteus vulgaris</i> , <i>Pseudomonas</i> spp., <i>Salmonella paratyphi-A</i> , <i>S. aureus</i>	(Masurkar et al., 2024)
<i>Talaromyces purpureogenus</i>	30–60 nm, spherical	Endophytic, extracellular	<i>L. monocytogenes</i>	(Sharma et al., 2022)

			, <i>E. coli</i> , <i>S. dysenteriae</i> , <i>S. typhi</i>	
<i>Aspergillus melleus</i> SSS-10	87.3 nm, irregular	Extracellular, color change in 24 h	<i>S. aureus</i> , <i>E. coli</i>	(Skanda et al., 2022)
<i>Alternaria alternata</i>	66–88.6 nm, spherical	Endophytic, 24 h incubation	<i>B. cereus</i> , <i>K. pneumoniae</i> , <i>P. aeruginosa</i> , <i>E. coli</i> , <i>S. aureus</i> , <i>P. mirabilis</i>	(M et al., 2022)
<i>Penicillium oxalicum</i>	60-80 nm, Spherical	Fungal metabolites, extracellular	<i>S. aureus</i> , <i>Shigella dysenteriae</i> , <i>S. typhi</i>	(Feroze et al., 2020)
<i>Alternaria alternata</i>	100 nm, Spherical	Endophytic, extracellular	<i>Staphylococcus aureus</i> , <i>Pseudomonas syringae</i> , <i>Bacillus subtilis</i> , <i>Knoellia sinensis</i> , <i>Pseudomonas aeruginosa</i> , and <i>Escherichia coli</i> ,	(Pooja et al., 2020)
<i>Gynura procumbens</i> + <i>fungal chitosan</i>	<100 nm, spherical	Encapsulated, phytogenic	<i>B. cereus</i> , <i>S. aureus</i> , <i>L. monocytogenes</i>	(Sathiyaseela n et al., 2020)

			, <i>E. coli</i> , <i>S. enterica</i>	
<i>Endophytic fungus (Tinospora cordifolia)</i>	25–35 nm, spherical	Extracellular	<i>Escherichia coli</i> , <i>Klebsiella pneumonia</i> , and <i>P. aeruginosa</i> , and <i>Staphylococcus aureus</i>	(Bagur et al., 2022)
<i>Penicillium polonicum</i>	10–15 nm	Extracellular, optimized pH/light	MDR <i>Acinetobacter baumannii</i>	(Neethu et al., 2018)
<i>Phoma exigua</i> var. <i>exigua</i> (MTTC 2321)	22 nm, Spherical	Encapsulated biomass, batch synthesis	<i>Escherichia coli</i> (ATCC 14948) and <i>Staphylococcus aureus</i> (ATCC 33591)	(Shende et al., 2017)
<i>Schizophyllum radiatum</i>	10–40 nm, various shapes	Extracellular	<i>S. aureus</i> , <i>B. subtilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i>	(Gudikandula et al., 2015)
<i>Fusarium oxysporum</i>	10–40 nm, spherical	1 mM AgNO ₃ , extracellular	<i>E. coli</i> , <i>S. aureus</i>	(Bawskar et al., 2015)
<i>Helminthosporium tetramera</i>	17–33 nm, spherical	Cell-free filtrate, 1 mM AgNO ₃	<i>Bacillus subtilis</i> , <i>Staphylococcus</i> sp.	(Shelar & Chavan, 2014)

<i>Penicillium</i> sp. (soil)	20-60 nm, Roughly spherical	1 mM AgNO ₃ , biomass exposure	<i>S. aureus</i> , <i>B. subtilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i>	(Swathi et al., 2014)
<i>Neurospora intermedia</i> (PTCC 5291)	30 nm, Spherical	Cell-free filtrate, varied ratios, light/dark	<i>E. coli</i> BL21	(Hamedi et al., 2014)
<i>Beauveria bassiana</i>	10–50 nm, mixed shapes	pH 6.0, 25 °C	<i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	(Tyagi et al., 2019)
<i>Pestalotia</i> sp.	10–40 nm, spherical	Endophytic, extracellular	<i>S. aureus</i> , <i>S. typhi</i>	(Raheman et al., 2011)

2.1.9 Mechanism of Action of Silver Nanoparticles Against Bacteria

AgNPs have strong antimicrobial action, and this may be attributed to their various, interconnected bactericidal activities which can act simultaneously on bacterial cell envelopes, metabolism, oxidative stress responses and quorum sensing (QS) networks. In contrast to traditional antibiotics, which usually target a single molecular mechanism, AgNPs utilize a complex mechanism of action (Figure 2.1.3), and the experience of resistance is therefore much less probable. The nanoparticles have demonstrated robust and profound antibacterial efficacy against multidrug-resistant (MDR) and carbapenem-resistant Enterobacterales (CRE) such as *E. coli* and *K. pneumoniae* (Joshi et al., 2020). Direct membrane and cell wall damage is usually the initial process in AgNP antimicrobial activity. AgNPs interact with bacterial surface proteins, peptidoglycans, and in Gram-negative bacteria, lipopolysaccharides (LPS), destabilizing the cell envelope and raising membrane permeability. In *E. coli*, AgNPs use the outer membrane porins like OmpF and OmpC to enter them; deletion mutant lacking these porin has a 4-8-fold increase in resistance, indicating the relevance of porin-mediated uptake of AgNPs (Ramalingam et al., 2016). The exposure to AgNPs has been found to cause cell shrinkage, blebbing of the membrane, and the extracellular

release of the cytoplasmic contents in CRE and MDR strains as observed by scanning electron microscopy (SEM) studies. After being taken up, AgNPs induce oxidative stress, which is a key element of their bactericidal action. They cause production of reactive oxygen species (ROS) such as hydroxyl radicals ($\bullet\text{OH}$), superoxide anions, and hydrogen peroxide (H_2O_2) that cause massive oxidative destruction of bacterial DNA, proteins, and membrane lipids. This causes an imbalance of the membrane potential and ionic gradients which interferes with the homeostasis of the cell. Bacterial mutants sensitive to oxidative stress (e.g., *mutY*, *mutM*, *mutT*) are more prone to the effects of AgNPs, which further indicates the role of ROS in the destruction of bacteria. AgNPs also interfere with iron homeostasis by preventing iron-binding proteins, which affect respiration and ATP synthesis, as was seen in *P. aeruginosa* (Rohde et al., 2021).

Concurrently, AgNPs have intracellular interference on metabolism and genetics. Transcriptomic and proteomic evidence show that AgNPs down-regulate DNA polymerases, helicases, and recombination repair proteins, which result in stalled replication forks and genomic instability. AgNPs in *K. pneumoniae* were reported to produce a triclosan-like effect by inhibiting type II fatty acid biosynthesis, which is required to produce phospholipid membranes. In addition, AgNPs combine with 30S ribosomal subunit, preventing protein synthesis and resulting in the arrest of translation. It is accompanied by the suppression of cell division proteins (e.g., *FtsZ*) and replication initiator proteins (*DnaA*) that further suppresses the growth of bacteria (Sura Saadoun Khalaf & Hanaa Abdellatif Yassin, 2021). AgNPs are also important in quorum sensing and biofilm disruption that are key in pathogenicity of MDR and CRE organisms. QS mediated bacterial communication, biofilm formation and virulence factor production occur through autoinducers such as acyl-homoserine lactones (AHLs) in Gram negative bacteria. AgNPs interfere with LuxR/LuxI-type QS systems by down-regulating biofilm-associated genes and preventing exopolysaccharide production (Singh et al., 2019). Reduced production of violacein pigment in *Chromobacterium violaceum* has been validated in support of this interference (Saeki et al., 2022; Sharma et al., 2024). The significant property of AgNPs is their synergistic effect with antibiotics and inactivation of resistance enzymes that can have a tremendous impact on their clinical potential. The combination of AgNPs with carbapenems imipenem and meropenem has

been demonstrated to decrease the minimum inhibitory concentration (MIC) of carbapenem-resistant *E. coli* and *K. pneumoniae* up to 1024-fold (2023). They also synergize with colistin, cefixime, and antimicrobials of vegetal origin, such as thymol and carvacrol, increasing the activity of antibiofilm and antibacterial against KPC-producing strains (Solomon et al., 2025). AgNPs specifically block β -lactamases like KPC, NDM, and OXA-48, which interfere with the hydrolysis of carbapenems, and the activity of the antibiotics is restored. Clinically, these mechanisms imply interesting applications and future perspectives. Medical devices with AgNP, including catheters, endotracheal tubes, and orthopedic implants, are capable of a substantial decrease in the hospital-acquired infections due to the prevention of the development of biofilms. AgNP-based wound dressing has shown to have better wound healing and antimicrobial results in MDR infected burns and surgical wounds (Stojanoski et al., 2021). AgNPs in combination with current antibiotic regimens can be used to reduce doses yet maintain performance, which may reduce selective pressure promoting resistance. Due to their wide-spectrum activity, antibiofilm efficacy, and the possibility to inhibit enzymes, AgNPs offer an important solution to the problem of carbapenem-resistant infections worldwide. Finally, AgNPs have antimicrobial activities mediated through a complex of complementary effects, such as the disruption of the cell envelope, the induction of oxidative stress, the interference of DNA replication and protein synthesis, the disruption of QS, the dismantling of biofilms, and the inhibition of β -lactamase. Such a multi-target strategy is especially effective against MDR and CRE related pathogens, minimizing the risk of resistance development and providing the flexible options of being integrated into the infection control policies. In future, the optimization of biocompatibility, large-scale production of green synthesis, and long-run safety profiles should be investigated to make them widely applicable in clinical practice in the post-antibiotic era.

occupational risks of manufacture (Husain et al., 2023). The fact that they have favorable safety margins, the potential to sustain high antimicrobial activity at sub-toxic doses, makes them good candidates as additives in wound dressings, medical coatings, and in combination antimicrobial agents. Although more studies are required to complete the mapping of their long-term biodistribution and clearances in higher animal models, the available preclinical evidence undoubtedly places fungal-mediated AgNPs as a safe, renewable and clinically promising AgNPs alternative to chemically modified ones.

2.1.11 Bibliometric Insights into Scientific Focus on Carbapenem Resistance *Escherichia coli* and *Klebsiella pneumoniae*

The analysis of scientific publication trends (Figure 2.1.4 & Figure 2.1.5) related to carbapenem-resistant *E. coli* and *K. pneumoniae* offers critical insight into the global research landscape surrounding antimicrobial resistance (AMR). However, it is essential to clarify that a higher publication frequency from a particular country does not inherently equate to a higher clinical burden or disease incidence within that region. Publication volume reflects the extent of academic engagement with the topic, which is shaped by multiple interrelated factors such as national research infrastructure, scientific funding, institutional priorities, data accessibility, and the emphasis placed on AMR within public health policy. Countries such as China, the United States, and India dominate the publication landscape, not necessarily because they experience the highest number of resistant infections, but because they possess robust research ecosystems, larger networks of active microbiologists and clinicians, better access to international collaborations, and often stronger incentives to publish in peer-reviewed journals. Furthermore, the observed publication trends are also influenced by external stimuli such as global health alerts, emergence of novel resistance mechanisms (e.g., NDM-1, KPC), WHO-led surveillance initiatives, and the alignment of national goals with global AMR frameworks. While this data cannot replace epidemiological surveillance or clinical reporting, it nonetheless provides valuable meta-analytical insights into how nations prioritize and respond to carbapenem resistance at the scientific level. It helps identify not only the countries actively producing knowledge but also those that are

underrepresented in the literature, potentially pointing to gaps in global data collection, scientific capacity, or public health reporting. The temporal increase in publications, particularly on *K. pneumoniae* post-2015, reflects a growing awareness and urgency within the research community, possibly due to rising treatment failures and hospital outbreaks linked to this pathogen. Thus, bibliometric mapping serves as a powerful complementary tool to understand the scientific response to AMR offering a window into where intellectual resources are being concentrated, how research agendas evolve over time, and which geographic regions may require strengthened capacity for surveillance and academic output. These insights are crucial for informing not only future research directions but also international funding strategies and collaborative policymaking efforts in combating the spread of carbapenem-resistant organisms.

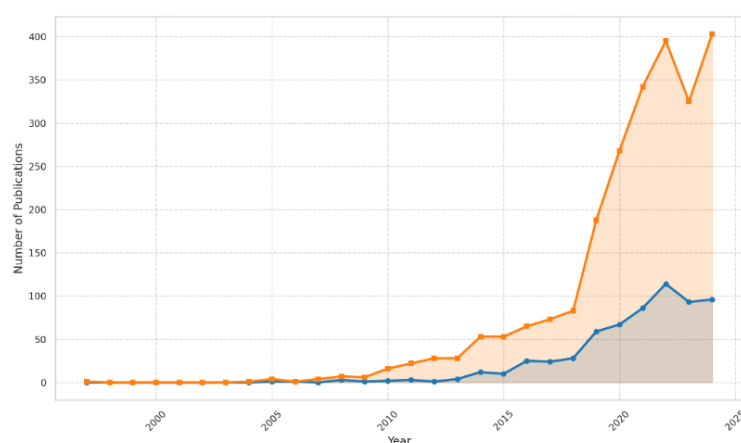


Figure 2.1.4: Comparative annual publication trend on carbapenem-resistant *E. coli* (Orange line) and *K. pneumoniae* (Blue line) (1997–2024). The analysis shows a sharp rise in publications after 2015, with *K. pneumoniae* consistently receiving higher research attention than *E. coli*, reflecting its dominant clinical and epidemiological impact in global CRE studies.

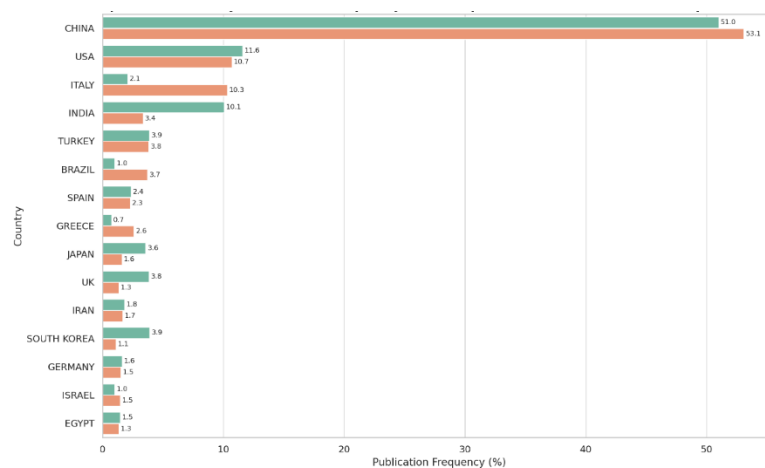


Figure 2.1.5: Top 15 countries by publication frequency on carbapenem-resistant *E. coli* (Green bar) and *K. pneumoniae* (Orange bar). China and the USA lead global research output, followed by Italy, India, and Turkey, indicating concentrated research activity in regions facing a high burden of antimicrobial resistance.

2.2 Hypothesis

The rapid rise of antimicrobial resistance in Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae*, has become a major global health concern. These pathogens are responsible for severe infections such as bloodstream infections, wound infections, urinary tract infections, and pneumonia, and have acquired multiple resistance mechanisms that undermine the efficacy of most available antibiotics. The situation is especially critical for carbapenem-resistant Enterobacteriaceae (CRE), as carbapenems are considered the last line of defense for treating multidrug-resistant Gram-negative infections. Due to their high mortality rates, treatment failures, and rapid spread, the World Health Organization (WHO) has classified CRE under the “Critical Priority” pathogens requiring immediate attention and urgent development of new therapeutic strategies.

Despite the severity of this threat, there is limited research specifically exploring biosynthesized silver nanoparticles as an alternative strategy against carbapenem-resistant *E. coli* and *K. pneumoniae*. Although silver nanoparticles are widely recognized for their broad-spectrum antimicrobial properties, very few studies focus on fungal-mediated (biogenic) synthesis for targeting CRE. Fungi represent a promising source of reducing and stabilizing biomolecules, and published studies increasingly highlight their potential to generate eco-friendly, stable, and highly active nanoparticles. However, their application against carbapenem-resistant Enterobacteriaceae remains underexplored, creating a clear gap that this study aims to address.

2.3 Objective of proposed research work

1. Biosynthesis of silver nanoparticles using soil fungi and their preliminary characterization.
2. Assessment of antibacterial activity of biosynthesized silver nanoparticles against carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae*.
3. Exploration of the mechanism of antimicrobial activity of the synthesized nanoparticles and toxicity evaluation of it.
4. Polyphasic characterization of potential fungi and physiochemical characterization of potential silver nanoparticles.

Chapter 3

3. Methodology

3.1. Soil Sample Collection

Six soil samples were collected for the purpose of isolating fungal strains with potential for silver nanoparticle biosynthesis. Soil was selected as the environmental source due to its well-documented microbial richness and the established capacity of soil fungi to facilitate nanoparticle formation through biologically driven reduction processes (Sonbol et al., 2022). Sampling was conducted at six distinct agricultural sites located in and around Phagwara, Punjab.

Each soil sample was collected aseptically from a depth of 10–15 cm, a layer known to be rich in organic matter and ideal for supporting fungal growth. Approximately 500 grams of soil per site were gathered using sterile augers and spatulas to prevent cross-contamination. The samples were then sealed in pre-labelled, sterile polyethylene bags, clearly marked with the date, sample code, and geographic coordinates. To preserve microbial integrity, the samples were transported to the laboratory under cooled conditions using insulated containers. Sample were processed immediately for fungus isolation and remaining samples were stored at 4°C (Aneja, 2007; FAO , 2020). Detailed information of all six soil sampling sites, including sample codes, soil type, time of collection and GPS coordinates, is presented in Table 3.1, while representative photographs of selected agricultural farmlands are shown in Figure 3.1.

Table 3.1. Geographical details and classification of soil sampling sites.

Sample Number	Code	Soil Type	Month & Year	Location
1	S1	Sandy loamy soil	October 2022	Latitude: -31.241042, Longitude: -75.700281
2	S2	Sandy loamy soil	October 2022	Latitude: -31.242014, Longitude: -75.697948

3	S3	Loamy soil	October 2022	Latitude: -31.247731, Longitude: -75.692343
4	S4	Clay soil	October 2022	Latitude: 31.217714, Longitude: -75.779846
5	S5	Loamy soil	December 2022	Latitude: 31.216595, Longitude: -75.777411
6	S6	Sandy loamy soil	December 2022	Latitude: 31.241098, Longitude: -75.700305



Figure 3.1: Soil sampling sites at agricultural farmlands in Phagwara, Punjab: (a) site located at Latitude 31.217714° N, Longitude 75.779846° E; (b) site located at Latitude 31.216595° N, Longitude 75.777411° E.

3.2. Isolation of Fungi

Fungi were isolated from the six collected soil samples using a combination of serial dilution and spread plate technique, which is a standard method for isolating fungal colonies from soil sample (Aneja, 2007; Emmanuel & Igoche, 2022). For each soil sample, 1 g of soil was aseptically transferred into a sterile test tube containing 9 mL

of sterile distilled water, producing an initial 10^{-1} dilution. The suspension was vigorously mixed to disperse soil particles and associated microorganisms uniformly. Serial dilutions were prepared by transferring 1 mL of the 10^{-1} suspension into a fresh test tube containing 9 mL of sterile distilled water, yielding a 10^{-2} dilution. This procedure was repeated sequentially up to a 10^{-5} dilution, with each tube mixed thoroughly before further dilution. From the prepared dilutions, 100 μ L aliquots of the 10^{-3} , 10^{-4} , and 10^{-5} suspensions were spread onto sterile potato dextrose agar (PDA) plates supplemented with chloramphenicol (100 μ g/mL) to suppress bacterial growth. The inoculum was spread evenly across the agar surface using a sterile glass spreader. Plates were incubated at 28 °C for 5–7 days in an inverted position to allow fungal colony development. Following incubation, distinct fungal colonies were carefully picked and sub-cultured onto fresh PDA plates to obtain pure isolates. Sub-culturing was performed under aseptic conditions using sterile inoculating loops, and the plates were incubated again at 28 °C for 5–7 days to confirm purity and viability of the fungal cultures.

3.3. Preparation of Intracellular and Extracellular Extracts from Purified Fungal Isolates

100 ml of Potato dextrose broth (PDB) was prepared in a conical flask by dissolving the 2.4 gm of medium in distilled water. The pH was adjusted to 5.5 with sterile 1N HCl or NaOH, and sterilized by autoclaving. PDA slants were prepared and inoculated with fungal isolates and incubated at 28 °C for 5 days. Spore suspensions were obtained by adding 10 mL of sterile distilled water containing 0.1% Tween 20 to PDA slants and gently scraping the fungal surface with a sterile loop to release spores into suspension. One milliliter of this suspension was inoculated into each 100 mL flask of sterile PDB under aseptic conditions. The inoculated flasks were incubated at 28 °C for 5 days under static conditions, during which fungal biomass developed as a mat on the broth surface. For the preparation of the extracellular extract, the fungal biomass was separated from the broth by filtration through sterile muslin cloth. The clear culture filtrate, containing secreted extracellular metabolites and enzymes, was collected in sterile containers and stored at 4 °C for further use (Moghaddam et al., 2015).

For the preparation of the intracellular extract, the retained fungal mat was washed thoroughly with sterile deionized water to remove residual medium and spores, then transferred to a fresh flask containing 100 mL of sterile deionized water. This suspension was incubated at 28 °C on a shaker at 150 rpm for 24 hours, during which the fungal cells underwent osmotic stress, leading to leakage of intracellular metabolites into the water. After incubation, the biomass was removed by filtration through sterile muslin cloth, and the resulting filtrate containing the intracellular extract was collected and refrigerated at 4 °C until further use (Moghaddam et al., 2015).

3.4. Biosynthesis of Silver Nanoparticles and its preliminary characterization

The biosynthesis of silver nanoparticles was carried out using both intracellular and extracellular fungal extracts derived from purified fungal isolates. Silver nitrate (1 mM) was used as the precursor solution. For each setup, the reaction mixture consisted of 19 mL of 1 mM AgNO₃ solution mixed with 1 mL of fungal extract (intracellular or extracellular, depending on the experiment). A parallel control was prepared under the same conditions, containing 19 mL of 1 mM AgNO₃ solution and 1 mL of sterile deionized water in place of fungal extract, in order to monitor any abiotic reduction of silver ions. All preparations were performed under aseptic conditions. The reaction mixtures were maintained in sterile 100 mL Erlenmeyer flasks incubated at 28 ± 2 °C on a rotary shaker at 100 rpm upto 48 hours and periodically observed to identify the bioreduction of silver ions by fungal metabolites (Malik et al., 2024).

Preliminary confirmation of silver nanoparticle formation was carried out using color changes and UV–Visible spectrophotometry. As mentioned above the samples were observed periodically for any change in color from colourless to light to brown. Following this the sample were subjected to UV–Visible spectrophotometry analysis. 1 mL aliquots were withdrawn from the reaction mixtures and was diluted 10-fold with sterile deionized water to reduce background absorbance. The diluted samples were transferred into quartz cuvettes (1 cm path length), and absorbance spectra were recorded using a Shimadzu UV-1800 spectrophotometer. Scanning was performed in the wavelength range of 200–800 nm, with a spectral resolution of 1 nm and scanning

speed of 200 nm/min. Sterile deionized water was used as the blank for baseline correction (Tończyk et al., 2023).

3.5. Evaluation of Antimicrobial Activity of Silver Nanoparticles against Carbapenem Resistant *Escherichia coli* and *Klebsiella pneumoniae*

3.5.1 Collection of bacterial isolates

Bacterial isolates of carbapenem-resistant *E. coli* and *K. pneumoniae* were obtained from clinical samples collected from the Punjab Institute of Medical Sciences (PIMS), Jalandhar, following prior approval from the Institutional Bioethics and Biosafety Committee (IBSC approval code: LOVRDAMR0236). Clinical specimens were collected under sterile conditions in compliance with institutional biosafety guidelines to maintain sample integrity. The isolates were transported to the research laboratory under cold conditions and samples were stored at 4 °C.

For the re-characterization, the clinical isolates were streaked on EMB under aseptic conditions and incubated at 37 °C for 24 hours. Initial identification was performed using Gram staining to determine Gram reaction and cell morphology, followed by a series of standard biochemical tests, including the indole test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test, and triple sugar iron (TSI) agar test (Aneja, 2007). These tests confirmed the identity of the isolates as *E. coli* and *K. pneumoniae*.

3.5.2 Confirmation of Isolates for Carbapenem Resistance

The confirmation of carbapenem resistance in *E. coli* and *K. pneumoniae* isolates was carried out using the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2025). Fresh bacterial colonies were obtained by sub-culturing isolates on nutrient agar and incubating at 37 °C for 18–24 hours. A standardized bacterial suspension was prepared by emulsifying a single colony in sterile saline and adjusting turbidity to match the 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL; Appendix IV). Sterile Mueller–Hinton agar plates were inoculated with the standardized suspension using sterile cotton swabs. Carbapenem antibiotic disks

(imipenem 10 µg and meropenem 10 µg) were aseptically placed on the agar surface using sterile forceps. Plates were incubated at 37 °C for 16–18 hours in an inverted position. After incubation, the zones of inhibition around each disk were measured in millimeters using a calibrated ruler. Results were interpreted according to CLSI breakpoints: isolates with ≥ 23 mm were classified as *susceptible*, 20–22 mm as *intermediate*, and ≤ 19 mm as *resistant*. These zone diameter criteria ensured standardized determination of carbapenem resistance in the tested isolates.

3.5.3 Evaluation of Antimicrobial Activity of Silver Nanoparticles

The antimicrobial activity of biosynthesized AgNPs was evaluated against carbapenem-resistant *E. coli* and *K. pneumoniae* isolates using the agar well diffusion method (CLSI, 2025). Test isolates were cultured overnight on nutrient agar, and a bacterial suspension equivalent to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL) was prepared in sterile saline. Sterile MHA plates were prepared, solidified, and uniformly swabbed with the standardized inoculum using sterile cotton swabs. For the initial screening assay, six wells (8 mm diameter) were aseptically punched into each MHA plate using a sterile cork borer. Prior to antimicrobial evaluation, the AgNP suspension was sonicated and vortexed to ensure uniform dispersion and prevent aggregation. The freshly prepared suspension was used immediately to maintain nanoparticle stability and consistency during the assay. Each well was loaded with 100 µL of AgNP suspension (1 mg/mL) synthesized from six different fungal isolates. Each treatment was performed in triplicate. Plates were incubated at 37 °C for 18–24 hours, and the diameters of inhibition zones were measured in millimetres.

Based on the screening results, the most effective AgNP (from the best-performing fungal isolate) was selected for a dose-dependent assay. In this experiment, five wells (8 mm diameter) were prepared per MHA plate. Three wells were filled with 100 µL of the selected AgNP suspension at concentrations of 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL, respectively. The fourth well contained the positive control (100 µL of meropenem at 100 µg/mL), while the fifth well served as the negative control containing 100 µL of sterile distilled water. Each treatment was performed in triplicate.

Plates were incubated at 37 °C for 18–24 hours, after which inhibition zones were recorded.

3.5.4. Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of biosynthesized silver nanoparticles (AgNPs) was determined using a microbroth dilution method, following established guidelines with minor modifications (CLSI, 2025). A stock solution of silver nanoparticles was prepared by mixing the biogenic fungal mediated silver nanoparticle in 1% deionized water to obtain a concentration of 10,000 µg/mL. A 0.1 mL aliquot of this solution was further diluted with 0.9 mL of sterile double-distilled water to generate a secondary stock of 1,000 µg/mL. From this sterile stock, working concentrations of 10, 20, 50, 100, 150, 200, 250, 300, 350 and 400 µg/mL were initially prepared each at double the desired final concentration. Upon mixing 1:1 with the inoculated medium in 96-well plates, the final test concentrations were adjusted to 5, 10, 25, 50, 75, 100, 125, 150, 175, and 200 µg/mL, respectively. A similar concentration series of the antibiotic meropenem was prepared as the positive control using the same dilution protocol.

Carbapenem-resistant isolates of *E. coli* and *K. pneumoniae*, were used for the MIC assay. Each strain was revived and cultured in tryptic soy broth (TSB) for 24 hours at 37°C. The bacterial suspension was standardized to match the turbidity of 0.5 McFarland (approx. 1.5×10^8 CFU/mL) using fresh, uninoculated broth. A 0.1 mL volume of this standardized culture was diluted in 9.9 mL of sterile double-strength TSB to achieve a 1:100 dilution. Upon mixing with an equal volume of AgNPs solution in the plate wells, the final bacterial load was standardized to approximately 5×10^5 CFU/mL, in line with CLSI and EUCAST recommendations (CLSI, 2025; EUCAST, 2025).

A sterile 96-well flat-bottom microtitre plate was used for the assay. To each well, 100 µL of the AgNP solution was dispensed, arranged in descending order of concentration. 100 µL of the prepared bacterial inoculum was then added to each well, ensuring a final volume of 200 µL per well. Negative control wells contained broth with silver nanoparticles but no bacterial inoculum, while sterility control wells had broth

and sterile distilled water only. Meropenem-treated wells served as positive controls for bacterial inhibition. The layout included separate sets for each test organism (*E. coli* and *K. pneumoniae*) treated with AgNPs and meropenem. Each treatment was performed in triplicate. The plates were incubated at $37 \pm 1^\circ\text{C}$ for 18–20 hours, and bacterial growth was assessed visually. The lowest concentration with no visible turbidity was recorded as the MIC.

3.5.5 Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) of the synthesized silver nanoparticles was determined by sub culturing from MIC assay wells that showed no visible bacterial growth (CLSI, 2025). A loop full culture was carefully withdrawn from the well before MIC, MIC and after MIC and streak inoculated onto freshly prepared MHA plates. The plates were incubated at $37 \pm 1^\circ\text{C}$ for 20 hours under aerobic conditions. After incubation, the presence or absence of bacterial colonies was recorded. The lowest concentration of silver nanoparticles at which no bacterial growth was observed on the agar surface was defined as the MBC. All experiments were performed in triplicate. This procedure was carried out in accordance with established bactericidal evaluation protocols.

3.6. Evaluation of Antibacterial Mechanism of Action

To elucidate the antibacterial mechanism of the synthesized silver nanoparticles (AgNPs), a series of mechanistic assays were conducted to assess their impact on bacterial physiology and cellular structures. The investigation aimed to determine how AgNPs influence bacterial growth, membrane integrity, cell wall stability, and genomic DNA. These mechanistic evaluations were conducted in four parts:

3.6.1 Growth Curve Analysis

The effect of biosynthesized AgNPs on bacterial proliferation was assessed through a growth curve assay with minor modifications from established protocols (Hall et al., 2014). A stock solution of AgNPs (1,000 $\mu\text{g/mL}$) was prepared in sterile deionized water, sonicated for 10 minutes to ensure complete dispersion. Four 100 ml conical

flask were taken each having 100 ml Muller Hinton broth was prepared according to the manufacturer's instructions (HiMedia Laboratories Pvt. Ltd., India), and autoclaved at 121 °C for 15 minutes. After cooling, one flask left blank (uninoculated), second flask as bacterial control, third flask was added with meropenem at half of MIC and fourth flask added with AgNPs at half of MIC. After this second, third and fourth flasks were inoculated with 100 µl of test bacteria adjusted to a 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). Same setup used for other test bacteria (*K. pneumoniae*).

All flasks were incubated at 37 ± 1 °C in a shaking incubator at 120 rpm. Bacterial growth was monitored by recording optical density (OD) at 600 nm at 2-hour intervals for 30 hours using a UV–visible spectrophotometer, and the growth curves were generated by plotting OD values against incubation time. All tests were performed in triplicate. The average OD values were plotted against time to observe bacterial growth kinetics and to compare the inhibitory effects of AgNPs relative to controls. Alterations in lag phase, exponential growth rate, and stationary phase were evaluated to assess the impact of AgNPs on bacterial proliferation.

3.6.2 Nucleotide Leakage Assay (Assessment of Membrane Integrity)

The impact of biosynthesized silver nanoparticles (AgNPs) on bacterial cell membrane integrity was assessed using a nucleotide leakage assay, based on previously established protocols with minor modifications (Sharma et al., 2021). Carbapenem-resistant *E. coli* and *K. pneumoniae* strains were cultured overnight in MHB at 37 ± 1 °C with continuous shaking for 20 hours. Following incubation, 1.5 mL of each bacterial suspension was transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting supernatants were discarded, and the bacterial pellets were washed twice with sterile phosphate buffer saline (PBS, pH 7.4) by repeating the centrifugation step under the same conditions, the washed bacterial pellets were resuspended in sterile PBS to achieve a final inoculum concentration of approximately 1.5×10^8 CFU/mL. The bacterial suspension was standardized using 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL).

The standardized bacterial suspensions were treated with AgNPs at concentrations equivalent to the previously determined MIC for each isolate. Meropenem was used as a positive control, while the negative control received only sterile double-distilled water. The mixtures were incubated at $37 \pm 1^\circ\text{C}$ and sampled at hourly intervals over a period of 8 hours (i.e., at 1, 2, 3, 4, 5, 6, 7, and 8 hours). At each time point, samples were centrifuged at 10,000 rpm for 10 minutes at 4°C , and the resulting supernatants were transferred into clean cuvettes. The release of nucleotides into the extracellular environment was measured by recording absorbance at 260 nm using a UV–Visible double beam spectrophotometer (Lasany LI-2800 Ex). Fresh uninoculated PBS served as the blank. All measurements were conducted in triplicate, and the mean absorbance values were calculated for each treatment group and time point.

3.6.3 Evaluation of Cell Wall Inhibition by Field Emission Scanning Electron Microscopy (FE-SEM)

The effect of biosynthesized AgNPs on bacterial cell wall integrity was investigated by analyzing morphological alterations in treated cells using Field Emission Scanning Electron Microscopy (FE-SEM), as per protocols adapted from (Havrdova et al., 2014; Vinothkanna et al., 2022). Carbapenem-resistant *E. coli* and *K. pneumoniae* were cultured in sterilized broth prepared according to the manufacturer's instructions. After incubation at $37 \pm 1^\circ\text{C}$ for 20 hours, the bacterial cultures were standardized to a 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL. Each bacterial suspension was then treated with AgNPs at their previously determined MIC values, while control samples received only sterile double-distilled water. The treated and control cultures were incubated at $37 \pm 1^\circ\text{C}$ for 3 hours. Post-incubation, 2 mL aliquots of each sample were transferred to labeled sterile Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C . The supernatants were discarded, and the pellets were resuspended in PBS (pH 7.4) and washed three times under identical centrifugation conditions.

Washed bacterial cells were fixed in 2.5% glutaraldehyde (prepared in PBS) for 2 hours at 4°C to preserve cellular ultrastructure. After fixation, a final wash with PBS

was performed to remove residual fixative. The samples were then subjected to graded ethanol dehydration steps: 30%, 50%, 70%, 80%, 95%, and 100% (twice), each for 10 minutes, followed by critical point drying (CPD) using 50% and 100% hexamethyldisilazane (HMDS), also for 10 minutes each. Following CPD, 20 μ L of each sample (AgNP-treated and control) was carefully spread onto clean coverslips to prepare thin smears. The smears were left to air dry at ambient temperature for 24 hours. Dried samples were then coated with an 8 nm layer of gold using a sputter coater to render the surface conductive for electron microscopy. The prepared samples were examined using a JEOL Field Emission Scanning Electron Microscope (Model: JEOL SM-74190UEC), operated at an accelerating voltage of 10 kV, with a back-scattered electron detector and probe current of 5 nA. The imaging and analysis were conducted at the Central Instrumentation Facility (CIF), Lovely Professional University, Phagwara, Punjab, India. Comparative analysis between control and AgNP-treated cells allowed for visual assessment of membrane distortion, cell wall collapse, surface roughness, and potential nanoparticle-induced morphological damage.

3.6.4 Nucleic Acid Damage Assay

To evaluate the potential of AgNPs to induce DNA damage in bacterial cells, a nucleic acid degradation assay was performed. Genomic DNA from AgNP-treated and untreated control bacterial cultures was isolated, purified, and analyzed by agarose gel electrophoresis. The protocol followed was adapted from (Green et al., 2012) with appropriate modifications. On Day 1, carbapenem-resistant *E. coli* and *K. pneumoniae* strains were inoculated into freshly prepared Luria–Bertani (LB) broth and incubated overnight at 37 ± 1 °C with shaking at 120 rpm. On Day 2, overnight cultures were used to prepare fresh LB broth cultures and grown to mid-logarithmic phase ($OD_{600} \approx 0.5$). These cultures were then treated with silver nanoparticles (AgNPs) at their respective minimum inhibitory concentration (MIC) values, while the negative control received sterile double-distilled water. All cultures were incubated further at 37 ± 1 °C for 20 hours with shaking. After treatment, 10 mL of each culture was transferred into sterile 15 mL falcon tubes and centrifuged at 7,500 rpm for 10 minutes. The supernatant was discarded, and the resulting pellets were washed once with 1 mL of Tris-EDTA (TE)

buffer (pH 8.0), then resuspended in 500 μ L of TE buffer in sterile 1.5 mL microcentrifuge tubes. Cell lysis was carried out by adding 30 μ L of 10% sodium dodecyl sulfate (SDS), followed by gentle mixing. Next, 3 μ L of proteinase K (20 mg/mL in TE buffer) was added, and the tubes were incubated at 37 °C for 1 hour. Following lysis, 100 μ L of 5 M NaCl and 80 μ L of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) were added, gently inverted, and incubated at 65 °C for 10 minutes in a water bath. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was then added, mixed thoroughly, and centrifuged at 8,000 rpm for 10 minutes. The aqueous phase was carefully transferred to a fresh tube. This extraction step was repeated once for improved purity. To remove RNA, RNase A (final concentration: 50 μ g/mL) was added, and samples were incubated at 37 °C for 30 minutes. A final phenol:chloroform extraction was performed, and the aqueous phase was collected in a new sterile tube. DNA was precipitated by adding 1/10 volume of 5 M NaCl and 2 volumes of ice-cold 100% ethanol (−20 °C), followed by incubation at −20 °C for 1 hour. The mixture was centrifuged at 12,000 rpm for 20 minutes at 4 °C, and the DNA pellet was washed with 1 mL of 70% ethanol. After centrifugation at 12,000 rpm for 10 minutes, ethanol was removed, and the pellet was air-dried at room temperature for 15–20 minutes. The dried DNA pellet was finally resuspended in 30 μ L of TE buffer (pH 8.0) and stored at −20 °C until electrophoresis.

A 1% agarose gel was prepared by dissolving 1 g of molecular-grade agarose in 100 mL of 1× Tris-Borate-EDTA (TBE) buffer. The solution was heated until fully molten and cooled to approximately 50°C. Ethidium bromide (EtBr) 5 μ L was added to a final concentration of 0.5 μ g/mL, and the gel solution was poured into a casting tray with a comb in place. The gel was allowed to solidify at room temperature. Prior to loading, 6× DNA loading dye was mixed with each DNA sample in a 1:5 ratio (1 μ L dye per 5 μ L DNA sample). A DNA ladder (250–10,000 bp) was loaded alongside 10 μ L of each DNA sample into separate wells. The gel was then submerged in 1× TBE buffer, and electrophoresis was carried out at 100 V for 60–90 minutes, until the tracking dye migrated approximately 75–80% of the gel length. DNA bands were visualized under UV illumination using a NuGenius/Syngene gel documentation system.

3.7. Toxicity Evaluation of Silver Nanoparticles Using MTT Assay

The potential toxicity of biosynthesized AgNPs was assessed using the MTT assay on 3T3 L1 mouse fibroblast cells, a standard model for evaluating in vitro biocompatibility. This assay was performed to evaluate the cytotoxic effects of AgNPs on normal mammalian cells. The method followed was based on established protocols (Mosmann, 1983; Riss et al., 2004), and aligned with safety standards for nanomaterial exposure (ASTM, 1987; Sosa et al., 2024). The 3T3 fibroblast cell line (*Mus musculus*) was cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, USA). To maintain sterility, the medium was fortified with penicillin (100 µg/mL), streptomycin (100 µg/mL), and amphotericin B (5 µg/mL) (Himedia, India). Cultures were maintained in 25 cm² flasks at 37 ± 1°C in a humidified incubator with 5% CO₂, and subcultured upon reaching ~80% confluence. A stock solution of AgNPs (1,000 µg/mL) was prepared in 0.1% DMSO, followed by serial dilution in serum-free EMEM to obtain 400, 200, 100, 50, and 25 µg/mL working concentrations.

Cells were harvested and diluted to a concentration of 1×10^5 cells/mL in EMEM containing 10% FBS. 100 µL of the cell suspension was seeded into each well of a 96-well plate and incubated for 24 hours at 37°C and 5% CO₂ to allow adherence. Following incubation, the culture medium was removed and cells were washed with 1× PBS (200 µL). Each well was treated with 200 µL of AgNP solutions (prepared in EMEM medium) at varying concentrations (400, 200, 100, 50, and 25 µg/mL). Experimental control wells received EMEM without silver nanoparticles, while blank wells contained medium only without cells. The plate was incubated for 72 hours under standard conditions. After treatment, the wells were washed with PBS and incubated with 200 µL of 0.5 mg/mL MTT solution (in PBS) for 4 hours. Formazan crystals formed by viable cells were dissolved in 100 µL DMSO, and absorbance was recorded at 570 nm using a microplate reader. The percentage of viable cells was calculated to assess the toxicological safety of the AgNPs:

$$\text{Percent (\%) viable cell} = (\text{Abs treated cell} / \text{Abs control cell}) * 100$$

Where:

- **Abs treated cells** = Absorbance of AgNP-treated wells
- **Abs control cells** = Mean absorbance of untreated control cells

The experiment was performed in triplicate, and the mean viability percentage was plotted against AgNP concentration to determine dose-dependent cellular response.

3.8. Molecular Identification of Fungal Isolate via Internal Transcribed Spacer (ITS) Sequencing

To molecularly characterize the potential fungal isolate, ITS gene sequencing was performed. The sequencing and downstream analysis were outsourced to a commercial sequencing facility. The steps carried out for molecular identification are outlined below: Genomic DNA was extracted from the selected fungal isolate using a modified cetyltrimethylammonium bromide (CTAB) method (Green et al., 2012), which is widely used for fungi owing to their rigid cell wall composition. Fresh fungal biomass was obtained by cultivating the isolate in PDB at 28 ± 1 °C for 5–7 days under static conditions, followed by harvesting the mycelium through sterile muslin cloth and washing twice with sterile distilled water to remove medium residues. Approximately 200 mg of mycelium was blotted dry, frozen in liquid nitrogen, and ground to a fine powder using a pre-chilled sterile mortar and pestle. The powdered tissue was immediately suspended in 700 μ L of pre-heated CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, and 0.2% β -mercaptoethanol added freshly) and incubated at 65 °C for 30 minutes with occasional mixing to facilitate complete lysis. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently, and centrifuged at 12,000 rpm for 10 minutes at 4 °C, and the aqueous phase was carefully transferred into a fresh sterile tube; this extraction step was repeated once for improved purity. DNA was precipitated by adding 0.6 volume of ice-cold isopropanol and incubating the mixture at -20 °C for 1 hour, followed by centrifugation at 12,000 rpm for 10 minutes at 4 °C. The resulting pellet was washed twice with 70% ethanol, air-dried at room temperature, and dissolved in 50 μ L of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). To remove RNA contamination, 2 μ L

of RNase A (10 mg/mL) was added and the mixture was incubated at 37 °C for 30 minutes. The integrity of the extracted DNA was verified by electrophoresis on a 1.0% agarose gel containing ethidium bromide (0.5 µg/mL), while purity and concentration were assessed spectrophotometrically using a NanoDrop instrument, with acceptable A260/A280 ratios between 1.7 and 2.0. The presence of a single high molecular weight DNA band with no observable degradation confirmed the suitability of the extracted DNA for downstream ITS amplification and sequencing.

Amplification of the fungal gene region was performed using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') universal primers, which target conserved regions flanking the internal transcribed spacer (ITS) regions of fungal ribosomal DNA. The PCR reaction yielded a single discrete amplicon, as confirmed by gel electrophoresis. The PCR amplicon was subjected to column-based purification to eliminate residual primers, nucleotides, and buffer salts. The purified product was used for sequencing reactions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on the ABI 3500xl Genetic Analyzer. The resulting nucleotide sequence of the ITS gene was used as a query for a BLASTn (Basic Local Alignment Search Tool) search against the NCBI (National Center for Biotechnology Information) GenBank database. The top ten sequences showing maximum identity were selected for comparative analysis. These sequences were aligned and phylogenetic tree was plotted through MEGA 12 (Molecular Evolutionary Genetics Analysis), and phylogenetic similarity was assessed to infer the taxonomic identity of the fungal isolate.

3.9 Characterization of Synthesized Silver Nanoparticles

The biosynthesized AgNPs were subjected to a series of physicochemical characterization techniques to confirm their formation, evaluate structural and morphological attributes, and determine surface properties. The methods used are described below:

3.9.1 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) analysis was performed to determine the hydrodynamic diameter and polydispersity index (PDI) of the synthesized silver nanoparticles. Measurements were carried out using a Malvern Zetasizer Nano ZS equipped with a 633 nm He-Ne laser, operating at a fixed backscatter angle of 173°. The colloidal nanoparticle solution was diluted 1:10 with ultrapure deionized water to reduce multiple scattering effects. From this diluted sample, 1 mL was transferred into a disposable polystyrene cuvette (Malvern DTS0012), taking care to avoid air bubbles. Samples were equilibrated at 25 °C for 120 seconds prior to measurement. Each sample was analyzed with measurement consisting of 15 runs. Data including the z-average diameter and PDI were recorded using Zetasizer Software version 7.13. After each measurement, cuvettes were cleaned with ethanol, rinsed with deionized water, and dried with lint-free wipes to prevent cross-contamination (Djahaniani et al., 2017).

3.9.2 Zeta Potential

The surface charge (zeta potential) of the synthesized silver nanoparticles was measured using a Malvern Zetasizer Nano ZS, equipped with a 633 nm laser and electrophoretic light scattering (ELS) capability. Measurements were carried out in folded capillary cells (DTS1070). Prior to measurement, the nanoparticle suspension was diluted 1:10 with ultrapure deionized water to maintain conductivity within the instrument's optimal range. The diluted sample was then transferred into the capillary cell using a disposable syringe, ensuring there were no air bubbles or leaks. The temperature was maintained at 25 °C, and each sample was allowed to equilibrate for 2 minutes before measurement. Zeta potential values recorded with each measurement consisting of at least 20 runs. Data acquisition and analysis were performed using Zetasizer Software version 7.13, and the mean zeta potential was calculated automatically by the instrument using Smoluchowski's model (Rajendrachari, 2013).

3.9.3 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to identify the functional groups present on the surface of the synthesized silver nanoparticles. The dried silver nanoparticle powder was obtained by centrifuging the colloidal suspension at 10,000

rpm for 15 minutes, followed by washing the pellet with deionized water and drying it at room temperature. Approximately 2 mg of the dried AgNP powder was finely ground and mixed with 200 mg of spectroscopic-grade potassium bromide (KBr). The mixture was then compressed into a thin, transparent pellet using a hydraulic press under moderate pressure. FTIR spectra were recorded using a PerkinElmer Spectrum Two FTIR spectrometer in the range of 4000–400 cm^{-1} , with a spectral resolution of 4 cm^{-1} and 32 scans per sample. A background spectrum was recorded using a pure KBr pellet and automatically subtracted. Spectral processing, including baseline correction and peak identification, was performed using the instrument's built-in software (Karthik et al., 2014).

3.9.4 X-ray Diffraction (XRD)

X-ray diffraction (XRD) analysis was performed to determine the crystalline structure and phase composition of the synthesized silver nanoparticles. The dried nanoparticle sample was obtained by centrifugation of the colloidal solution at 10,000 rpm for 15 minutes, followed by washing with deionized water and drying at room temperature. The resulting powder was finely ground using an agate mortar and pestle. XRD measurements were carried out using a Rigaku Ultima IV X-ray diffractometer operated at 40 kV and 30 mA, with Cu-K α radiation ($\lambda = 1.5406 \text{ \AA}$) as the X-ray source. The diffraction patterns were recorded over the 2θ range of 20° to 80° , using a step size of 0.02° and a scan speed of $2^\circ/\text{min}$. The obtained diffraction data were analyzed using OriginPro 2021 and Match! Phase Identification Software to identify characteristic peaks corresponding to crystalline silver (Karthik et al., 2014).

3.9.5 Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) was employed to analyze the morphology, size, and dispersion of the synthesized silver nanoparticles. Imaging was performed using a Delong Instruments LVEM 5 TEM operating at an electron beam energy of 5 keV. Sample preparation involved dropping 5 μL of the colloidal AgNP solution onto a carbon-coated copper grid (200 mesh) using a micropipette. The grid was allowed to air-dry under dust-free conditions for 15 minutes at room temperature. No staining was

applied. Once dried, the grids were mounted on the specimen holder and inserted into the microscope chamber. Images were acquired at varying magnifications, and particle size measurements were conducted using the integrated imaging software. Field of view (FOV) parameters and scale bars were calibrated internally by the instrument prior to acquisition. Multiple fields were imaged to ensure representative nanoparticle analysis (Bankura et al., 2012; Loiseau et al., 2019).

3.9.6 Field Emission Scanning Electron Microscopy (FESEM)

Surface morphology of the synthesized silver nanoparticles was analyzed using a JEOL Field Emission Scanning Electron Microscope (FESEM) equipped with a Schottky Field Emission Gun and in-lens objective lens system. Approximately 10 μ L of the colloidal nanoparticle solution was dropped onto a clean glass slide and allowed to dry under ambient conditions for 20 minutes. The dried sample was then mounted on a standard aluminum stub using carbon adhesive tape. Since the sample was non-conductive, a thin gold sputter coating ($\sim$ 5 nm) was applied using a sputter coater (e.g., Quorum Q150R) prior to imaging to prevent charging. Imaging was performed at an accelerating voltage of 10 kV under high vacuum mode. The secondary electron detector was used to capture high-resolution surface morphology at various magnifications. The working distance was adjusted to optimize focus and image clarity, typically maintained between 5–10 mm. All images were processed and analyzed using the built-in imaging software (Nayak et al., 2016; Zare-Bidaki et al., 2022).

3.9.7 Energy Dispersive X-ray Spectroscopy (EDX)

Elemental analysis of the silver nanoparticles was conducted using the EDS detector integrated with the FESEM system. The same gold-coated sample prepared for SEM was used for EDX analysis. Spectra were acquired at an accelerating voltage of 15 kV, and the system was calibrated using a standard reference sample prior to measurement. Multiple points and area scans were recorded to confirm the elemental composition and ensure homogeneity across the sample. Elements were identified based on characteristic X-ray peaks, and the elemental weight and atomic percentages were quantified using the system's native EDS analysis software (H. Singh et al., 2018).

Chapter 4

4. Results and Discussion

This chapter presents the results of the study, highlighting the successful isolation of diverse soil fungi and their subsequent application in the eco-friendly synthesis of AgNPs. The biosynthesized AgNPs were evaluated for their antibacterial efficacy against clinically significant carbapenem-resistant *E. coli* and *K. pneumoniae*, both categorized as “critical priority pathogens” by the World Health Organization. Comprehensive mechanistic investigations including growth kinetics analysis, nucleotide leakage assay, DNA fragmentation, and cell wall damage assessment were conducted to elucidate the mode of antibacterial action. In parallel, cytotoxicity assays were performed using the 3T3-L1 mouse fibroblast cell line, thereby addressing a key limitation in the biomedical translation of metallic nanoparticles.

Advanced physicochemical characterization techniques, including UV–Visible spectroscopy, Dynamic Light Scattering (DLS), Zeta potential measurement, Fourier-Transform Infrared Spectroscopy (FTIR), X-Ray Diffraction (XRD), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and Energy-Dispersive X-Ray Spectroscopy (EDX), were employed to define the structural, morphological, and functional attributes of the biosynthesized AgNPs. Furthermore, molecular identification of the most potent fungal isolate through Internal Transcribed Spacer (ITS) sequencing revealed its genetic novelty and biosynthetic potential, positioning it as a promising candidate for scalable antimicrobial nanoparticle production. Collectively, these results contribute to the growing body of evidence supporting green nanotechnology as a sustainable and biocompatible strategy to combat multidrug-resistant bacterial infections.

4.1 Isolation of Fungi from Agricultural Soil

Soil samples were collected from six agricultural locations in the Phagwara region of Punjab, India, and subjected to fungal isolation using the standard serial dilution and spread plate combination method on PDA supplemented with chloramphenicol to

suppress bacterial growth (Habeeb et al., 2023; Wild, 1947). Plates were incubated at $28 \pm 1^\circ\text{C}$ for 5–7 days, during which time a range of morphologically distinct fungal colonies emerged. During this study a total of 25 morphologically distinct isolates were obtained. Sample 1 resulted into isolation of six culture, sample 2 resulted into isolation of three isolate, sample 3 resulted into isolation of three isolate, sample 4 resulted into isolation of five isolate, sample 5 resulted into isolation of four isolate and sample 6 resulted into isolation of four isolate. Each isolate was coded according to a systematic key combining the sample site and isolate number (e.g., S1I1; here S- Sample number and I- Isolate number) (Table 4.1).

Table 4.1: Distribution of fungal isolates obtained from different soil samples (S1–S6) along with their respective isolate codes and total number of isolates recovered.

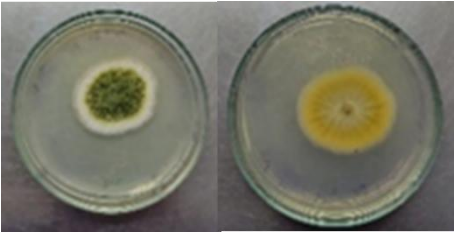
Sample Name	Number of Isolates	Fungal Isolate Code
S1	6	S1I1, S1I2, S1I3, S1I4, S1I5, S1I6
S2	3	S2I1, S2I2, S2I3
S3	3	S3I1, S3I2, S3I3
S4	5	S4I1, S4I2, S4I3, S4I4, S4I5
S5	4	S5I1, S5I2, S5I3, S5I4
S6	4	S6I1, S6I2, S6I3, S6I4

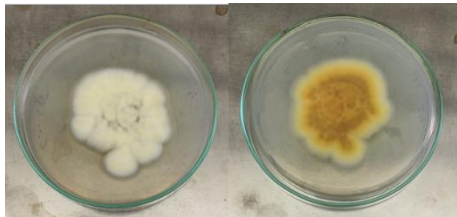
Colonies were initially screened based on their macroscopic features, including colony pigmentation, margin structure, surface texture, and radial expansion. Images of purified fungal colonies (front and back side) isolates are provided in the Table 4.2, along with description of colony morphology. This method of isolation is consistent with prior studies that highlight agricultural soil as a rich reservoir of diverse fungal taxa, especially filamentous fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Cladosporium* (Azmi & Hisham, 2021) (Ghosh et al., 2017). These genera are frequently reported in cultivated soils and are known for their ecological versatility and secondary metabolite production. The observed fungal diversity reflects the ecological richness of cultivated soil environments, which are often amended with organic matter

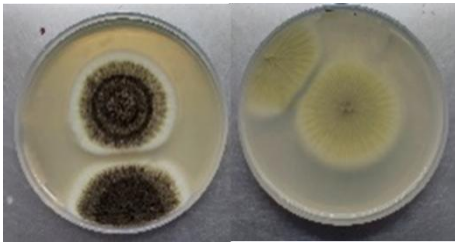
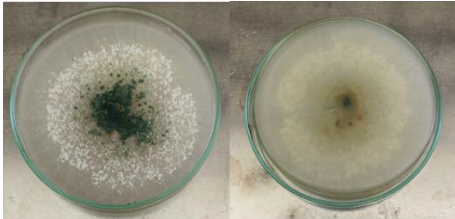
and fertilizers, creating nutrient-rich niches for fungal colonization and metabolic expression (Muhsen & Al-Dossary, 2023).

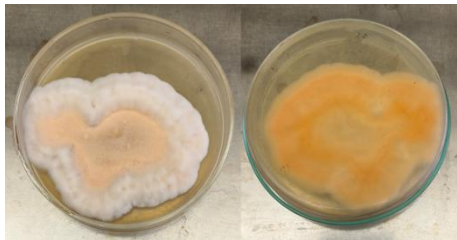
Fungi isolated from agricultural soils are known to possess diverse enzymatic systems that support applications such as bioremediation and nanomaterial synthesis; however, only a limited subset exhibit functional capabilities like nanoparticle biosynthesis, highlighting the importance of broad-spectrum isolation strategies to capture this functional diversity (Adeleke et al., 2024). The diversity of isolated fungi across different soil types suggested the rich and varied mycobiota present in these agricultural fields. These purified isolates were subsequently subjected to screening for their potential to biosynthesize silver nanoparticles.

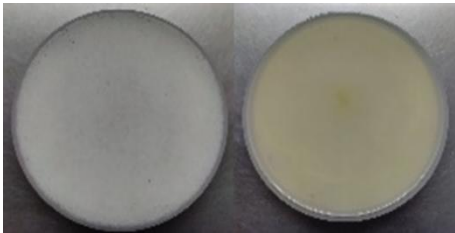
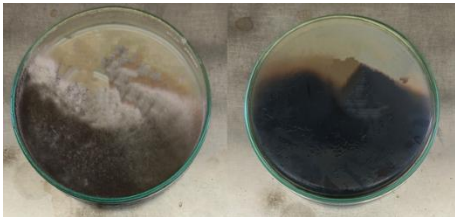
Table 4.2: Colony morphology of fungal isolates obtained from agricultural soil samples

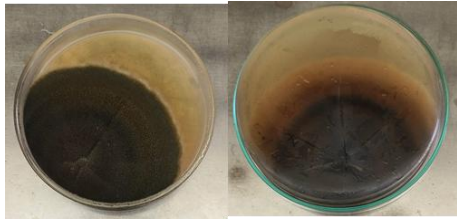
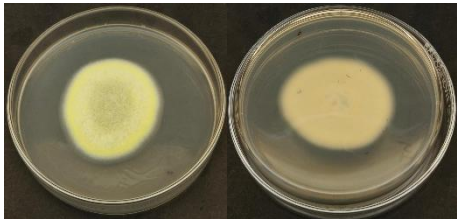
Sample Code	Purified Isolate Code	Purified Isolate Picture		Colony characterization
		Front View	Reverse View	
S1	S1I1			Colonies exhibit a circular, centrally dense growth with a velvety to powdery texture; the obverse displays a deep olive-green pigmentation transitioning into a narrow, well-defined white periphery. The reverse side reveals a bright yellow to mustard hue with a pronounced radial furrowed pattern, and a compact, smooth

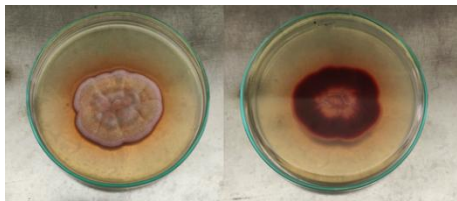
			basal texture suggestive of sporulating mycelia.
	S1I2		Colonies display irregular spreading growth with distinct zonation; the surface is dark bluish-green with granular, powdery conidial tufts and sharply demarcated white margins. The reverse side shows a pale to dull yellow pigmentation with uneven distribution and diffuse boundaries, suggesting heterogeneous metabolic activity.
	S1I3		Colonies are irregularly shaped with a cottony to floccose texture, predominantly white with subtle central elevation and lobed edges indicating active radial expansion. The reverse side exhibits a rich amber to rust-brown pigmentation

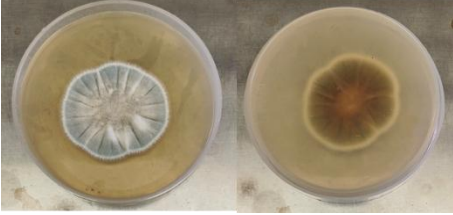
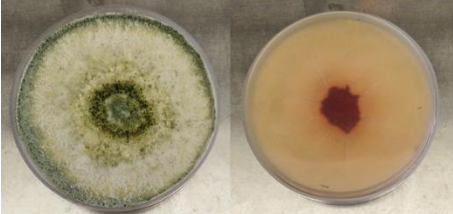
			concentrated at the center, gradually fading toward the margins with a translucent halo.
	S1I4		Colonies are circular with well-defined concentric zonation, featuring a dense, dark brown to black central region surrounded by a pale outer ring with velvety texture and low relief. The reverse side reveals a pale yellow background with a radially striated pattern and slightly raised center, indicating active sporulation.
	S1I5		Colonies exhibit compact, centrally pigmented growth with a dark green core and are surrounded by dense white tufts of aerial mycelium in a finely granular, radial distribution. The reverse side is pale with a faint olive tint radiating from the center, where a dark

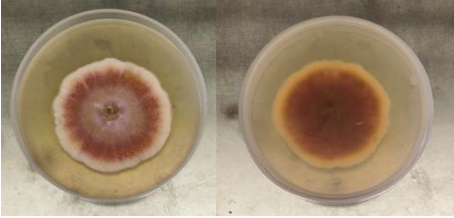
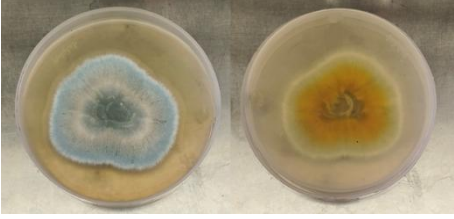
			pinpoint core suggests localized sporulation.
	S1I6		Colonies are circular with a distinctive concentric zonation pattern, exhibiting a central purple core that fades into pink and then white toward the edges, with a powdery surface and evenly scalloped margins. The reverse side displays intense reddish pigmentation with a darkened center and radiating bands, indicating strong secondary metabolite production.
S2	S2I1		Colonies exhibit irregular spreading growth with a dense, cottony white periphery enclosing a central peach to light salmon-colored zone, with undulating margins and a layered surface suggesting aerial mycelial development. The reverse side is diffusely pigmented with

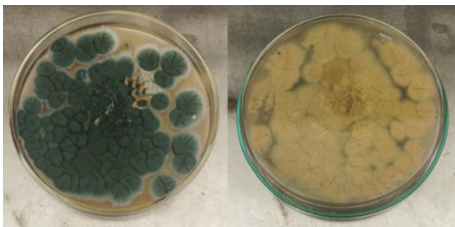
			a uniform orange hue, intensifying toward the center and fading at the edges.
	S2I2		Colonies exhibit uniform, circular growth with a smooth, powdery white surface lacking visible zonation or aerial structures, indicative of sparse sporulation. The reverse side is pale cream to off-white with minimal pigmentation and no distinct pattern, reflecting a homogenous mycelial mat.
	S2I3		Colonies show asymmetrical, spreading growth with a dark gray to black surface, interspersed with patches of white aerial mycelium and occasional violet undertones, giving a mottled appearance. The reverse side is intensely pigmented, ranging from dark brown to near-black, with sharp contrast at the margins,

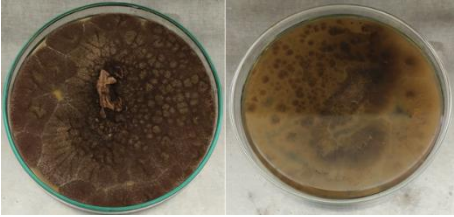
			suggesting strong pigment secretion into the medium.
S3	S3I1		Colonies are dark olive to nearly black with a dense, velvety texture and sharply defined circular margins; surface appears zonate with subtle radial grooves extending from the center. The reverse side is deeply pigmented with concentric brown to black rings and visible radial fissures, indicative of compact hyphal growth and high pigment diffusion.
	S3I2		Colonies exhibit compact, circular growth with a finely velvety surface texture. The upper surface displays a pale yellow to light green pigmentation that intensifies toward the center, producing a subtle zonation pattern. Margins remain smooth and well-defined,

			indicating uniform radial expansion. The reverse side shows a dull yellow to ochre pigmentation with a slightly darker central region, suggesting moderate pigment diffusion into the medium and balanced metabolic activity.
	S3I3		Colonies are circular with a lobed, wrinkled surface and a central pinkish-purple pigmentation that gradually diffuses into a translucent beige periphery; surface appears compact with low aerial mycelium. The reverse side displays intense maroon to wine-red pigmentation concentrated centrally, radiating outward into paler reddish zones, indicating strong pigment exudation into the medium.
	S4I1		Colonies are centrally raised with a distinctive

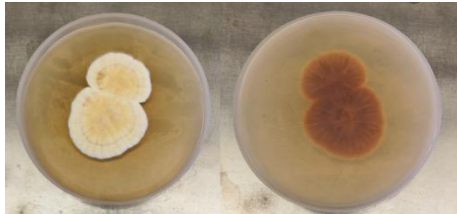
S4			radial furrowing pattern; the surface is bluish-gray with a dense, velvety texture surrounded by a white marginal zone, giving a starburst appearance. The reverse side shows a central reddish-brown pigmentation with radiating lines that mirror the obverse furrows, suggesting coordinated hyphal growth and pigment deposition.
	S4I2		Colonies are circular with a central dark green sporulating zone, gradually transitioning into a floccose white outer ring; the surface is textured with a dense mat of aerial mycelium and sharply defined margins. The reverse side exhibits intense central maroon pigmentation radiating into a peach-colored background, indicative of strong diffusible pigment production.

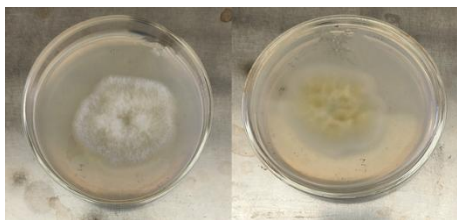
	S4I3		Colonies are circular with a velvety surface, exhibiting a central reddish-purple zone surrounded by pink to white concentric bands, and a well-defined, scalloped margin. The reverse side displays a uniform reddish-brown pigmentation fading into a light peach periphery, indicating consistent pigment diffusion across the medium.
	S4I4		Colonies are irregularly circular with a central raised zone; the surface displays bluish-green pigmentation encircled by a white outer ring and subtle concentric ridging, giving a zonated appearance. The reverse side shows a bright yellow to orange gradient with deeper central pigmentation, indicative of metabolite secretion into the medium.

	S4I5		Colonies exhibit dense, velvety dark green growth with prominent radial folds and lobed sectoring, interspersed with light tan zones and sharply scalloped margins. The reverse side shows yellowish-brown pigmentation with visible sector lines and pale halos, reflecting compartmentalized metabolic activity.
S5	S5I1		Colonies are circular with a floccose, cottony white surface and well-defined radial grooves; sectoring is evident between the two growth centers, suggesting co-inoculation or mixed morphology. The reverse side presents rich orange to ochre pigmentation with radiating zones, corresponding to the surface furrows and indicating active metabolite diffusion.

	S5I2		Colonies display a dense, cottony white surface with pronounced sectoring and wrinkled folds, forming a cerebriform pattern especially near the central region. The reverse side is marked by intense reddish-brown pigmentation with circular patches and darker zones centrally, suggesting strong pigment production and zonated metabolite diffusion.
	S5I3		Colonies exhibit a dark brown, leathery surface with prominent radial cracking and raised central tissue, giving a crust-like texture; the surface is matte and zonated with concentric folds. The reverse side presents uneven brown pigmentation with darker blotches and sectoring, reflecting dense substrate

			invasion and pigment secretion.
	S5I4		Colonies are circular with a fluffy, cottony white surface and a denser central tuft, gradually spreading to form a uniform mat with indistinct margins. The reverse side displays a light orange background with a concentrated yellow-brown spot in the center, indicating localized pigment production.
S6	S6I1		Colonies are centrally compact with a star-like, wrinkled structure exhibiting pinkish-white margins and a central orange zone, surrounded by diffuse mycelial outgrowth. The reverse side shows a deep reddish-brown core with irregular orange blotches radiating outward, suggesting active

			pigment secretion and zonal sporulation.
	S6I2		Colonies display dual circular growths with a floccose surface, cream-colored centers, and white lobed margins showing prominent radial furrows; the surface is compact and slightly raised. The reverse side exhibits rich reddish-brown pigmentation with well-defined sectoring, mirroring the surface zonation and indicating active sporulation in both regions.
	S6I3		Colonies exhibit uniform, densely textured growth with concentric zonation and radial grooves; the surface is brownish-olive with a velvety texture and a prominent central ridge likely from initial inoculation. The reverse side reveals consistent dark brown pigmentation

			with mirrored radial striations, indicating uniform hyphal penetration and pigment diffusion.
	S6I4		Colonies are compact with a low, woolly white surface and uneven circular margins, showing faint radial zoning and a slightly elevated central zone. The reverse side presents a translucent cream to pale yellow pigmentation with diffuse boundaries, indicating minimal pigment secretion into the agar.

4.2 Biosynthesis of Silver Nanoparticles and Preliminary Characterization

The biosynthesis of AgNPs was evaluated using both extracellular and intracellular fungal extracts derived from 25 morphologically distinct soil isolates. For each reaction, 19 mL of 1 mM AgNO₃ was mixed with 1 mL of fungal extract, while controls were prepared using 1 mL sterile distilled water in place of the fungal component. Visual monitoring of the reaction mixtures revealed early evidence of nanoparticle formation through distinct color changes (brown to dark brown), which are known to result from surface plasmon resonance (SPR) excitation associated with silver nanoparticle synthesis. Figure 4.1-4.4 illustrates representative vials showing clear differences between controls and test reactions.

Among the 50 samples (25 Intracellular & 25 Extracellular) tested, only 8 samples including S1I4 (intracellular), S1I4 (extracellular), S2I2, S3I1, S3I3, S4I3 and S5I1 (intracellular), S5I1 (extracellular) exhibited positive biosynthesis responses (Color change) indicating the silver nanoparticle formation. Out of a total of 50 reactions performed from 25 fungal isolates, two isolates (S1I4 and S5I1) produced silver nanoparticles from both extracellular and intracellular extracts, while four other isolates (S2I2, S3I1, S3I3, S4I3) showed nanoparticle synthesis only with intracellular extracts. Based on above observation it can be emphasised that the silver nanoparticle formation is highly strain specific and method dependent. This variation may be attributed to differences in the metabolic and enzymatic profiles of the fungal isolates. Only certain strains possess sufficient reductase enzymes and bioactive metabolites required for the reduction of silver ions and stabilization of nanoparticles. Additionally, variations in protein secretion and redox potential among isolates may influence their nanoparticle synthesis capability. The following figures (Figures 4.1–4.4) present the visual outcomes of silver nanoparticle biosynthesis experiments conducted with all 25 fungal isolates under intracellular and extracellular conditions. Each panel shows three vials per isolate: (A) 1 mM AgNO_3 solution (control), (B) fungal extract (Intracellular or Extracellular), and (C) the corresponding reaction mixture (1 mM AgNO_3 + Fungal Extract) after 48 h. The appearance of yellow to brown coloration in vial C represents positive AgNP formation, while no change in color confirms the absence of silver nanoparticles formation.

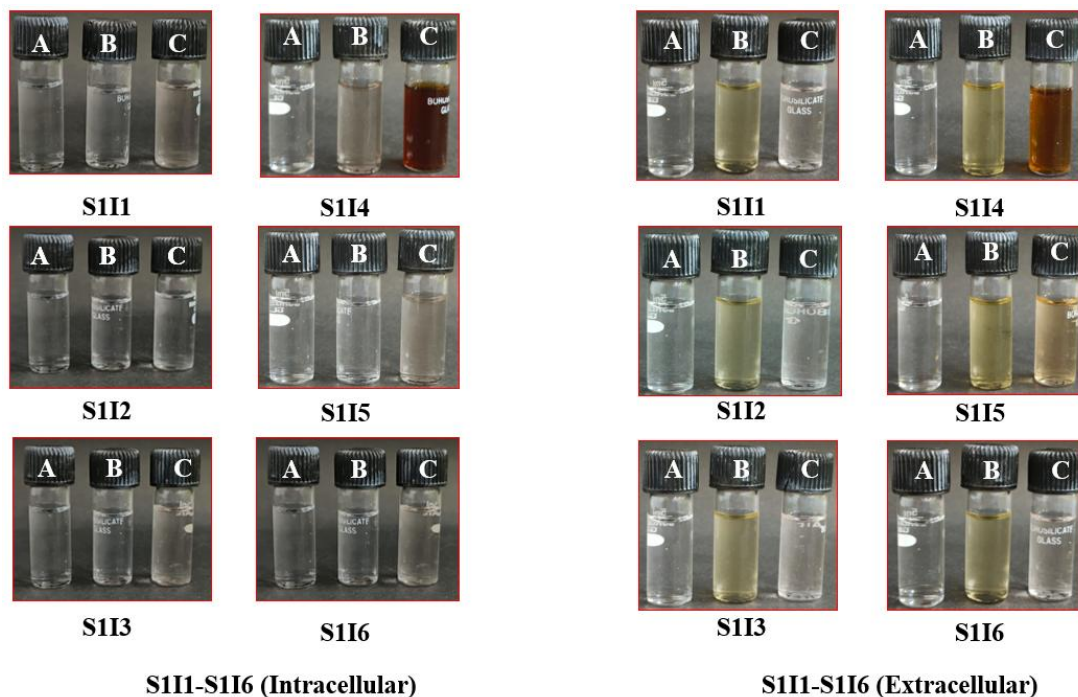


Figure 4.1: Biosynthesis of AgNPs by fungal isolates S1I1–S1I6 under intracellular and extracellular conditions. For each isolate, three vials are shown: (A) 1 mM AgNO₃ solution (control), (B) fungal extract (intracellular or extracellular), and (C) reaction mixture after 48 h. The distinct change from colorless to brown/yellow in vial C confirms nanoparticle formation via fungal-mediated bioreduction.

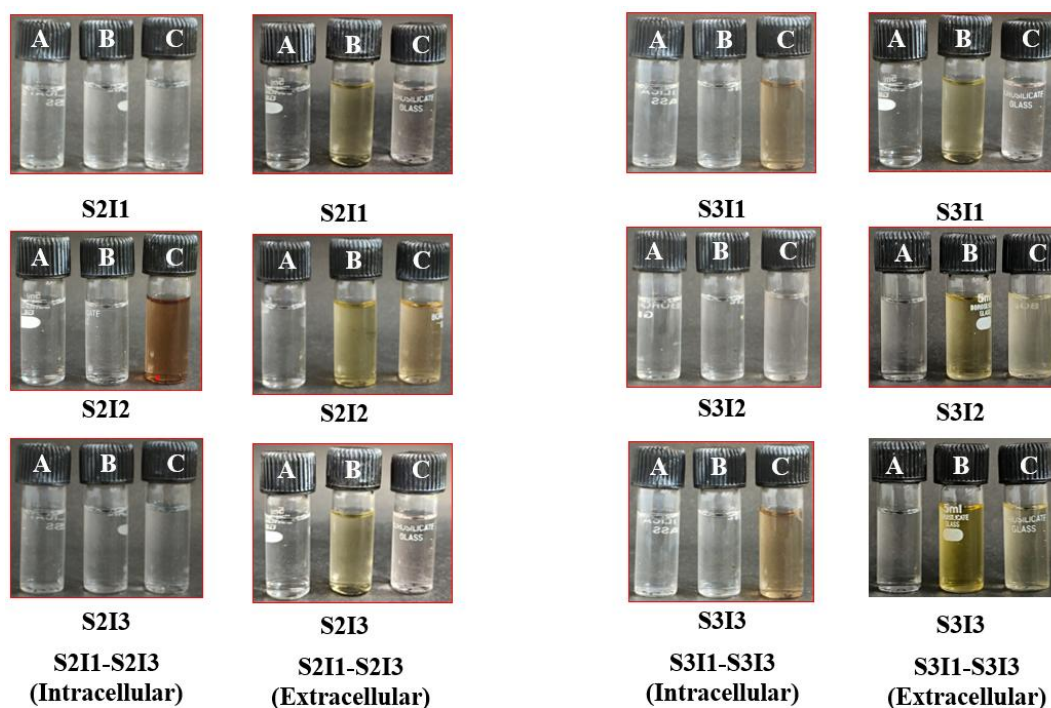


Figure 4.2: Silver nanoparticle biosynthesis by fungal isolates S2I1–S2I3 and S3I1–S3I3 under intracellular and extracellular conditions. For each isolate, three vials are displayed: (A) AgNO_3 solution (1 mM) as control, (B) fungal extract, and (C) reaction mixture after 48 h. The visible shift from colorless to brown or yellow in vial C confirms successful fungal-mediated bio-reduction of silver ions into silver nanoparticles.

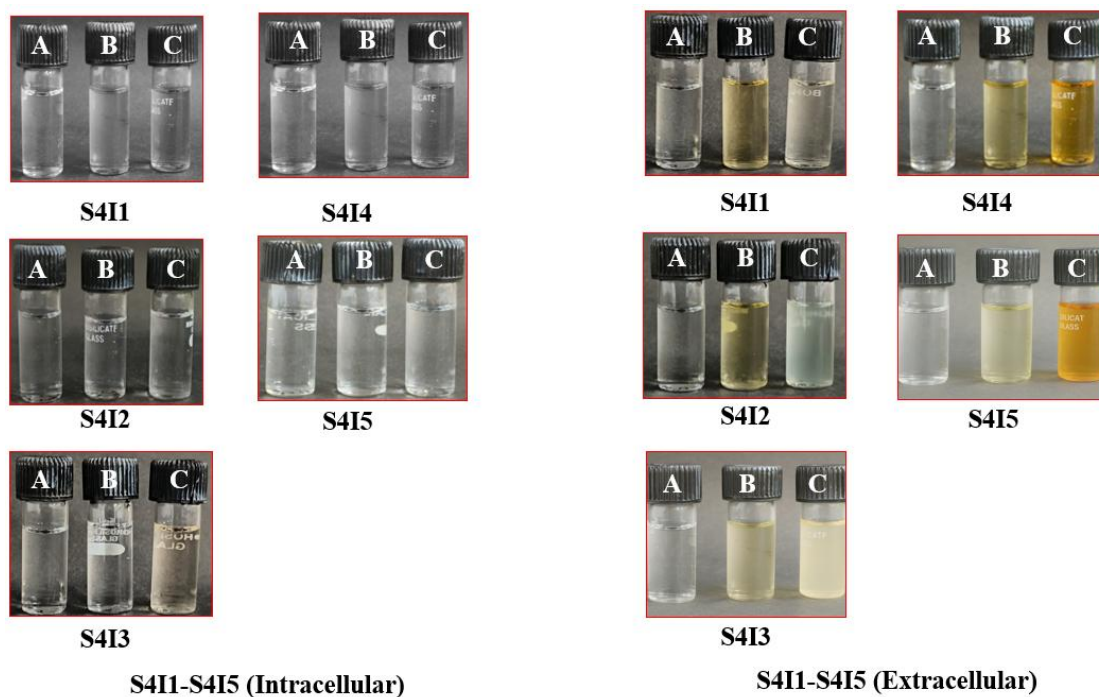


Figure 4.3: Silver nanoparticle biosynthesis by fungal isolates S4I1–S4I5 under intracellular and extracellular conditions. Each isolate is represented with three vials: (A) AgNO_3 solution (1 mM) as control, (B) fungal extract, and (C) reaction mixture after 48 hour. The visible color change in vial C from colorless to yellow or brown indicates successful fungal-mediated reduction of silver ions and confirms AgNP formation.

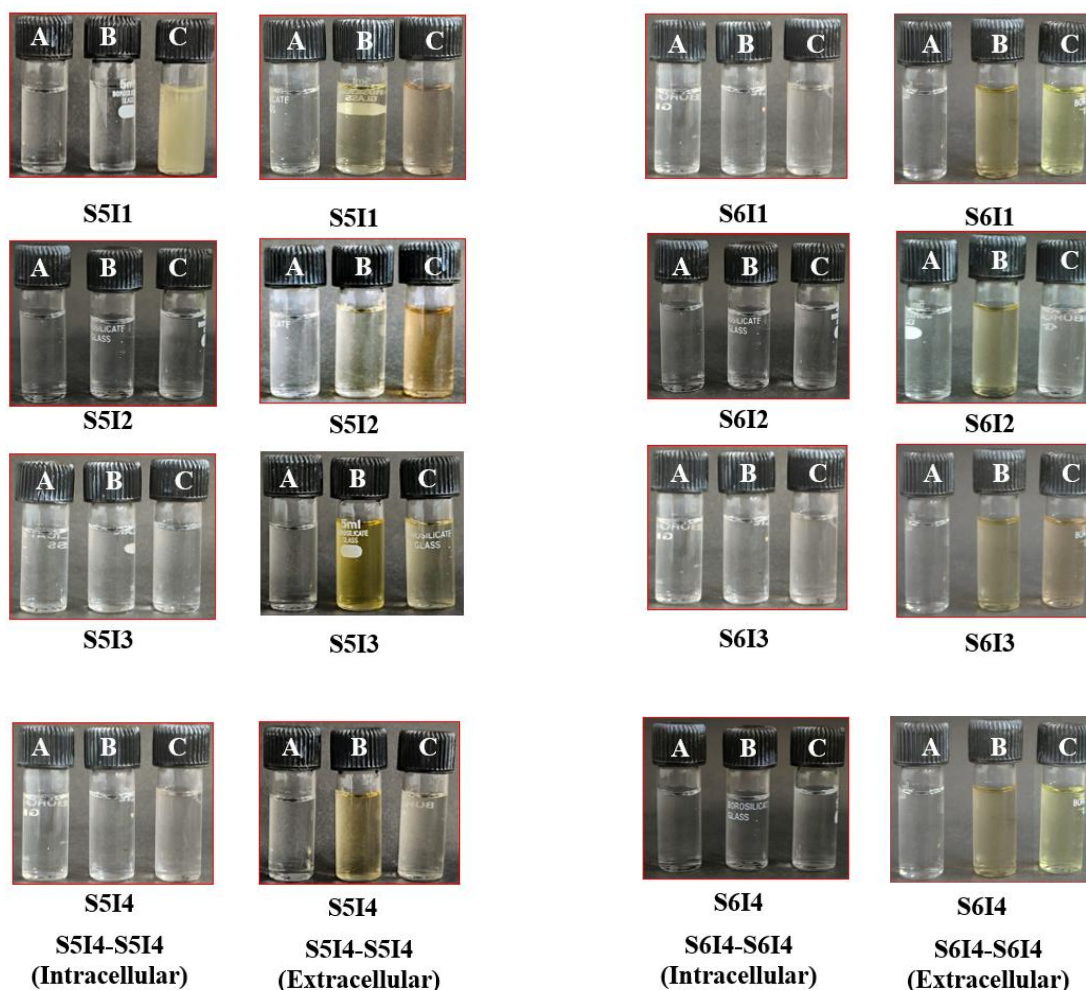


Figure 4.4: Silver nanoparticle biosynthesis by fungal isolates S5I1–S5I4 and S6I1–S6I4 under intracellular and extracellular conditions. For each isolate, three vials are presented: (A) AgNO_3 solution (1 mM) as control, (B) fungal extract, and (C) reaction mixture after 48 hours. The observed color change in vial C from colorless to yellow or brown indicates fungal-mediated reduction of silver ions, confirming the successful formation of silver nanoparticles.

UV–Visible spectroscopy further confirmed the successful synthesis of AgNPs across all eight fungal isolates, as evidenced by visible color changes and distinct absorption peaks ranging from 391 to 450 nm. These peaks correspond to the surface plasmon resonance (SPR) bands typically associated with AgNPs, consistent with earlier reports on fungal-mediated synthesis (Elamawi et al., 2018; Gupta et al., 2022; Hashem et al., 2022). Variations in SPR peak position are known to correlate with nanoparticle size

and distribution, where a blue shift (lower wavelength) typically indicates smaller particle size, while a red shift (higher wavelength) suggests larger or aggregated particles. Thus, the observed SPR range reflects differences in particle size among the synthesized nanoparticles. Notably, the presence of single, sharp SPR peaks in most samples suggests the formation of relatively monodispersed nanoparticles, which is advantageous for biomedical and antimicrobial applications. Among biosynthesized AgNPs, isolates S1I4 and S5I1 demonstrated positive biosynthetic activity via both extracellular and intracellular mechanisms, highlighting their high redox potential and the likely involvement of robust fungal enzymatic systems. This aligns with previous reports which reported AgNP synthesis by *Beauveria bassiana* under optimized pH and temperature conditions, with peak UV-Vis absorbance between 420–454 nm attributed to the action of extracellular enzymes facilitating metal ion reduction (Soleimani et al., 2022). Similarly, recent studies confirmed that *Cephalosporium aphidicola*-derived AgNPs exhibit SPR peaks at ~420 nm and possess strong antimicrobial and antioxidant activity, further validating the efficiency of fungal routes in generating functionally stable AgNPs (Barabadi et al., 2025). These observations suggest that fungal systems, particularly isolates with dual-route biosynthetic capacity, could serve as versatile nanofactories for producing AgNPs with high monodispersity, yield, and biological activity. Table 4.3 summarizes the spectrometric responses and UV–Vis absorption data for all tested fungal isolates.

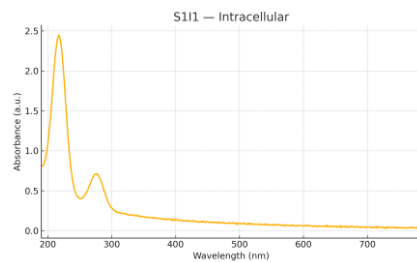
Table 4.3: Summary of biosynthesis of silver nanoparticles by fungal isolates, color change, and UV-Vis spectroscopic analysis.

Sample Name	Extract	Color Change	Characteristics AgNPs Peak
S1I1	Intracellular	-	NA
	Extracellular	-	NA
S1I2	Intracellular	-	NA
	Extracellular	-	NA
S1I3	Intracellular	-	NA
	Extracellular	-	NA

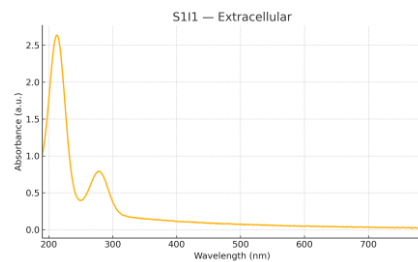
S1I4	Intracellular	Dark Brown	418 nm
	Extracellular	Dark Brown	426 nm
S1I5	Intracellular	-	NA
	Extracellular	Light Brown	NA
S1I6	Intracellular	-	NA
	Extracellular	-	NA
S2I1	Intracellular	-	NA
	Extracellular	Light Brown	NA
S2I2	Intracellular	Brown	417 nm
	Extracellular	Light Brown	NA
S2I3	Intracellular	-	NA
	Extracellular	Light Brown	NA
S3I1	Intracellular	Light Brown	427 nm
	Extracellular	-	NA
S3I2	Intracellular	-	NA
	Extracellular	-	NA
S3I3	Intracellular	Light Brown	427 nm
	Extracellular	-	NA
S4I1	Intracellular	-	NA
	Extracellular	Light Yellow	NA
S4I2	Intracellular	-	NA
	Extracellular	Light Green	NA
S4I3	Intracellular	Light Brown	391 nm
	Extracellular	Whitish Yellow	NA
S4I4	Intracellular	-	NA
	Extracellular	Yellow	NA
S4I5	Intracellular	-	NA
	Extracellular	Yellow	NA
S5I1	Intracellular	Whitish Yellow	436 nm
	Extracellular	Brown	450 nm
S5I2	Intracellular	-	NA

	Extracellular	Light Brown	NA
S5I3	Intracellular	-	NA
	Extracellular	Light Yellow	NA
S5I4	Intracellular	-	NA
	Extracellular	-	NA
S6I1	Intracellular	-	NA
	Extracellular	Light Yellow	NA
S6I2	Intracellular	-	NA
	Extracellular	-	NA
S6I3	Intracellular	-	NA
	Extracellular	Light Brown	NA
S6I4	Intracellular	-	NA
	Extracellular	Light Yellow	NA

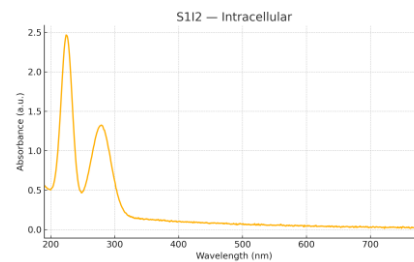
Figure 4.5-4.10 displays UV–Vis spectra for all 50 reactions, positive sample highlighting the spectral peaks indicative of successful nanoparticle synthesis. These findings collectively reinforce the idea that fungal-mediated AgNP biosynthesis is influenced not only by species-level traits, but also by mode of extract (intra vs. extra), biomass condition, and redox potential of the medium. Importantly, the variability observed supports the need for broad screening and optimization in selecting potent fungal candidates for scalable green nanomaterial production (Qamandar & Shafeeq, 2017).



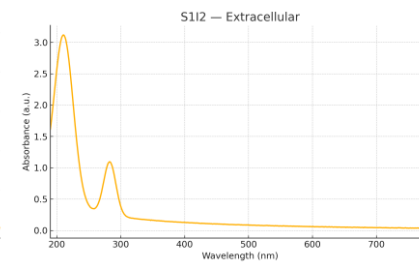
(a) S1I1 Intracellular



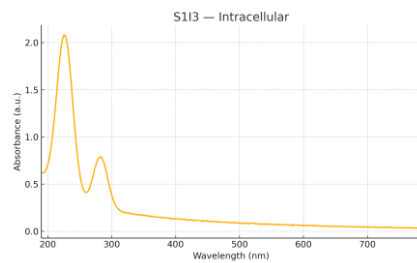
(b) S1I1 Extracellular



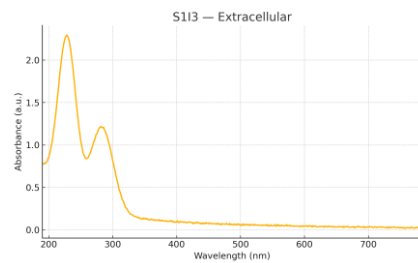
(c) S1I2 Intracellular



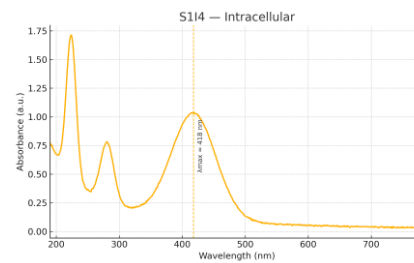
(d) S1I2 Extracellular



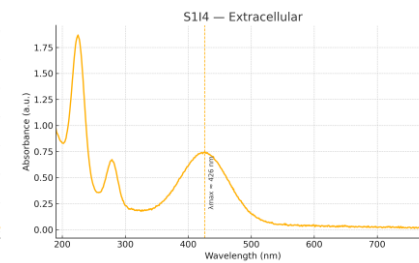
(e) S1I3 Intracellular



(f) S1I3 Extracellular



(g) S1I4 Intracellular



(h) S1I4 Extracellular

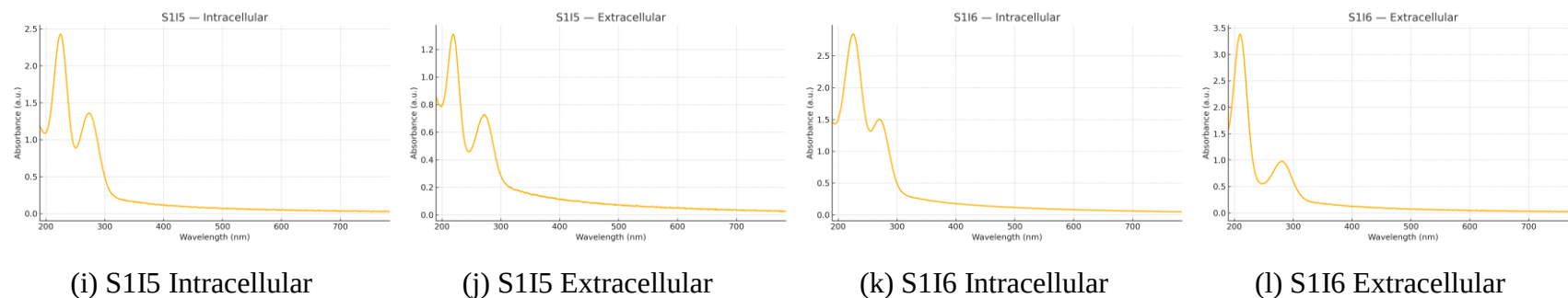


Figure 4.5: UV–Vis spectra for S1 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S1I1 Intracellular; (b) S1I1 Extracellular; (c) S1I2 Intracellular; (d) S1I2 Extracellular; (e) S1I3 Intracellular; (f) S1I3 Extracellular; (g) S1I4 Intracellular; (h) S1I4 Extracellular; (i) S1I5 Intracellular; (j) S1I5 Extracellular; (k) S1I6 Intracellular; (l) S1I6 Extracellular.

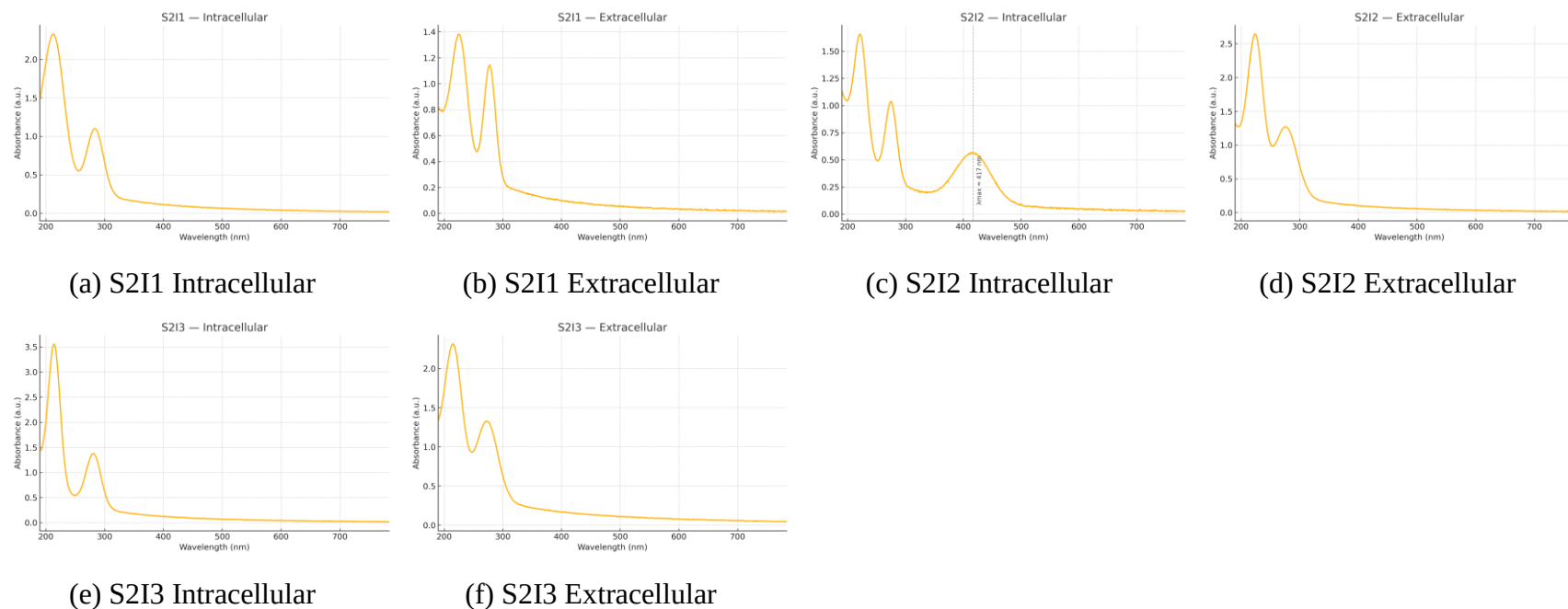


Figure 4.6: UV–Vis spectra for S2 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S2I1 Intracellular; (b) S2I1 Extracellular; (c) S2I2 Intracellular; (d) S2I2 Extracellular; (e) S2I3 Intracellular; (f) S2I3 Extracellular.

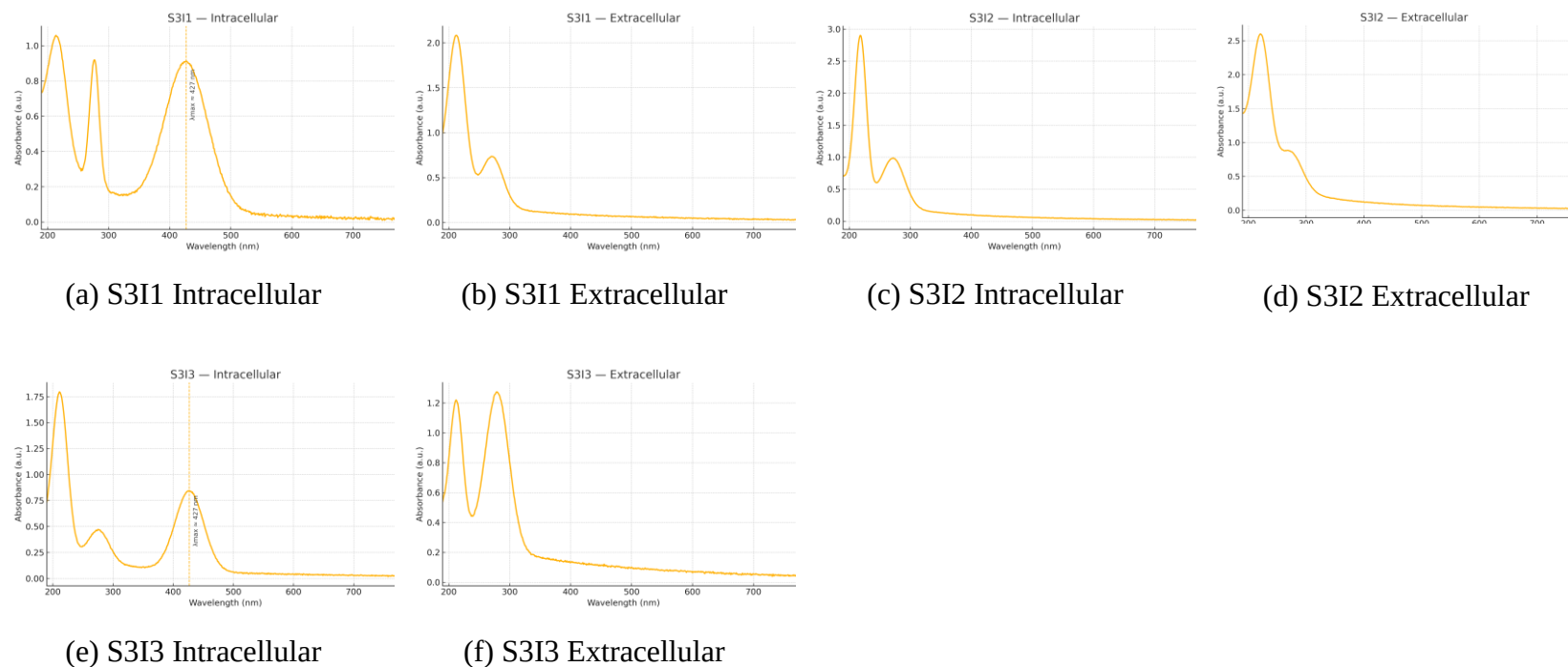
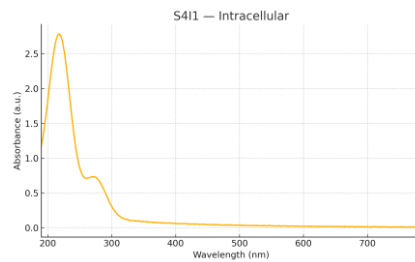
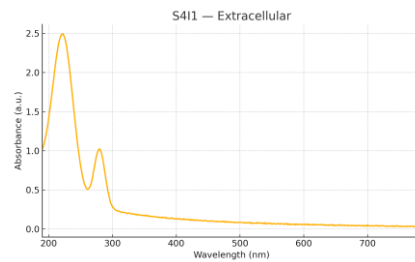


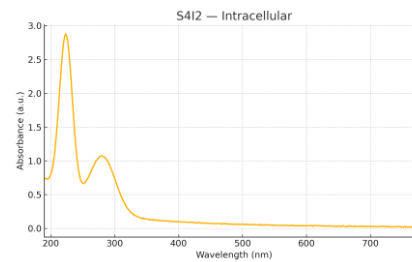
Figure 4.7: UV–Vis spectra for S3 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S3I1 Intracellular; (b) S3I1 Extracellular; (c) S3I2 Intracellular; (d) S3I2 Extracellular; (e) S3I3 Intracellular; (f) S3I3 Extracellular.



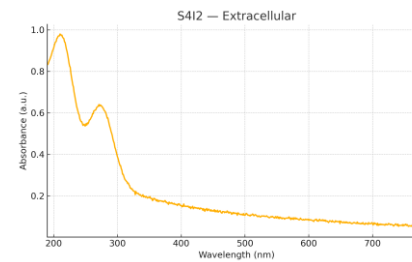
(a) S4I1 Intracellular



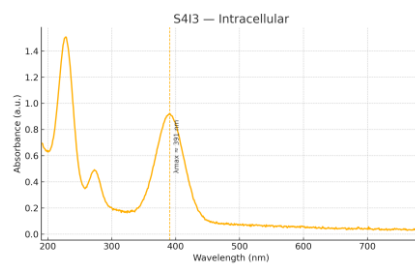
(b) S4I1 Extracellular



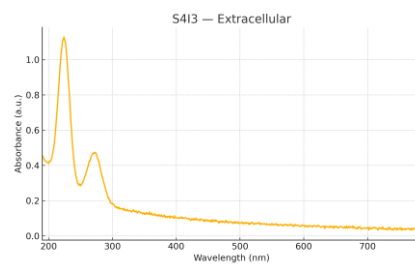
(c) S4I2 Intracellular



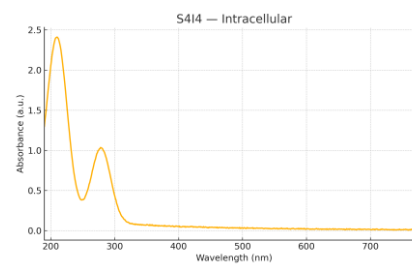
(d) S4I2 Extracellular



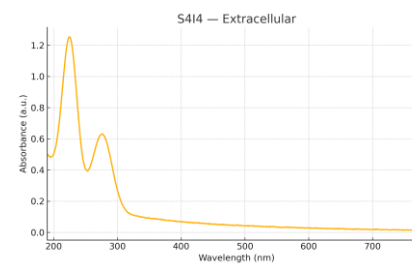
(e) S4I3 Intracellular



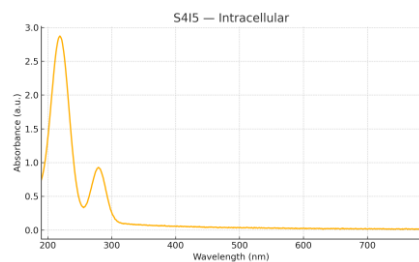
(f) S4I3 Extracellular



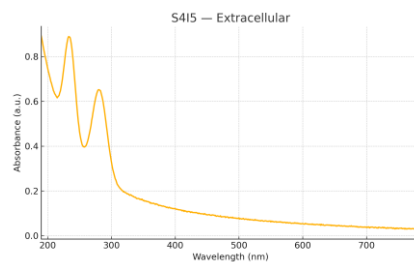
(g) S4I4 Intracellular



(h) S4I4 Extracellular



(i) S4I5 Intracellular



(j) S4I5 Extracellular

Figure 4.8: UV–Vis spectra for S4 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S4I1 Intracellular; (b) S4I1 Extracellular; (c) S4I2 Intracellular; (d) S4I2 Extracellular; (e) S4I3 Intracellular; (f) S4I3 Extracellular; (g) S4I4 Intracellular; (h) S4I4 Extracellular; (i) S4I5 Intracellular; (j) S4I5 Extracellular.

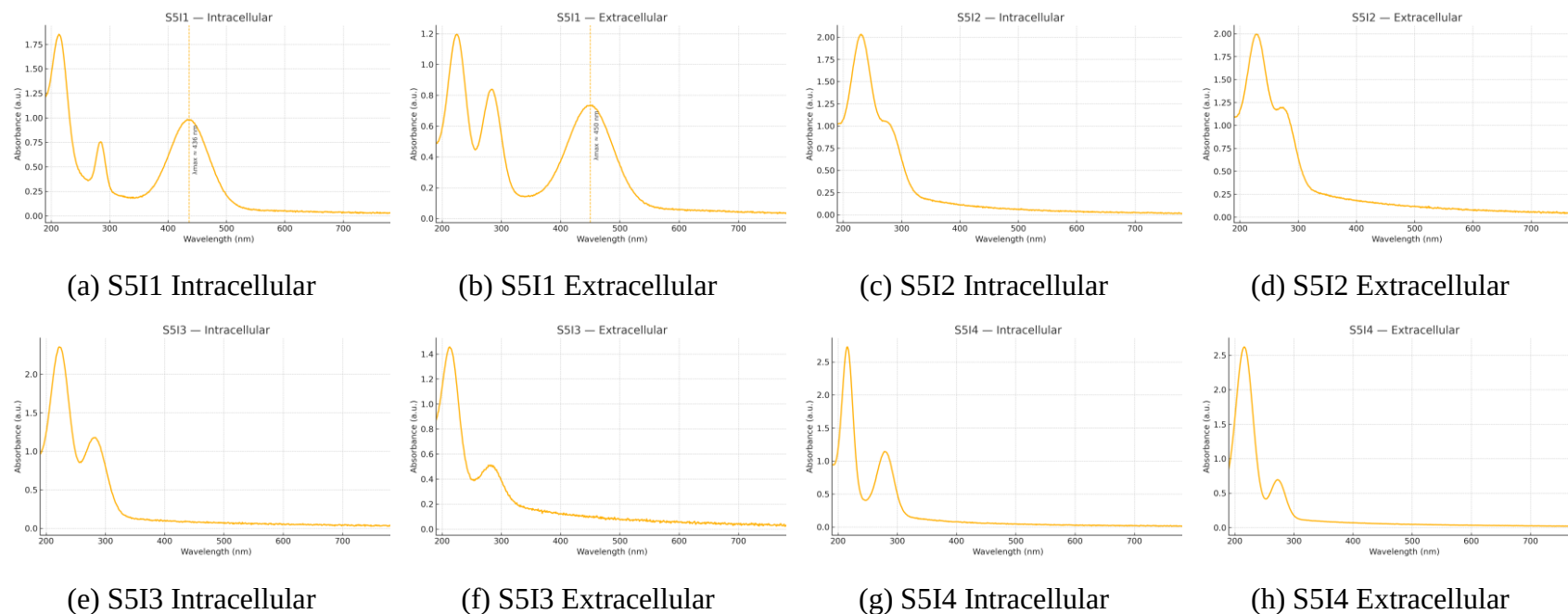


Figure 4.9: UV–Vis spectra for S5 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S5I1 Intracellular; (b) S5I1 Extracellular; (c) S5I2 Intracellular; (d) S5I2 Extracellular; (e) S5I3 Intracellular; (f) S5I3 Extracellular; (g) S5I4 Intracellular; (h) S5I4 Extracellular.

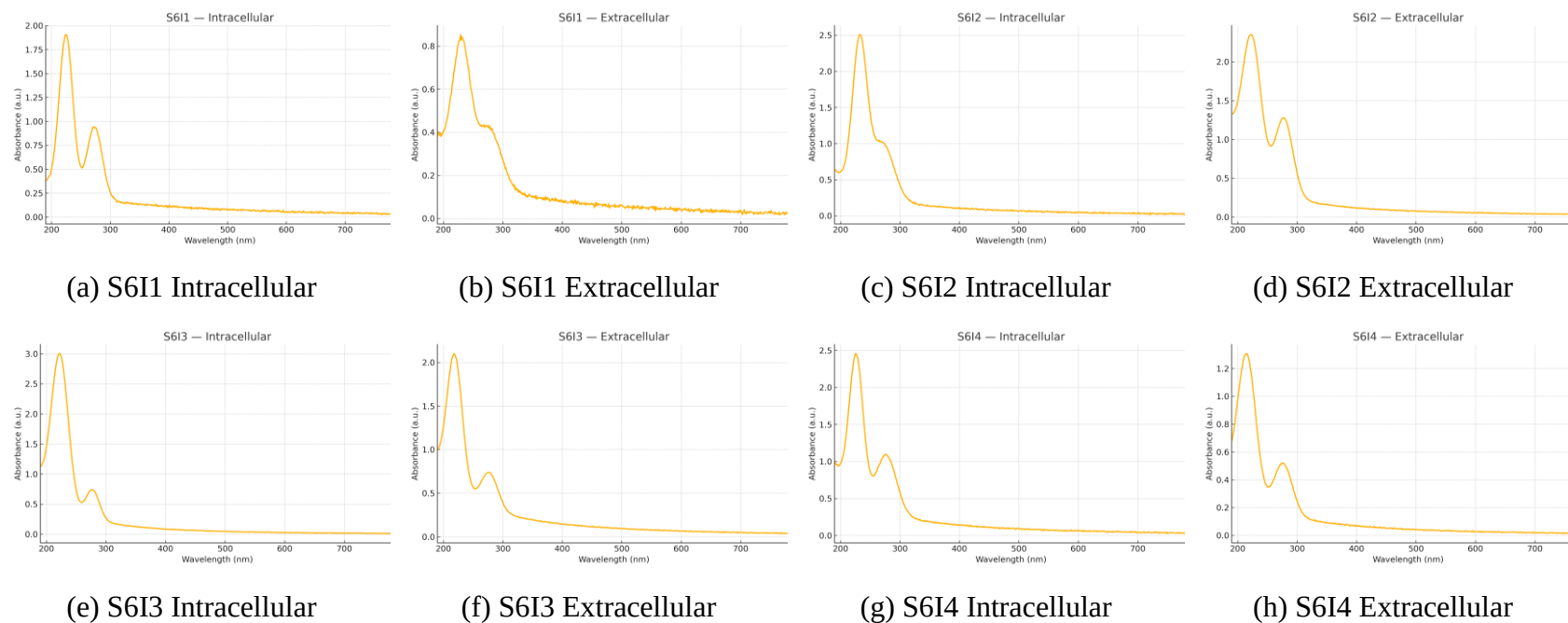


Figure 4.10: UV–Vis spectra for S6 isolates. Each graph shows absorbance profiles between 200–800 nm. Prominent surface plasmon resonance (SPR) peaks observed between 390–450 nm confirms successful AgNP formation, with peak intensity and sharpness varying by isolate and extract type. (a) S6I1 Intracellular; (b) S6I1 Extracellular; (c) S6I2 Intracellular; (d) S6I2 Extracellular; (e) S6I3 Intracellular; (f) S6I3 Extracellular; (g) S6I4 Intracellular; (h) S6I4 Extracellular.

4.3 Collection and Identification of Clinical Isolates

Clinical isolates of carbapenem-resistant *E. coli* and *K. pneumoniae* were obtained from patient specimens collected at the Punjab Institute of Medical Sciences (PIMS), Jalandhar. These isolates were re-characterized to confirm their identity, beginning with culture on Eosin Methylene Blue (EMB) agar (Figure 4.11). On EMB, *E. coli* produced characteristic dark purple colonies with a metallic green sheen, indicative of strong lactose fermentation and acid production (Left). In contrast, *K. pneumoniae* exhibited large, pink, mucoid colonies without metallic sheen, consistent with slower lactose fermentation and the presence of a polysaccharide capsule (Right). These observations are in agreement with classical colony morphology of *E. coli* and *K. pneumoniae* (Kale & Moon, 2024).

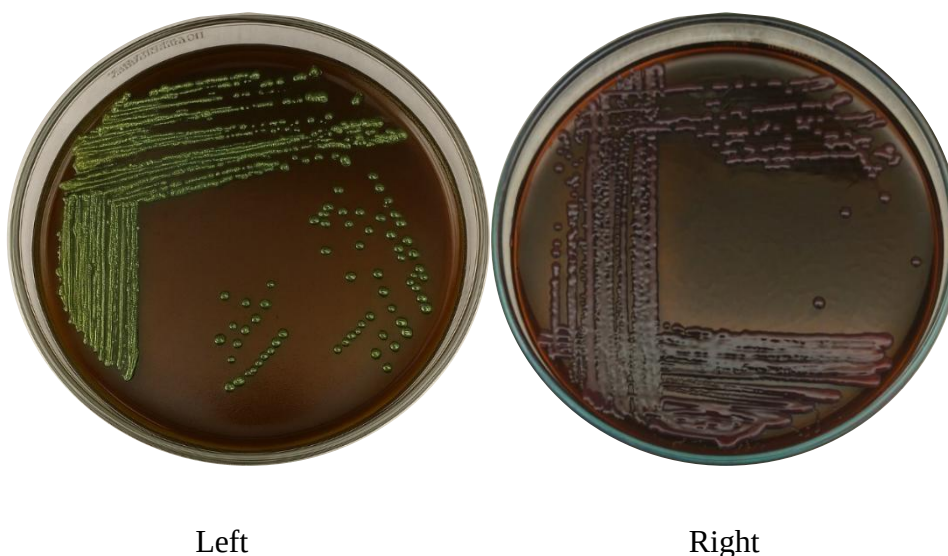


Figure 4.11: Identification of clinical isolates on EMB agar. (Left) Colonies of *E. coli* exhibiting characteristic dark purple coloration with metallic green sheen. (Right) Colonies of *K. pneumoniae* displaying large, mucoid, pink-coloured appearance without metallic sheen

Subsequently, a panel of biochemical tests was performed for definitive confirmation (Table 4.4). *E. coli* was indole positive, methyl red (MR) positive, and negative for citrate, urease, and Voges-Proskauer (VP). Its TSI profile showed acid/acid reaction with gas production but no H₂S, while oxidase was negative and catalase positive. In

contrast, *K. pneumoniae* was indole and MR negative but VP, citrate, and urease positive. Its TSI profile also revealed acid/acid with gas but no H₂S, with oxidase negative and catalase positive. These complementary biochemical signatures are consistent with established diagnostic patterns for these enteric pathogens (Pirko, 2015). The combined use of EMB morphology and biochemical testing confirmed the identity of the isolates as carbapenem-resistant *E. coli* and *K. pneumoniae*. This rigorous re-characterization was critical to ensure accuracy in selecting clinically relevant strains for subsequent evaluation of the antifungal-derived silver nanoparticles.

Table 4.4: Biochemical characterization of clinical isolates.

Test	<i>E. coli</i>	<i>K. pneumoniae</i>
Gram Staining	Gram-negative rods	Gram-negative rods
Indole	Positive (+)	Negative (–)
Methyl Red (MR)	Positive (+)	Negative (–)
Voges-Proskauer (VP)	Negative (–)	Positive (+)
Citrate	Negative (–)	Positive (+)
Urease	Negative (–)	Positive (+)
Triple Sugar Iron (TSI)	Acid/Acid, Gas (+), No H ₂ S	Acid/Acid, Gas (+), No H ₂ S
Oxidase	Negative (–)	Negative (–)
Catalase	Positive (+)	Positive (+)

The colony morphology and biochemical test results confirmed the identities of the isolates as *E. coli* and *K. pneumoniae*. The identification strategy used aligns with classical microbiological protocols and remains a cornerstone in clinical diagnostics due to its cost-effectiveness and reliability (Spiga et al., 2021). These phenotypic approaches not only aid in rapid preliminary identification but also reflect the conserved metabolic and structural traits of the organisms, often linked to their pathogenic mechanisms.

4.3 Screening for Carbapenem Resistance

The antibiotic resistance profile of the clinical isolates was assessed using the disk diffusion method to determine susceptibility to carbapenems by using meropenem and imipenem. The clinical isolates of *E. coli* and *K. pneumoniae*, previously confirmed by morphological and biochemical tests, were cultured on Mueller-Hinton agar, and antibiotic-impregnated disks [10 µg each of meropenem and imipenem (HiMedia)] were applied to evaluate their susceptibility patterns (Figure 4.12). Following 18 hours of incubation at $35 \pm 2^\circ\text{C}$, zones of inhibition were measured and interpreted according to the guidelines issued by the Clinical and Laboratory Standards Institute (CLSI, 2023). For both tested antibiotics, a zone diameter of ≥ 23 mm was categorized as susceptible, 20–22 mm as intermediate, and ≤ 19 mm as resistant. In the present study, both *E. coli* and *K. pneumoniae* clinical isolates exhibited markedly reduced susceptibility to carbapenem antibiotics. *E. coli* showed inhibition zones of 8 mm for both imipenem and meropenem, while *K. pneumoniae* demonstrated no detectable inhibition zone with imipenem and only a 12 mm zone with meropenem. These measurements fall well below the CLSI susceptibility breakpoints typically ≥ 23 mm for meropenem and ≥ 22 mm for imipenem in Enterobacteriaceae confirming the isolates' classification as carbapenem-resistant. These results, underscore the multidrug-resistant profiles of both strains. Representative antibiotic inhibition patterns are illustrated in Figure 4A and 4B. These results were in line with published reports confirming the emergence and rapid spread of carbapenem-resistant strains of *Enterobacteriaceae*, particularly in clinical settings (Grundmann et al., 2010; Li et al., 2018; Olowo-okere et al., 2020). Previous studies have documented that disk diffusion, though simple, remains a highly effective preliminary method for phenotypic detection of carbapenem resistance, especially in resource-limited environments (Niu & Chen, 2018; Sekar, 2015). Moreover, the findings align with molecular studies that correlate phenotypic resistance with the presence of carbapenemase genes, reinforcing the clinical importance of such phenotypic assays as frontline diagnostic tools. The evident resistance in both isolates thus validates their classification as carbapenem-resistant *E. coli* and *K. pneumoniae*, providing a suitable model for evaluating the antimicrobial potential of synthesized silver nanoparticles in subsequent experiments.

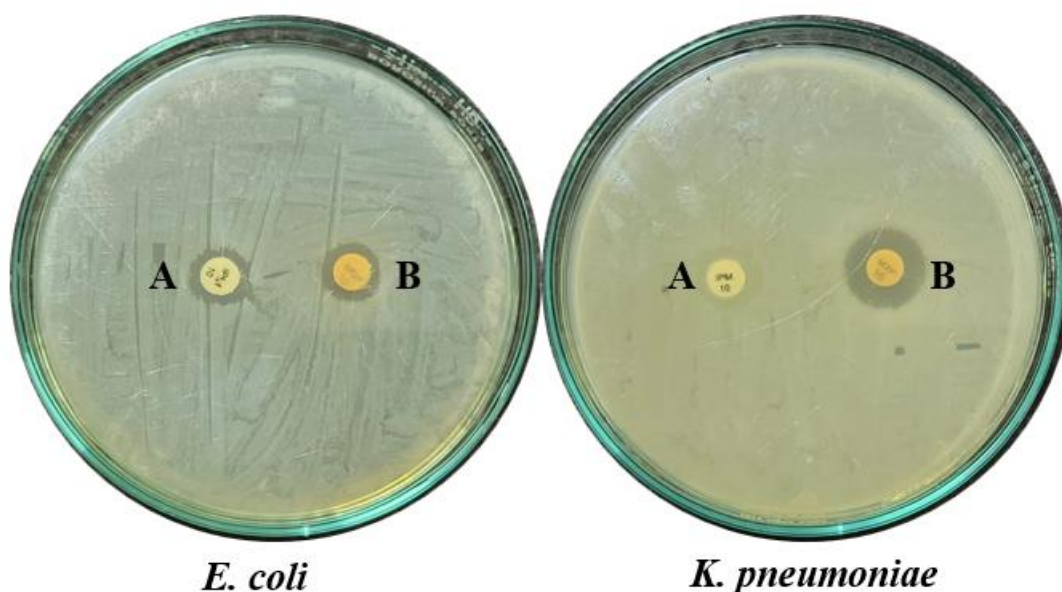


Figure 4.12: Phenotypic confirmation of carbapenem resistance in clinical isolates by disk diffusion assay. Left: *E. coli* showing reduced inhibition zones to imipenem (10 µg/disk A) and meropenem (10 µg/disk B). Right: *K. pneumoniae* exhibiting complete resistance to imipenem (10 µg/disk A) and only a minimal inhibition zone with meropenem (10 µg/ disk B).

4.4 Antibacterial Activity of Biosynthesized AgNPs (Agar Well Diffusion Method)

To comprehensively evaluate the antibacterial efficacy of the biosynthesized silver nanoparticles, a two-step validation strategy was employed. First, six AgNP preparations were screened at 1 mg/ml against a single representative strain each of carbapenem-resistant *E. coli* and *K. pneumoniae* using the agar well diffusion assay. Based on superior performance in this preliminary screening, S1I4 was selected as the most effective candidate. In the second phase, the antibacterial potential of S1I4 was rigorously validated by testing it against a broader panel of ten clinical isolates each of carbapenem resistant *E. coli* and *K. pneumoniae*. This systematic approach ensured that the observed activity was not strain-specific but reproducible across multiple clinical isolates. Subsequent experiments were designed to investigate its dose-dependent efficacy, followed by determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), thereby providing robust validation of the nanoparticle's antibacterial potential.

4.4.1 Initial Screening of AgNPs Against Carbapenem resistant Isolates

The initial antibacterial screening aimed to evaluate the efficacy of six biosynthesized AgNPs formulations against clinically confirmed carbapenem-resistant *E. coli* and *K. pneumoniae* isolates (Figure 4.13). These multidrug-resistant (MDR) strains pose a significant clinical challenge due to limited therapeutic options and complex resistance mechanisms, such as carbapenemase expression, efflux pumps, and porin mutations. All AgNPs were tested at a fixed concentration of 1 mg/mL using the agar well diffusion method. The mean zones of inhibition (mm $\pm$ SD, n = 3), which served as the primary parameter for antibacterial activity, are presented in Table 4.5, and representative inhibition patterns are shown in Figure 4.13. Among the formulations, AgNPs synthesized by isolate S1I4 exhibited the strongest antibacterial activity, with mean inhibition zones of 24.33 ± 1.52 mm against *E. coli* and 25.33 ± 2.08 mm against *K. pneumoniae*. Other AgNPs showed moderate to mild activity. For *E. coli*, inhibition zones ranged from 17.33 ± 0.57 mm (S2I2) to 11.66 ± 0.57 mm (S4I3), while for *K. pneumoniae*, most formulations resulted in narrower inhibition zones, between 9.66 ± 0.57 mm and 6.66 ± 0.57 mm. To statistically validate these differences, a one-way ANOVA was applied to the inhibition zone data for each bacterial species. ANOVA was selected because it enables comparison of mean inhibition zones across multiple nanoparticle groups to determine whether the observed differences were statistically significant. The analysis revealed highly significant variation in antibacterial activity among the groups for both *E. coli* and *K. pneumoniae* (**p < 0.001). Statistical significance is denoted in Table 4 by asterisks next to the values of S1I4, indicating that its mean inhibition zones differed significantly from those of other AgNP formulations. This confirms that the differences observed are not due to experimental variation but reflect true differences in antimicrobial performance.

These findings align with numerous studies demonstrating the potent antibacterial action of AgNPs against MDR Gram-negative bacteria. For example, biosynthesized AgNPs have shown effective inhibition of resistant *E. coli* and *K. pneumoniae* strains via mechanisms such as membrane disruption, generation of reactive oxygen species, and interference with essential biomolecules (Shinde et al., 2021a). The superior efficacy of S1I4-derived AgNPs may be attributed to their smaller

size, enhanced surface reactivity, and active biological capping agents from the fungal synthesis process. These properties likely improve their interaction with bacterial membranes and help overcome conventional resistance pathways. Overall, the screening results identified S1I4-derived AgNPs as the most effective formulation against both carbapenem-resistant strains, laying the foundation for more detailed concentration-dependent and mechanistic evaluations in subsequent experiments.

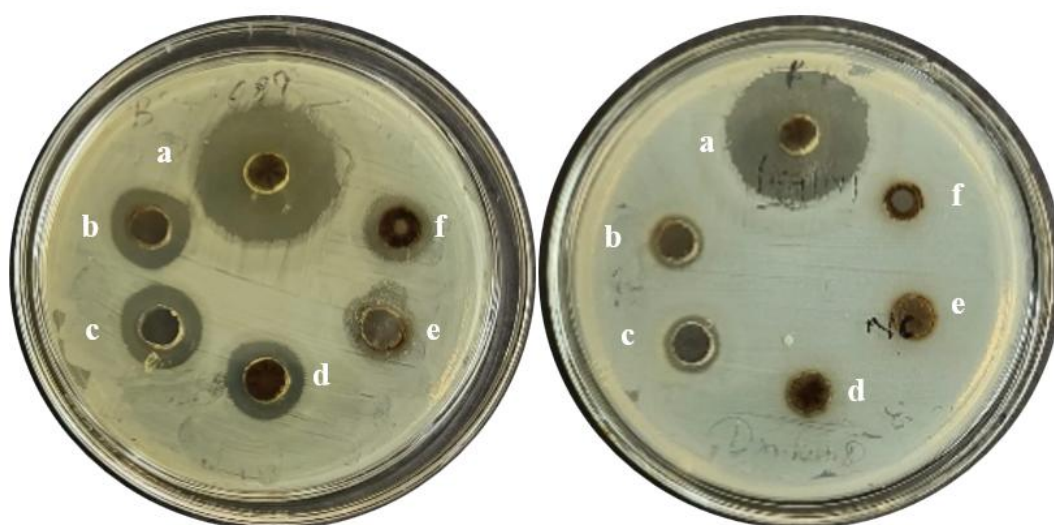


Figure 4.13: Agar well diffusion assay showing zones of inhibition produced by biosynthesized silver nanoparticles against carbapenem-resistant clinical isolates. (Left) *E. coli* and (Right) *K. pneumoniae*. Wells a–f represent AgNPs synthesized from fungal isolates: a = S1I4, b = S2I2, c = S3I1, d = S3I3, e = S4I3, and f = S5I1, each tested at 1 mg/mL. Notably, S1I4 (well a) exhibited the largest zones in both organisms, indicating superior antibacterial potency.

Table 4.5: Mean zones of inhibition (mm $\pm$ standard deviation, n = 3) exhibited by biosynthesized silver nanoparticles from six fungal isolates (S1I4, S2I2, S3I1, S3I3, S4I3, S5I1) against carbapenem-resistant *E. coli* and *K. pneumoniae* at a fixed concentration of 1 mg/mL, assessed via agar well diffusion assay.

Isolate	Concentrations	Mean zone of inhibition (mm) $\pm$ SD (N= 3)					
		S1I4	S2I2	S3I1	S3I3	S4I3	S5I1

<i>E. coli</i>	1 mg/ml	24.33 ± 1.52***	17.33 ± 0.57	15.66 ± 1.15	13.66 ± 0.57	11.66 ± 0.57	12.00 ± 1.00
<i>K. pneumoniae</i>	1 mg/ml	25.33 ± 2.08***	9.66 ± 0.57	8.66 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Here, statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test. Values marked with *** indicate $p < 0.001$ compared with all other AgNP groups.

4.4.2 Strain-Specific Antibacterial Activity of S1I4-AgNPs

Following the initial screening, the most potent AgNP formulation (S1I4) was selected to evaluate its antibacterial efficacy across ten clinical strains each of *E. coli* and *K. pneumoniae*, collected from PIMS Jalandhar, Punjab. Unlike conventional antibiotic testing, the aim here was not only to assess concentration-dependent inhibition but also to determine inter-strain variability in response to biosynthesized silver nanoparticles among carbapenem-resistant isolates. Each bacterial strain was exposed to S1I4-AgNPs at three concentrations (0.50, 0.25, and 0.125 mg/mL). Zone of inhibition (ZOI) data were recorded and compared with positive controls (meropenem, 0.1 mg/mL) and negative controls (sterile water). Mean ZOI values for each isolate and treatment group are presented in Table 4.6 and Table 4.7, with representative inhibition patterns shown in Figure 4.14.

For *E. coli*, clear differences in susceptibility were noted. At 0.50 mg/mL, the mean ZOI ranged from 20.0 to 22.3 mm, reflecting uniformly strong inhibition across all strains. At 0.25 mg/mL, inhibition zones decreased slightly (16.0–20.0 mm), while at 0.125 mg/mL, activity remained observable (9.3–12.3 mm), indicating that even the lowest concentration retained measurable inhibitory potential. The meropenem control produced an average ZOI of 13.7 ± 0.6 mm, consistently lower than all AgNP concentrations tested. Statistical analysis (one-way ANOVA) was applied to evaluate inter-strain variability at each concentration. Significant differences were observed at 0.25 mg/mL ($*p < 0.05$) and 0.125 mg/mL ($p < 0.01$), while no significant variability

was detected at 0.50 mg/mL (ns). These results confirm that although all *E. coli* strains were susceptible, the degree of inhibition varied significantly at lower concentrations.

Table 4.6: Strain-specific antibacterial activity of S1I4-AgNPs against ten clinical isolates of carbapenem-resistant *Escherichia coli*

Isolate	0.50 mg/mL	0.25 mg/mL	0.125 mg/mL	Positive control	Negative control
Mean $\pm$ SD (n= 3)					
<i>E. coli</i> 1	22.33 $\pm$ 0.57	17.33 $\pm$ 0.57	12.33 $\pm$ 0.57	13.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>E. coli</i> 2	21 $\pm$ 2	17.66 $\pm$ 2.08	10.33 $\pm$ 1.52	13.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<i>E. coli</i> 3	20.66 $\pm$ 0.57	20 $\pm$ 1.00	12.00 $\pm$ 0.00	14.66 $\pm$ 1.52	0.00 $\pm$ 0.00
<i>E. coli</i> 4	20.33 $\pm$ 1.52	16.66 $\pm$ 0.57	9.33 $\pm$ 1.52	14.00 $\pm$ 1.00	0.00 $\pm$ 0.00
<i>E. coli</i> 5	21.00 $\pm$ 1.00	18.00 $\pm$ 1.00	11.66 $\pm$ 0.57	14.33 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>E. coli</i> 6	20.00 $\pm$ 0.00	18.33 $\pm$ 1.52	11.33 $\pm$ 0.57	14.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>E. coli</i> 7	22.00 $\pm$ 1.00	19.33 $\pm$ 1.15	11.00 $\pm$ 0.00	14.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<i>E. coli</i> 8	22.66 $\pm$ 2.08	18.33 $\pm$ 0.57	10.66 $\pm$ 0.57	13.33 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>E. coli</i> 9	20.33 $\pm$ 0.57	18.33 $\pm$ 1.15	10.33 $\pm$ 1.15	14.00 $\pm$ 2.00	0.00 $\pm$ 0.00
<i>E. coli</i> 10	20.33 $\pm$ 0.57	16.00 $\pm$ 1.00	12.00 $\pm$ 0.00	13.00 $\pm$ 0.00	0.00 $\pm$ 0.00
Mean $\pm$ SE (n= 10)	21.06 $\pm$ 0.30 (ns)	17.99 $\pm$ 0.37*	11.10 $\pm$ 0.30**	13.86 $\pm$ 0.19 (ns)	0.00 $\pm$ 0.00 (ns)

Here, results are expressed as mean $\pm$ SD for individual isolates (n = 3) and as mean $\pm$ SE in the final row. One-way ANOVA indicated significant variability at 0.25 mg/mL ($p < 0.05$) and 0.125 mg/mL ($p < 0.01$), while differences at 0.50 mg/mL were not significant (ns).

For *K. pneumoniae*, a more selective pattern was observed. At 0.50 mg/mL, ZOI_s ranged from 16.3 to 19.3 mm, while at 0.25 mg/mL, values decreased modestly (16.3–18.3 mm). However, at 0.125 mg/mL, no inhibition was recorded for any strain, indicating a clear minimum threshold for bacteriostatic efficacy. The meropenem control averaged 12.7 $\pm$ 0.6 mm, which was consistently lower than AgNPs at 0.50 and 0.25 mg/mL. ANOVA testing revealed no statistically significant variability among *K.*

pneumoniae isolates at any concentration (ns), indicating that all strains responded uniformly to S1I4-AgNPs.

Table 4.7: Strain-specific antibacterial activity of S1I4-AgNPs against ten clinical isolates of carbapenem-resistant *Klebsiella pneumoniae*

Isolate	0.50 mg/mL	0.25 mg/mL	0.125 mg/mL	Positive control	Negative control
Mean $\pm$ SD (n= 3)					
<i>K. pneumoniae</i> 1	18.00 $\pm$ 0.00	16.33 $\pm$ 1.52	0.00 $\pm$ 0.00	12.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 2	18.66 $\pm$ 0.57	17.33 $\pm$ 1.15	0.00 $\pm$ 0.00	12.66 $\pm$ 1.15	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 3	17.33 $\pm$ 2.51	16.66 $\pm$ 0.57	0.00 $\pm$ 0.00	12.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 4	18.00 $\pm$ 0.00	17.66 $\pm$ 0.57	0.00 $\pm$ 0.00	12.33 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 5	18.66 $\pm$ 0.57	18.66 $\pm$ 0.57	0.00 $\pm$ 0.00	12.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 6	16.33 $\pm$ 0.57	17.33 $\pm$ 0.57	0.00 $\pm$ 0.00	13.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 7	19.33 $\pm$ 1.52	16.33 $\pm$ 0.57	0.00 $\pm$ 0.00	12.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 8	17.33 $\pm$ 2.51	16.33 $\pm$ 1.15	0.00 $\pm$ 0.00	13.00 $\pm$ 0.00	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 9	18.33 $\pm$ 0.57	17.66 $\pm$ 1.52	0.00 $\pm$ 0.00	15.66 $\pm$ 0.57	0.00 $\pm$ 0.00
<i>K. pneumoniae</i> 10	17.66 $\pm$ 1.52	18.33 $\pm$ 0.57	0.00 $\pm$ 0.00	12.33 $\pm$ 0.57	0.00 $\pm$ 0.00
Mean $\pm$ SE	17.96 $\pm$ 0.27 (ns)	17.26 $\pm$ 0.27 (ns)	0.00 $\pm$ 0.00 (ns)	12.96 $\pm$ 0.31 (ns)	0.00 $\pm$ 0.00 (ns)

Here, results are expressed as mean $\pm$ SD for individual isolates ($n = 3$) and as mean $\pm$ SE in the final row. One-way ANOVA revealed no statistically significant variability among isolates at any concentration (ns).

This strain-wise analysis highlights a key feature of biosynthesized AgNPs and their ability to maintain potent antibacterial activity across genetically distinct, carbapenem-resistant clinical isolates. While *E. coli* strains responded positively at all tested concentrations, *K. pneumoniae* strains required higher nanoparticle loads for observable inhibition. These differences reflect known resistance mechanisms particularly reduced outer membrane permeability and robust efflux systems in *K. pneumoniae* rather than mere concentration gradients. Multiple studies have reported AgNPs as effective antimicrobial agents against multidrug-resistant Gram-negative bacteria. For instance, AgNPs synthesized from *Azadirachta indica* and *Fusarium spp.* have demonstrated strong inhibition of resistant *E. coli* and *K. pneumoniae* strains (Pareek et al., 2021; Shinde et al., 2021b). These nanoparticles operate via multiple mechanisms membrane destabilization, oxidative stress, protein inactivation, and DNA interference making resistance development less likely (Tawiah et al., 2025). The high efficacy of S1I4-AgNPs observed here may be attributed to their optimal particle size, stability, and surface-bound biomolecules from fungal origin, which enhance bacterial interaction and Ag^+ release kinetics. Notably, their superior performance over meropenem especially against *E. coli* underscores their clinical relevance in the fight against resistant Enterobacteriaceae. Further mechanistic studies and cytotoxicity assays are warranted to translate these in vitro findings into therapeutic strategies.

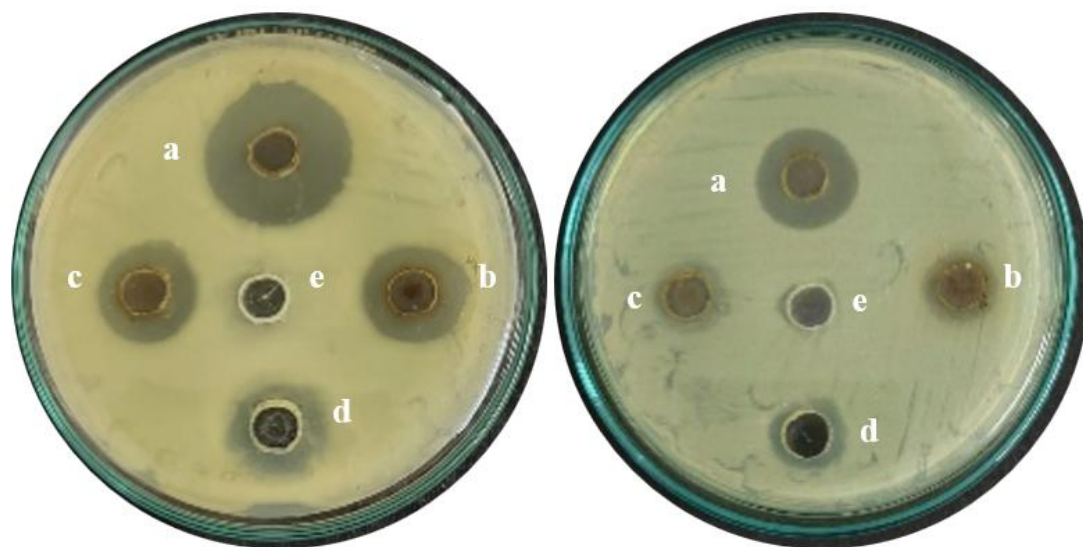


Figure 4.14: Agar well diffusion assay showing antibacterial activity of biosynthesized silver nanoparticles (S1I4-AgNPs). (Left) *Escherichia coli* and (Right) *Klebsiella pneumoniae* tested against different concentrations of AgNPs. Wells: (a) 0.5 mg/ml AgNPs, (b) 0.25 mg/ml AgNPs, (c) 0.125 mg/ml AgNPs, (d) positive control (Meropenem, 0.1 mg/ml), and (e) negative control (sterile distilled water). Clear inhibition zones indicate concentration-dependent antibacterial activity of S1I4-AgNPs, with higher concentrations producing stronger inhibitory effects.

4.4.3 Minimum Inhibitory and Bactericidal Concentration of AgNPs

The antibacterial potential of biosynthesized AgNPs derived from isolate S1I4 was quantitatively assessed through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) testing against carbapenem-resistant strains of *E. coli* and *K. pneumoniae* (Table 4.8). For *E. coli*, the AgNPs exhibited an MIC of $5 \pm 0.0 \mu\text{g/mL}$ and an MBC of $50 \pm 0.0 \mu\text{g/mL}$, while for *K. pneumoniae*, the MIC and MBC were $25 \pm 0.0 \mu\text{g/mL}$ and $75 \pm 0.0 \mu\text{g/mL}$, respectively. These results reveal significantly enhanced inhibitory potency of AgNPs over meropenem, whose MICs were $25 \pm 0.0 \mu\text{g/mL}$ (*E. coli*) and $60 \pm 0.0 \mu\text{g/mL}$ (*K. pneumoniae*), respectively. The MBC/MIC ratio further indicated that AgNPs exhibited bacteriostatic activity against *E. coli* (ratio = 10) and bactericidal activity against *K. pneumoniae* (ratio = 3), suggesting species-specific differences in killing efficiency. Importantly, the MIC values observed here for *E. coli* ($5 \mu\text{g/mL}$) are markedly lower than those reported in

earlier studies, such as Doudi & Setorki (2014), who found MIC and MBC values of 33.86 µg/mL and 63.23 µg/mL for multidrug-resistant *E. coli* strains (Doudi, 2014). Similarly, our findings compare favorably with Huq & Akter (2021), who reported MICs of 12.5 µg/mL and MBCs of 50 µg/mL for biosynthesized AgNPs against *K. pneumoniae* (Huq & Akter, 2021).

The differential MIC/MBC values between *E. coli* and *K. pneumoniae* suggest strain-specific resistance rather than solely structural factors such as capsule formation. Several recent studies attribute the reduced sensitivity of *K. pneumoniae* to multifactorial mechanisms including efflux pumps, porin mutations, and beta-lactamase overexpression (Li et al., 2024), rather than capsular thickness alone. Notably, biosynthesized AgNPs demonstrated superior inhibitory performance compared to meropenem across both strains, despite the higher bactericidal thresholds. This supports the hypothesis that AgNPs exert their antibacterial action through multiple mechanisms such as ROS generation, membrane destabilization, and protein/enzyme interaction resulting in both growth inhibition and eventual cell death (Mohammed et al., 2021).

Table 4.8: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of biosynthesized silver nanoparticles (S1I4) compared to the standard antibiotic meropenem against carbapenem-resistant *E. coli* and *K. pneumoniae*.

Test	<i>E. coli</i> (S1I4 AgNPs)	<i>E. coli</i> (Meropenem)	<i>K. pneumoniae</i> (S1I4 AgNPs)	<i>K. pneumoniae</i> (Meropenem)
MIC (µg/ml)	5 ± 0.0	25 ± 0.0	25 ± 0.0	60 ± 0.0
MBC (µg/ml)	50 ± 0.0	50 ± 0.0	75 ± 0.0	125 ± 0.0

Here, results indicate greater sensitivity of *E. coli* to AgNPs, with lower MIC and MBC values compared to *K. pneumoniae*, while both pathogens required higher concentrations of meropenem to achieve similar effects.

4.5. Evaluation of mode of Action of Biosynthesized Silver Nanoparticles

To gain a clearer understanding of the antibacterial properties of the biosynthesized AgNPs, their mechanism of action was systematically investigated. Although antimicrobial efficacy had already been demonstrated through MIC, MBC, and well diffusion assays, the specific cellular and molecular disruptions caused by AgNP exposure remained to be clarified. To address this, a series of mechanistic assays were designed to examine the interactions of AgNPs with bacterial systems at multiple levels. The evaluations focused on four key aspects of bacterial physiology: (i) growth kinetics, (ii) membrane integrity, (iii) DNA integrity, and (iv) cell wall architecture. These parameters were selected based on established evidence that AgNPs exert bactericidal effects through multiple converging pathways, including reactive oxygen species (ROS) generation, DNA damage, and physical disruption of bacterial membranes and cell walls (Lee et al., 2014; Pareek et al., 2021; Scandorieiro et al., 2022). All assays were carried out using the most potent AgNP formulation (S1I4) against clinically relevant carbapenem-resistant *E. coli* and *K. pneumoniae*. Collectively, these mechanistic studies were designed to delineate both structural and functional alterations induced by AgNPs, thereby providing deeper insight into their bactericidal potential and future therapeutic applicability.

4.5.1 Growth Kinetic Analysis

To understand the time-dependent bacteriostatic effects of biosynthesized AgNPs, the growth kinetics of *E. coli* and *K. pneumoniae* were monitored over 30 hours using spectrophotometer. Each bacterial culture tube was subjected to one of three treatments: AgNPs at half of the minimum inhibitory concentration (MIC), meropenem at half of the MIC, or no treatment (serving as the growth control). The growth curves for both strains are shown in Figure 4.15.

In *E. coli*, the untreated control exhibited a typical sigmoid growth curve with a distinct lag phase, followed by exponential and stationary phases. Treatment with meropenem caused a significant prolongation of the lag phase (6 hour), along with a shortened and delayed exponential phase and a reduced stationary phase compared to the untreated control. In contrast, AgNP-treated cells showed a marked extension of the lag phase, with complete growth suppression for the first 14 hours, followed by a shortened and

delayed exponential phase and a reduced stationary phase relative to both the untreated and meropenem-treated controls. In *K. pneumoniae*, the untreated control displayed a classical sigmoid growth curve, with a short lag phase followed by rapid exponential growth and a stable stationary phase. Meropenem-treated cells exhibited a noticeable extension of the lag phase (9 hour), with exponential growth commencing later and reaching a reduced stationary phase compared to the control. By contrast, AgNP-treated cells showed the most pronounced growth inhibition, characterized by a markedly prolonged lag phase extending to 14–16 hours, followed by a delayed and slower exponential phase. The stationary phase remained consistently reduced relative to both the untreated and antibiotic-treated groups, reflecting strong early bacteriostatic action and limited recovery during later growth stages.

These results confirm that AgNPs significantly impair bacterial proliferation by extending the lag phase and reducing growth rate, particularly during early exposure periods. The delay in growth resumption may be attributed to stress responses, ROS generation, cell wall damage, membrane damage, protein degradation and DNA damage (Vishwakarma, 2017; Theophel et al., 2014). The stronger and more prolonged growth inhibition seen in *K. pneumoniae* may indicate species-specific differences in nanoparticle susceptibility, possibly linked to cell envelope structure or efflux regulation. Together, the kinetic profiles highlight the effective early-phase suppression exerted by biosynthesized AgNPs on both pathogens, with potential for post-exposure regrowth under sub-lethal conditions an important consideration for dosing and delivery strategies in clinical settings.

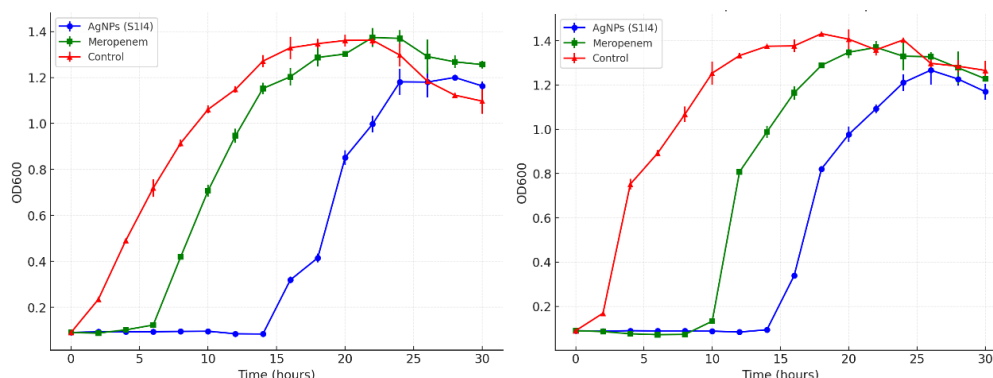


Figure 4.15: Growth kinetics of carbapenem-resistant *Escherichia coli* (Left) and *Klebsiella pneumoniae* (Right) in response to silver nanoparticles (AgNPs) synthesized by fungal isolate S1I4. Optical density (OD₆₀₀) was monitored for 30 hours under three conditions: untreated control, meropenem-treated (antibiotic control), and AgNP-treated cultures. AgNP exposure caused a pronounced extension of the lag phase, delayed and reduced exponential growth, and a lower stationary phase plateau compared to both controls, indicating potent bacteriostatic effects particularly during the exponential growth phase.

4.5.2 Nucleotide Leakage Assay

To evaluate membrane integrity as part of the antibacterial mechanism of biosynthesized silver nanoparticles (AgNPs), nucleotide leakage was assessed by monitoring absorbance at 260 nm (Figure 4.16). This assay detects the extracellular release of nucleic acids and related UV-absorbing molecules, which serves as a direct indicator of membrane compromise. In *E. coli*, the untreated control group showed negligible absorbance throughout the experiment, reflecting intact membrane barriers and the absence of nucleotide leakage, thereby confirming the baseline stability of the assay. In contrast, meropenem-treated cells exhibited a moderate but progressive increase in absorbance, which can be attributed to its primary mode of action inhibition of peptidoglycan biosynthesis leading indirectly to membrane weakening and autolysin activation, ultimately resulting in partial leakage of intracellular contents. The most pronounced effect, however, was observed in AgNP-treated cells, which displayed a sharp and early rise in absorbance indicative of extensive nucleotide leakage. This response is consistent with direct nanoparticle membrane interactions, including pore formation, lipid bilayer destabilization, and oxidative damage through reactive oxygen species (ROS), collectively explaining the significantly greater membrane disruption compared to both the control and antibiotic groups.

A similar pattern was evident in *K. pneumoniae*, untreated cells maintained consistently low absorbance values, confirming intact envelope integrity under normal conditions. Meropenem-treated cells again demonstrated moderate leakage, attributable to cell wall synthesis inhibition and consequent destabilization of the cell envelope. However, the

extent of leakage was comparatively lower than in *E. coli*, likely due to structural factors such as the presence of a thick capsule and altered porins, which restrict antibiotic penetration. In sharp contrast, AgNP-treated *K. pneumoniae* cells exhibited the highest and most sustained nucleotide release, underscoring the potent ability of nanoparticles to compromise even the more resistant Gram-negative barriers. The strong leakage effect may be explained by interactions of AgNPs with lipopolysaccharides and cytoplasmic membranes, as well as potential accumulation within the capsular matrix, amplifying their disruptive potential. Statistical analysis confirmed that differences among treatment groups were highly significant at all time points (one-way ANOVA, *** $p < 0.001$), validating that the observed patterns were treatment-specific and not due to experimental variation. These results strongly suggest that biosynthesized AgNPs compromise the bacterial cytoplasmic membrane, resulting in uncontrolled efflux of intracellular components such as nucleotides, as reflected by elevated OD260 values. This finding aligns with numerous studies where AgNPs induced significant membrane disruption and subsequent leakage of cellular materials in both Gram-negative and Gram-positive bacteria (Bondarenko et al., 2018a; Saqib et al., 2022; Shalini et al., 2024). Notably, Singh et al (2016) study demonstrated that AgNP-treated *E. coli* showed rapid leakage of intracellular β -galactosidase, confirming pore formation in the membrane and significant cellular leakage (Singh, 2016). The observed faster and more intense OD260 increase in AgNP-treated groups compared to meropenem supports the hypothesis that nanoparticles act rapidly through multi-modal mechanisms, including direct membrane disruption, oxidative stress, and perturbation of membrane-bound enzymes (Ahmad et al., 2017). Moreover, the rapid onset of leakage within 2–3 hours post-treatment further underscores that membrane destabilization is an early bactericidal event, likely preceding downstream effects such as DNA or protein damage. Recent analyses emphasize that Gram-negative bacteria are particularly vulnerable due to their outer membrane's high negative surface potential and thinner peptidoglycan layer. Studies on *Pseudomonas aeruginosa* and *E. coli* showed significant K^+ leakage and membrane depolarization within seconds of AgNP exposure, indicating that even brief contact is sufficient to trigger bactericidal effects (Bondarenko et al., 2018b). In conclusion, the nucleotide leakage assay confirms that biosynthesized AgNPs exert a fast-acting, membrane-targeting antibacterial

mechanism, significantly outperforming meropenem under the tested conditions. This potent and rapid disruption of membrane integrity supports their role as promising antimicrobial agents against multidrug-resistant Gram-negative pathogens.

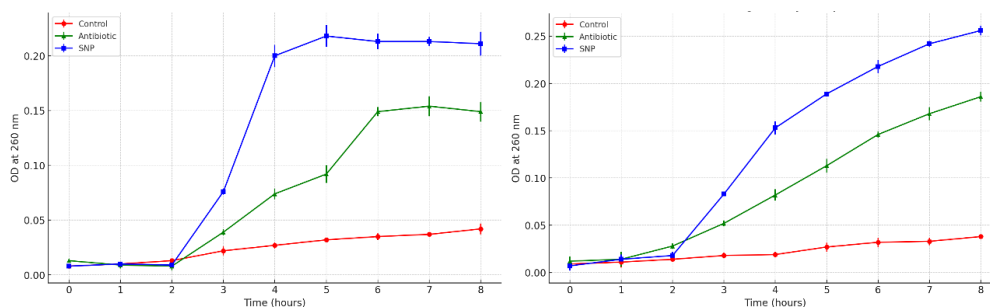


Figure 4.16: Nucleotide leakage assay of carbapenem-resistant *E. coli* (Left) and *K. pneumoniae* (Right) following treatment with biosynthesized silver nanoparticles (S1I4), meropenem (antibiotic control), and untreated control.

Here absorbance at 260 nm was measured over 8 hours to quantify extracellular nucleotide release as an indicator of membrane damage. AgNP treatment caused a rapid and pronounced increase in absorbance, reflecting severe membrane disruption and cytoplasmic leakage. Meropenem induced moderate leakage, while untreated controls exhibited negligible absorbance, confirming membrane integrity. Statistical analysis using one-way ANOVA revealed significant differences among treatment groups at all time points ($p < 0.001$), indicating that AgNPs compromised bacterial membrane integrity substantially more than meropenem or control treatments.

4.5.3 DNA Damage

Agarose gel electrophoresis was employed to assess the integrity of genomic DNA in carbapenem-resistant *E. coli* and *K. pneumoniae* following treatment with biosynthesized silver nanoparticles as shown in Figure 4.17 (AgNPs, isolate S1I4). In *E. coli*, the untreated control (Lane 1) displayed a distinct, intact, high-molecular-weight DNA band, consistent with preserved genomic integrity. By contrast, the AgNP-treated group (Lane 2) exhibited marked DNA degradation, characterized by smearing and fragmentation patterns, indicative of severe genotoxic stress. These findings clearly demonstrate that AgNPs disrupted genomic DNA in *E. coli*. For *K. pneumoniae*, the

untreated control (Lane 4) similarly showed intact genomic DNA. However, the AgNP-treated cells (Lane 5) did not display typical smearing but rather multiple discrete DNA bands. This banding pattern is attributable not to genomic degradation but to the co-isolation of large plasmids and accessory plasmids, which are frequently carried by *K. pneumoniae* alongside chromosomal DNA. Such plasmid-associated banding is a well-recognized phenomenon in clinical *K. pneumoniae* strains and reflects their role in harbouring resistance determinants.

The observed DNA fragmentation in *E. coli* following AgNP exposure is consistent with established mechanisms of silver nanoparticle mediated genotoxicity. AgNPs generate reactive oxygen species (ROS), interact with phosphate backbones, and bind directly to nucleobases, leading to both strand breaks and oxidative modification of DNA. Prior studies have demonstrated that AgNPs disrupt bacterial DNA integrity through multiple pathways, including oxidative stress, physical binding, and interference with repair processes (Lim et al., 2012; Rodriguez-Garraus et al., 2020). The clear smearing observed in treated *E. coli* thus provides strong experimental confirmation of nanoparticle-induced genotoxic effects. In *K. pneumoniae*, the absence of classical smearing but appearance of multiple bands after AgNP treatment highlights a species-specific difference. Clinical isolates of *K. pneumoniae* are known to carry multiple large plasmids that can co-purify during genomic DNA isolation, producing distinct bands on electrophoresis (Yang et al., 2016). These plasmids often encode extended-spectrum β -lactamases (ESBLs) or carbapenemase genes and can exceed 50–150 kb in size. Therefore, the banding observed in Lane 5 most likely reflects AgNP-induced destabilization of genomic DNA combined with co-isolated plasmid DNA, rather than exclusive genomic degradation. Taken together, the DNA integrity assay confirms that biosynthesized AgNPs exert potent genotoxic stress on bacterial cells, causing fragmentation in *E. coli* and distinctive plasmid-associated profiles in *K. pneumoniae*. The latter observation further underscores the role of plasmid biology in shaping resistance phenotypes in Enterobacteriaceae, and suggests that AgNPs may differentially affect genomic versus extrachromosomal DNA targets depending on species-specific genomic architecture.

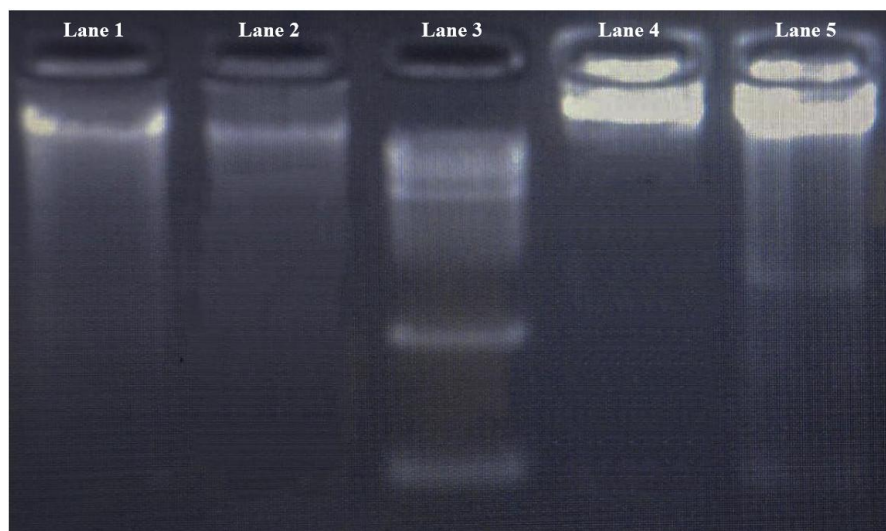


Figure 4.17: Agarose gel electrophoresis showing genomic DNA integrity of *Escherichia coli* and *Klebsiella pneumoniae* after treatment with silver nanoparticles synthesized by isolate S114. Lane 1: Untreated *E. coli* control, showing intact genomic DNA. Lane 2: AgNP-treated *E. coli*, exhibiting DNA fragmentation and smearing, consistent with nanoparticle-induced genotoxicity. Lane 3: 1 kb DNA ladder (molecular size marker). Lane 4: Untreated *K. pneumoniae* control, showing intact genomic DNA. Lane 5: AgNP-treated *K. pneumoniae*, displaying multiple DNA bands due to co-isolation of large plasmids alongside chromosomal DNA, a phenomenon well-documented in plasmid-rich clinical strains.

4.5.4 Evaluation of Cell Wall Damage by Field Emission Scanning Electron Microscopy (FE-SEM)

Field Emission Scanning Electron Microscopy (FE-SEM) provided direct ultrastructural evidence of cell envelope disruption in carbapenem-resistant *E. coli* and *K. pneumoniae* following exposure to biosynthesized silver nanoparticles (AgNPs) (Figure 4.18). Untreated control cells of both species (Figure 4.18A, 4.18D) retained their characteristic smooth, rod-shaped morphology with intact surfaces, indicative of viable Gram-negative architecture. In contrast, AgNP-treated cells displayed extensive surface alterations. Treated *E. coli* (Figure 4.18B) exhibited irregular contours, roughened textures, and collapsed cell walls, while *K. pneumoniae* cells (Figure 4.18E) showed deformation, wrinkling, and disrupted outer surfaces. At higher magnification

(Figure 4.18C, 4.18F), both pathogens demonstrated severe rupture of the envelope and loss of structural integrity, consistent with cell wall destabilization as a primary antimicrobial effect of AgNPs.

These findings align closely with previous SEM and TEM investigations that have established cell wall damage as an early and critical target of AgNPs. For instance, (Sondi & Salopek-Sondi, 2004) demonstrated pitting and perforation of *E. coli* cell walls caused by AgNP accumulation, leading to increased permeability and eventual collapse. Similarly, (Husak et al., 2020) observed morphological shortening, surface roughening, and impaired division in *E. coli* and *K. pneumoniae* following AgNP treatment, confirming envelope destabilization across Gram-negative pathogens. At a molecular level, (Mirzajani et al., 2011) provided biochemical evidence of peptidoglycan disruption in *Staphylococcus aureus*, marked by muramic acid release after AgNP exposure, reinforcing the direct interaction of nanoparticles with cell wall components. More recently, proteomic analyses revealed that AgNPs inhibit enzymes involved in peptidoglycan biosynthesis, further weakening the wall structure and accelerating lysis (Liu et al., 2023). Complementary high-resolution TEM studies confirmed thinning of the wall and permeabilization prior to complete rupture, highlighting cell wall destabilization as a precursor to cytoplasmic leakage and cell death (Romero-Urbina et al., 2015). Taken together, our FE-SEM results provide strong visual confirmation that biosynthesized AgNPs compromise bacterial cell walls in both *E. coli* and *K. pneumoniae*. This damage is not merely secondary to membrane disruption but represents a direct, early-acting structural effect, supported by extensive prior evidence across Gram-positive and Gram-negative species. The combination of wall destabilization, surface perforation, and collapse likely facilitates subsequent mechanisms of action such as nucleotide leakage and DNA damage, thereby reinforcing the multi-targeted antibacterial potential of AgNPs against multidrug-resistant pathogens.

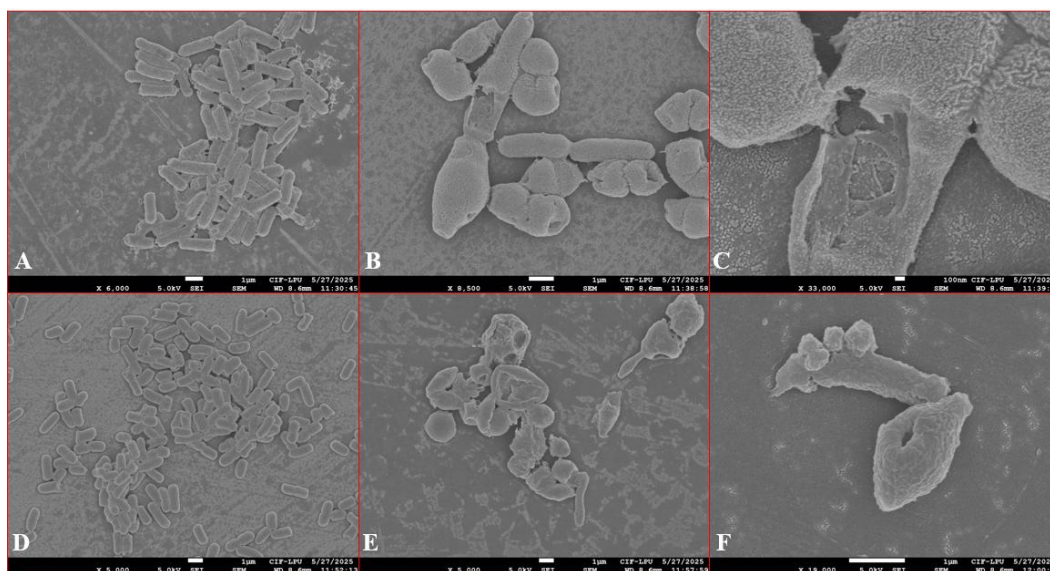


Figure 4.18: Scanning Electron Microscopy (SEM) images showing ultrastructural alterations in carbapenem-resistant *E. coli* and *K. pneumoniae* following treatment with silver nanoparticles synthesized by isolate S114. (A) Untreated *E. coli* exhibiting intact rod-shaped morphology with smooth surfaces. (B) AgNP-treated *E. coli* showing surface roughness, morphological distortion, and membrane rupture. (C) High-magnification view of AgNP-treated *E. coli* illustrating extensive membrane damage with cytoplasmic leakage. (D) Untreated *K. pneumoniae* displaying characteristic mucoid rod-shaped structures. (E) AgNP-treated *K. pneumoniae* showing surface collapse, shrinkage, and irregular morphology. (F) High-magnification image of AgNP-treated *K. pneumoniae* revealing severe cell wall disruption and complete structural disintegration, confirming nanoparticle-induced cellular damage.

To consolidate the mechanistic findings of this study, the major antibacterial effects of biosynthesized AgNPs against carbapenem-resistant *E. coli* and *K. pneumoniae* are summarized in Table 4.9. The results collectively demonstrate that AgNPs exert a multi-targeted mode of action, beginning with growth suppression, followed by membrane destabilization, genomic damage, and cell wall disintegration. Each mechanism contributes synergistically to the overall bactericidal effect, highlighting the broad-spectrum and potent antimicrobial potential of fungal-derived AgNPs.

Table 4.9: Summary of Mechanisms of Antibacterial Action of Biosynthesized AgNPs

Mechanism	Mode of Action	Key Observations
Growth Kinetics Suppression	AgNPs delay bacterial proliferation by inducing stress, extending the lag phase, and slowing replication.	Sub-MIC AgNPs caused a pronounced delay in growth initiation and reduced overall biomass compared to antibiotic and control groups.
Membrane Disruption (Leakage)	AgNPs compromise membrane integrity, leading to uncontrolled leakage of nucleotides and other intracellular components.	Treated cells exhibited significant nucleotide release, whereas antibiotic-treated cells showed moderate leakage and controls showed none.
Genomic DNA Damage	AgNPs cause genotoxic effects through direct interaction with DNA and ROS-mediated strand breaks.	Electrophoresis revealed smeared, fragmented DNA in <i>E. coli</i> , while <i>K. pneumoniae</i> displayed distinct banding linked to plasmid co-isolation rather than typical fragmentation.
Cell Wall Disintegration (FE-SEM)	AgNPs adhere to bacterial surfaces, disrupt structural integrity, and collapse the envelope architecture.	FE-SEM images revealed irregular shapes, ruptured surfaces, and collapsed cell walls in treated cells, while untreated bacteria retained smooth, intact morphology.

4.6 Toxicity Evaluation of AgNPs on 3T3-L1 Cells (MTT Assay)

The toxicological profile of the biosynthesized silver nanoparticles (AgNPs) was evaluated using the MTT assay on 3T3-L1 mouse fibroblast cells to determine their compatibility with mammalian systems. Cells were exposed to AgNP concentrations ranging from 25 to 400 $\mu\text{g/mL}$, while untreated cultures served as controls. The results (Figure 4.19 & 4.20) revealed a dose-dependent response, with minimal effects on cell viability at lower concentrations (≤ 50 $\mu\text{g/mL}$) and a pronounced decline at higher concentrations (≥ 100 $\mu\text{g/mL}$). The calculated IC_{50} value of 100.06 $\mu\text{g/mL}$ indicates that AgNPs possess moderate toxicity at elevated doses. Importantly, the concentration window required for antimicrobial activity (5 $\mu\text{g/mL}$ against *E. coli* and 25 $\mu\text{g/mL}$ against *K. pneumoniae*) lies well below the IC_{50} threshold. This overlap suggests a favorable therapeutic index, where effective bacterial inhibition can be achieved without compromising mammalian cell viability. These findings confirm that while AgNPs exert potent toxic effects against pathogens, their toxicity towards mammalian fibroblasts remains limited within clinically relevant concentration ranges, supporting their promise as safe and selective antimicrobial agents.

The toxicity of silver nanoparticles (AgNPs) is a multifactorial phenomenon influenced primarily by particle size, shape, dose, and surface chemistry, all of which determine their interaction with mammalian cells. In the present study, MTT assays performed on 3T3 fibroblasts revealed that the biosynthesized AgNPs exhibit dose-dependent toxicity, with minimal effects observed below 50 $\mu\text{g/mL}$ and an IC_{50} value near 100 $\mu\text{g/mL}$. Importantly, this concentration is substantially higher than the minimum inhibitory concentrations (MICs) required for antibacterial activity (5–25 $\mu\text{g/mL}$), underscoring a favorable therapeutic window. Previous studies consistently show that smaller AgNPs (<10 nm) are considerably more cytotoxic than larger particles, as their higher surface area enables greater cellular uptake and generation of reactive oxygen species (ROS). Ávalos (2016) demonstrated that 4.7 nm AgNPs were significantly more toxic to human dermal fibroblasts compared to 42 nm particles, with oxidative stress being a key driver of cell death (Avalos et al., 2016). Similarly, Carlson (2006) reported a clear size- and dose-dependent toxicity, where 15–30 nm AgNPs reduced mitochondrial function more strongly than larger 55 nm counterparts, correlating with decreased ATP production and cell viability (Carlson, 2006). Shape also modulates toxicity. Promptong (2015) showed that triangular AgNPs induced

stronger ROS-mediated toxicity and membrane damage compared to spherical ones of similar size, highlighting the role of geometry in biological interactions (Promtong, 2015). This is consistent with our finding that biologically synthesized AgNPs with spherical morphology and moderate size (~20–30 nm) caused only moderate toxicity to fibroblasts, while retaining strong antibacterial action. Dose remains the most critical determinant. Sambale (2015) observed clear concentration-dependent toxicity in NIH 3T3 fibroblasts, with IC₅₀ values varying across cell lines but consistently higher than antibacterial thresholds, suggesting selective toxicity (Sambale et al., 2015). Similarly, Saravanan et al. (2018) reported that biosynthesized AgNPs from *Phenerochaete chrysosporium* were non-toxic to fibroblasts up to 12.5 µg/mL while exhibiting bactericidal activity at higher doses, further supporting the therapeutic selectivity of biologically derived nanoparticles (Saravanan et al., 2018). Collectively, these findings emphasize that the moderate-sized, spherical AgNPs synthesized in this study occupy an optimal balance: sufficiently small to penetrate and disrupt bacterial cells, yet large and capped enough to limit excessive toxicity towards mammalian fibroblasts at therapeutic concentrations. The observed IC₅₀ aligns well with reported values for fibroblast models, reinforcing the biocompatibility of biosynthesized AgNPs within clinically relevant ranges.

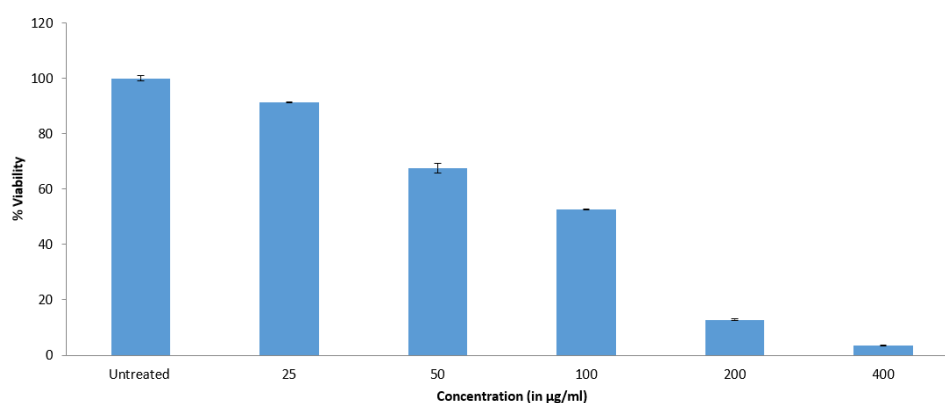


Figure 4.19: Toxicity of biosynthesized silver nanoparticles (S1I4) against 3T3-L1 fibroblast cells, determined by MTT assay after 72-hour exposure. Percentage cell viability decreased in a concentration-dependent manner, with significant reductions observed at ≥ 100 µg/mL.

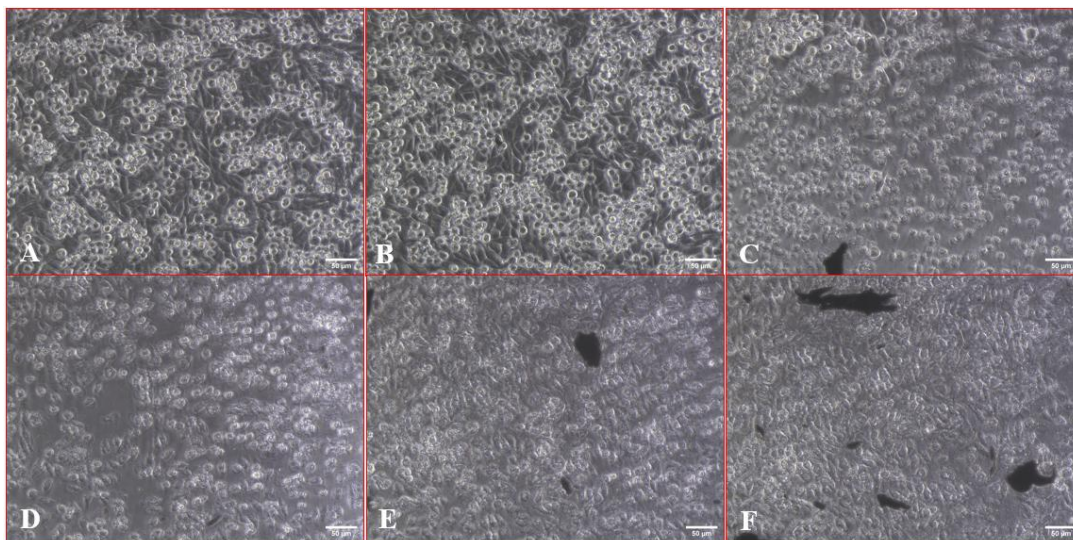


Figure 4.20: Phase contrast microscopy images showing dose-dependent cytotoxicity of silver nanoparticles synthesized by isolate S1I4 on 3T3-L1 fibroblast cells. (A) Untreated control cells exhibiting healthy, confluent morphology. (B–F) Cells treated with AgNPs at 25, 50, 100, 200, and 400 $\mu\text{g/mL}$, respectively, for 72 hours. A progressive reduction in cell density, along with morphological alterations such as rounding, membrane blebbing, and detachment, was observed at increasing concentrations, indicating dose-dependent cytotoxic effects. Scale bars: 50 μm .

4.7. Characterization of Most Potent Silver Nanoparticles (S1I4)

To elucidate the physicochemical attributes of the biosynthesized AgNPs, a comprehensive characterization was undertaken using multiple analytical techniques. The S1I4 exhibited best antimicrobial activity based on that criteria we selected it for further characterization. Light Scattering (DLS) was employed to determine the hydrodynamic diameter and polydispersity index (PDI), providing insights into particle size distribution and colloidal uniformity. Zeta potential analysis was performed to assess the surface charge characteristics, which directly influence nanoparticle stability and dispersion. Fourier-transform infrared spectroscopy (FTIR) was used to identify functional groups derived from fungal metabolites that acted as reducing and capping agents, thereby stabilizing the nanoparticles. X-ray diffraction (XRD) analysis confirmed the crystalline nature and phase purity of the AgNPs. Transmission Electron Microscopy (TEM) enabled nanoscale visualization of particle shape, morphology, and

size distribution, while Scanning Electron Microscopy coupled with Energy Dispersive X-ray spectroscopy (SEM-EDX) provided detailed surface topology and validated elemental composition. Together, these characterization techniques provided a holistic understanding of the S1I4 AgNPs, linking nanoscale features such as size, crystallinity, surface charge, and biomolecular capping to their overall physicochemical stability and quality.

4.7.1 Dynamic Light Scattering (DLS) Analysis

DLS analysis of the S1I4 AgNPs formulation revealed a sharp, unimodal distribution with an average hydrodynamic diameter of 24.55 nm, confirming the presence of uniformly distributed nanoparticles (Figure 4.21). The absence of secondary peaks in the intensity profile indicated a monodisperse population, and the low polydispersity index ($PDI < 0.3$) further validated the high degree of colloidal stability and synthesis control. Notably, biogenic synthesis of silver nanoparticles using fungal extracts frequently results in particle sizes ranging between 10–100 nm, with several studies reporting mean sizes in the 20–35 nm range (Bagur et al., 2022; Rodrigues et al., 2013), supporting the observed size of our S1I4 formulation. This size range is considered optimal for biological interactions, especially with microbial membranes, due to the enhanced surface area-to-volume ratio, which facilitates superior antimicrobial activity. Moreover, fungal-mediated synthesis often results in biomolecule-capped nanoparticles, which enhance stability, dispersion, and biological functionality (Guilger-Casagrande & Lima, 2019). Such features align with our findings of stable, well-dispersed particles in colloidal suspension. In conclusion, the DLS data confirm that the S1I4 formulation comprises nanoscale, monodisperse, and stable silver nanoparticles, consistent with those synthesized by fungal biogenic methods and highly suitable for biomedical and antimicrobial applications.

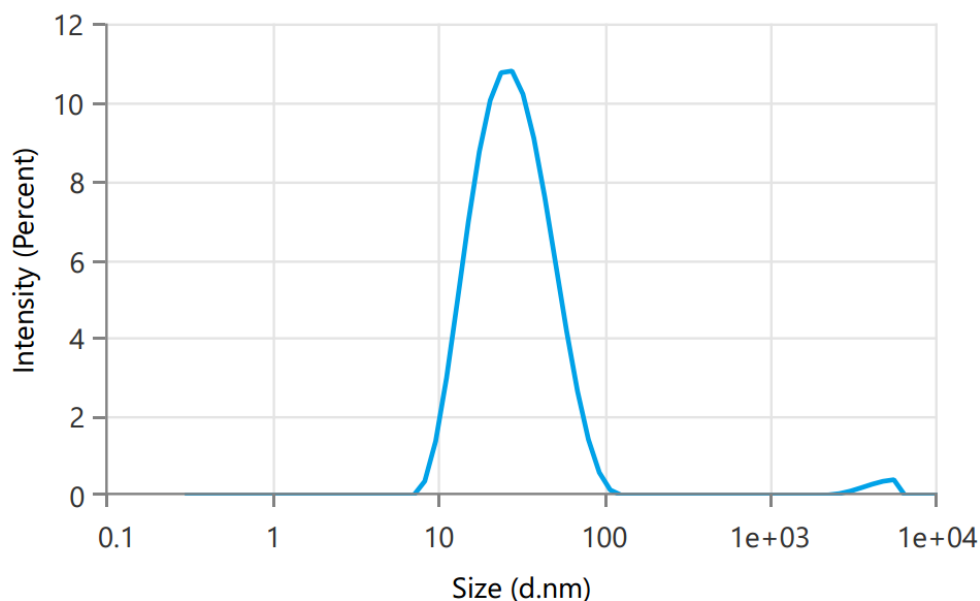


Figure 4.21: Dynamic Light Scattering (DLS) intensity profile of silver nanoparticles synthesized by isolate S1I4. The profile shows a sharp unimodal distribution with an average hydrodynamic diameter of 24.55 nm. The absence of secondary peaks confirms a predominantly monodisperse nanoparticle population with minimal aggregation.

4.7.2 Zeta Potential Analysis

Zeta potential measurement serves as a critical indicator of nanoparticle colloidal stability and surface charge behavior in suspension. In this study, the biosynthesized AgNPs (S1I4) exhibited a zeta potential of -23.66 mV, as shown by the sharp unimodal peak in the distribution plot (Figure 4.22). While this value is slightly below the conventional ± 30 mV stability threshold, it still suggests moderate electrostatic stability, especially when additional steric stabilization is provided by surface-bound biomolecules (Clogston & Patri, 2011), (Kulkarni et al., 2019). The negative surface charge is attributed to the adsorption of anionic functional groups such as carboxyl ($-\text{COOH}$), hydroxyl ($-\text{OH}$), and amide ($-\text{CONH}$) derived from fungal metabolites used during biogenic synthesis. These functional groups not only provide electrostatic repulsion but also contribute to steric hindrance, enhancing colloidal stability even at zeta potential values less than -30 mV (Savchenko & Velichko, 2019). This observation is consistent with previous findings on fungal-mediated silver nanoparticle synthesis, where extracellular proteins and polysaccharides act as both reducing and capping

agents, stabilizing the nanoparticles in aqueous media (Guilger-Casagrande & Lima, 2019).

A zeta potential of -23.66 mV indicates sufficient electrostatic stability of the S1I4-derived nanoparticles, limiting aggregation and supporting consistent dispersibility. The narrow zeta potential distribution confirms a homogeneous and monodispersed population, in agreement with DLS results. These surface characteristics support the suitability of the nanoparticles for biomedical and environmental applications.

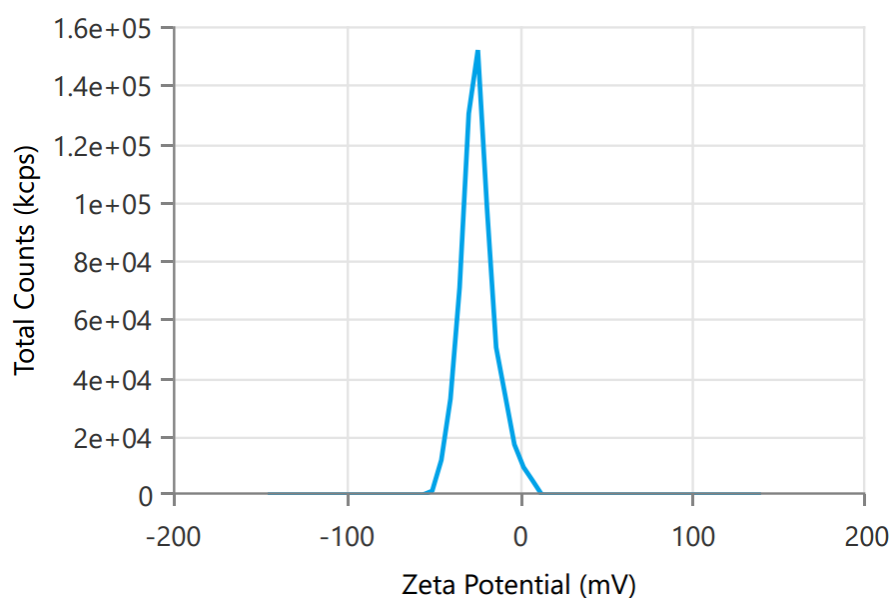


Figure 4.22: Zeta potential distribution of silver nanoparticles synthesized by isolate S1I4. The nanoparticles display a sharp unimodal peak centered at -23.66 mV, indicating a moderately stable colloidal suspension. The narrow distribution reflects a uniform surface charge, supporting good dispersion stability and reduced aggregation in aqueous media.

4.7.3 Fourier Transform Infrared (FTIR) Spectral Analysis

Fourier-transform infrared (FTIR) spectra of the fungal extract and biosynthesized silver nanoparticles (AgNPs) are shown in Figure 4.23. The fungal extract (green line) exhibited prominent absorption bands at ~ 3300 cm^{-1} (O–H stretching of hydroxyl groups), 2970 and 2855 cm^{-1} (C–H stretching of alkanes), 1560 cm^{-1} (N–H bending of

amides), 1380 cm^{-1} (C–N stretching of aromatic amines), and strong peaks at 1100 and 1050 cm^{-1} (C–O–C and C–O stretching vibrations of polysaccharides). Peaks in the region $500\text{--}600\text{ cm}^{-1}$ correspond to metal–oxygen vibrations. The AgNP spectrum (red line) retained all major functional peaks observed in the fungal extract, including $\sim 3300\text{ cm}^{-1}$ (O–H), $\sim 2970/2855\text{ cm}^{-1}$ (C–H), $\sim 1560\text{ cm}^{-1}$ (amide), $\sim 1380\text{ cm}^{-1}$ (amine), and $\sim 1100/1050\text{ cm}^{-1}$ (C–O–C/C–O). This confirmed the involvement of fungal biomolecules in the nanoparticle system. The presence of hydroxyl, amide, amine, and polysaccharide-associated vibrations strongly suggests that fungal metabolites acted as natural reducing agents for Ag^+ ions, while also serving as capping and stabilizing agents for the synthesized AgNPs. Such biomolecular capping not only stabilized the nanoparticles against aggregation but also enhanced their biological value, as functionalized surfaces are known to improve antimicrobial efficacy through synergistic effects of bioactive metabolites with silver. These findings align with earlier reports where fungal proteins, polysaccharides, and phenolic groups were implicated in both nanoparticle stabilization and their potent antimicrobial properties.

FTIR analysis of biosynthesized AgNPs revealed multiple functional groups from the fungal extract that remained associated with the nanoparticle surface, suggesting their dual role in reduction and stabilization of silver ions. The fungal extract spectrum showed characteristic peaks corresponding to hydroxyl ($\sim 3400\text{ cm}^{-1}$), C–H stretching ($2920\text{--}2850\text{ cm}^{-1}$), amide ($1650\text{--}1550\text{ cm}^{-1}$), and C–O stretching vibrations ($1380\text{--}1050\text{ cm}^{-1}$). In the AgNP spectrum, these functional groups were still evident, confirming their involvement as capping and stabilizing agents around the nanoparticles (Singh et al., 2024). Importantly, no significant disappearance of peaks was observed, which demonstrates that proteins, polysaccharides, and phenolic metabolites from the fungal extract likely acted as surface ligands rather than being consumed during nanoparticle formation. The persistence of these functional groups provides a biochemical rationale for the stability and bioactivity of the nanoparticles. Hydroxyl and carbonyl groups are often associated with electron donation during Ag^+ reduction, while amide and phenolic groups contribute to capping and colloidal stabilization, preventing aggregation (Ballottin et al., 2016). Moreover, FTIR studies consistently show that fungal proteins rich in cysteine, amines, and carboxyl groups form supramolecular interactions with AgNPs, reinforcing nanoparticle integrity and

enhancing their functional performance (Allothman & Abeer, 2019). This surface functionalization is also closely linked to antimicrobial action. Bioactive metabolites from fungi not only stabilize nanoparticles but also enhance their interaction with bacterial membranes through hydrogen bonding and electrostatic forces. For instance, phenolic and flavonoid residues contribute to ROS generation and cell wall destabilization, which synergistically improve antibacterial efficacy (Sathiyaseelan et al., 2020). Overall, the FTIR findings confirm that fungal-derived metabolites not only drive the reduction of silver ions but also provide a protective and biologically active capping layer. This dual function is likely responsible for the observed high antimicrobial activity of S1I4-mediated AgNPs, as the capped nanoparticles maintain structural stability, resist aggregation, and retain biofunctional ligands that can enhance their bactericidal potential.

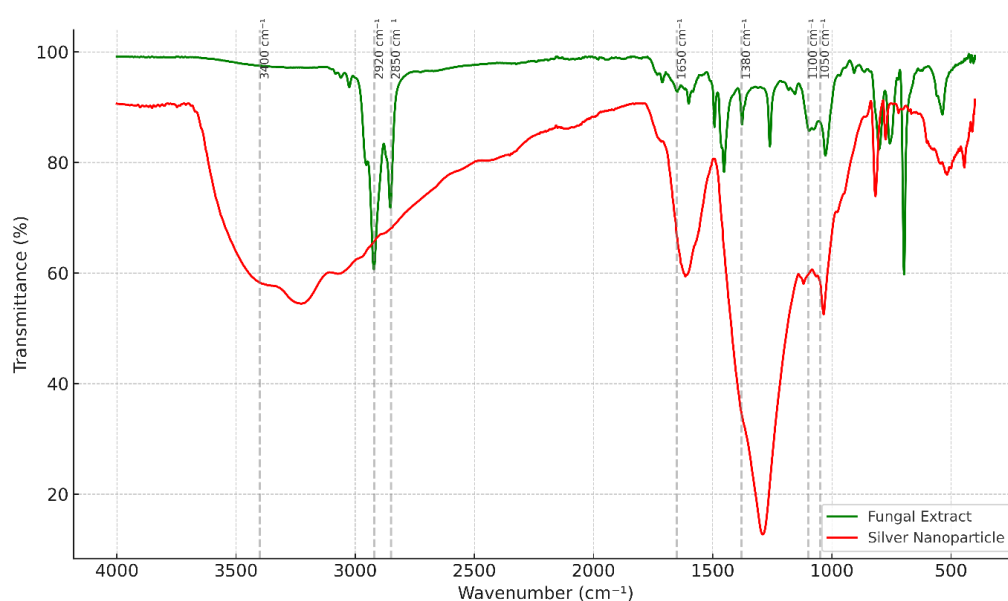


Figure 4.23: FTIR spectra of fungal extract (green) and silver nanoparticles (red) synthesized by isolate S1I4. The spectra highlight the involvement of biomolecules from the fungal extract in the reduction, capping, and stabilization of silver nanoparticles.

4.7.4 XRD Characterization of Biosynthesized Silver Nanoparticles

XRD analysis was employed to confirm the crystalline nature, structural phase, and purity of the biosynthesized AgNPs. The XRD spectrum (Figure 4.24) displayed sharp and well-resolved peaks at 2θ values of approximately 38.1° , 44.3° , 64.4° , and 77.4° , which correspond to the (111), (200), (220), and (311) lattice planes, respectively, of face-centered cubic (FCC) metallic silver (Figure 24). These reflections align precisely with the standard silver reference (JCPDS card No. 04-0783), confirming the formation of nanocrystalline elemental silver. The most intense peak at 38.1° for the (111) plane suggests a preferred orientation along this facet, a typical observation in biogenic silver nanoparticle synthesis. This enhanced (111) reflection has been correlated with higher surface energy and reactivity, which can enhance antimicrobial properties by increasing the exposure of reactive crystal facets (Jemal et al., 2017). The crystallite size of the AgNPs was further estimated using the Scherrer equation applied to the major diffraction peaks. The calculated crystallite sizes ranged between 11.3 and 14.0 nm, with an average size of ~ 12.8 nm. These nanoscale dimensions indicate high crystallinity and corroborate TEM-based size estimations, confirming the successful biosynthesis of small, stable, and crystalline AgNPs.

The presence of these specific peaks confirms the FCC crystal structure, which is a hallmark of high-purity silver nanoparticles. The absence of additional peaks suggests that no other crystalline phases or silver oxides are present, validating the chemical purity and successful reduction of silver ions. The sharpness and narrow width of the peaks reflect the small particle size and high degree of crystallinity. These findings are consistent with previous reports of fungal-mediated synthesis where similar peak patterns were observed in nanoparticles synthesized by *Penicillium aculeatum* and *Aspergillus brunneoviolaceus* (Mistry et al., 2021; Yaglioglu et al., 2022). Additionally, the dominance of the (111) plane in fungal-derived AgNPs may reflect the influence of surface-bound proteins and enzymes that direct anisotropic growth during biosynthesis. This strong preferential orientation, combined with the confirmed nanoscale crystallite size and absence of extraneous signals, emphasizes the reliability and reproducibility of fungal-mediated synthesis routes in producing phase-pure and crystalline nanoparticles. Such structural integrity is crucial for biomedical applications, as crystalline defects or secondary phases can adversely affect biological performance. In conclusion, the XRD data substantiate that the biosynthesized AgNPs

possess a high degree of crystallinity, purity, and a well-defined FCC structure, affirming their potential for applications where structural uniformity and functional consistency are essential (Mrs. N. K, 2024).

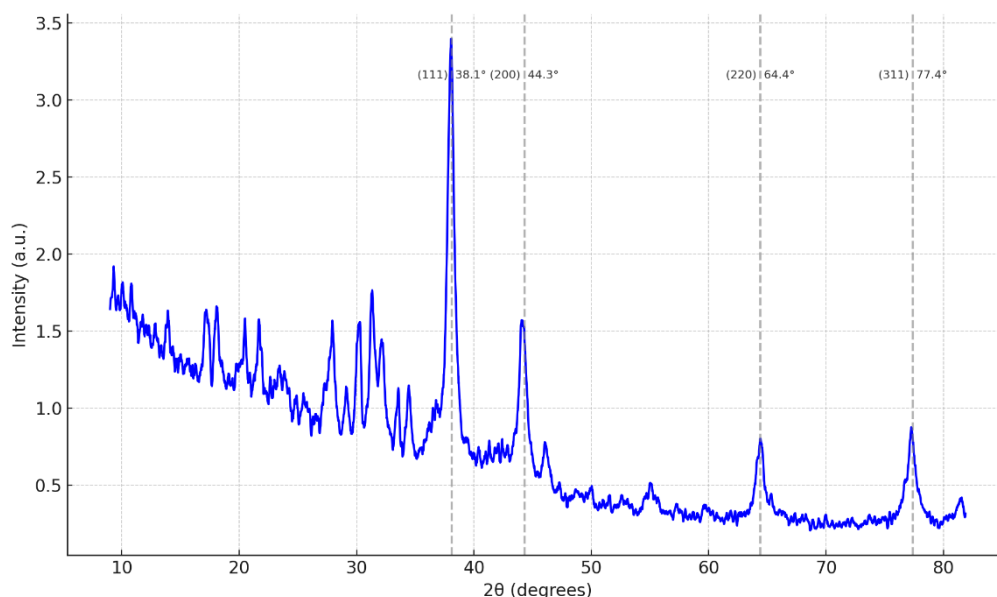


Figure 4.24: X-ray diffraction (XRD) pattern of silver nanoparticles synthesized by isolate S1I4. The diffraction profile confirms the face-centered cubic (FCC) structure of metallic silver with sharp reflections indicative of high crystallinity and the absence of secondary phases, validating the purity of the nanoparticles.

4.7.5 Transmission Electron Microscopy (TEM)

TEM was employed to assess the morphological features, dispersion characteristics, and nanoscale dimensions of the biosynthesized AgNPs derived from the most bioactive fungal isolate, S1I4 (Figure 4.25). At the lower magnification (200 nm scale), the nanoparticles appear well-dispersed and relatively monodispersed, confirming efficient nucleation and subsequent stabilization likely mediated by fungal biomolecules. The absence of prominent agglomeration suggests effective capping by bio-reducing and stabilizing agents such as proteins and phenolic compounds, commonly present in fungal filtrates. Higher magnification analysis (100 nm scale) enabled accurate measurement of particle size, with visible particles ranging between average diameter close 29 to 31 nm. This nanoscale size corresponds well with values

obtained from DLS and XRD analyses, reinforcing the structural consistency of the synthesized AgNPs. The nanoparticles predominantly exhibit spherical geometry with slight polydispersity, a characteristic frequently observed in biologically synthesized AgNPs due to inherent variability in the concentration and nature of bioactive metabolites. The observed size range is of particular interest for biomedical applications, as AgNPs below 40 nm are known to exhibit enhanced antimicrobial activity due to a higher surface-area-to-volume ratio, facilitating efficient interaction with microbial membranes and promoting intracellular uptake (Ma et al., 2017).

Similar spherical morphology and nanoscale dimensions have been reported in other fungal-mediated AgNP studies, including those synthesized by *Penicillium oxalicum* and *Fusarium hainanense*, where particles were consistently under 40 nm and demonstrated broad-spectrum antimicrobial potential (Netala et al., 2015). Furthermore, proteinaceous and phenolic compounds were found to be directly involved in the reduction and capping processes, contributing to the particles stability and uniformity (Netala et al., 2015). The minimal signs of clustering and consistent shape observed in the present study validate the role of fungal metabolites in maintaining colloidal stability through both electrostatic and steric mechanisms. Overall, the TEM findings conclusively demonstrate that the AgNPs synthesized by S1I4 are small, spherical, monodispersed, and non-agglomerated, with an average size of ~31 nm properties that are highly desirable for therapeutic and antimicrobial applications due to enhanced bioavailability, stability, and surface reactivity (Jaidev & Narasimha, 2010).

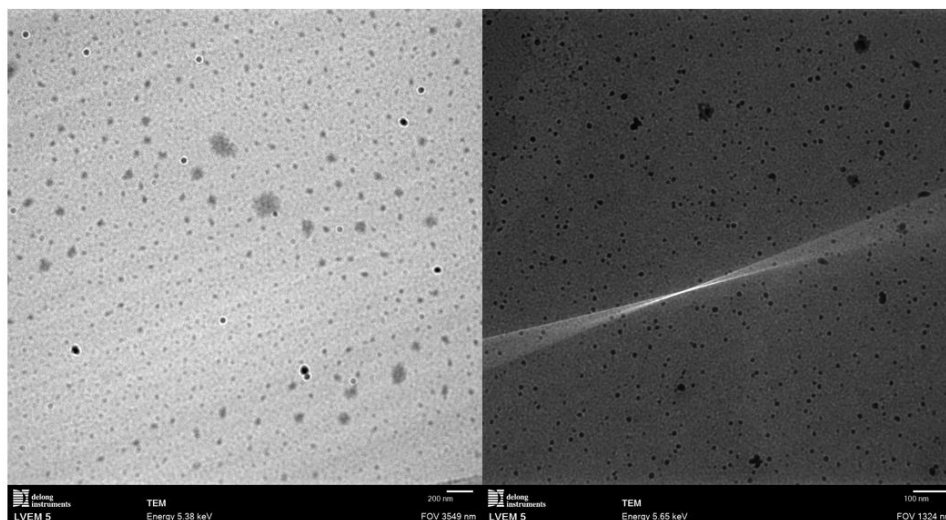


Figure 4.25: Transmission Electron Microscopy (TEM) images of silver nanoparticles synthesized by fungal isolate S1I4. (A) Low-magnification image showing a uniform dispersion of predominantly spherical nanoparticles with minimal agglomeration, indicating effective stabilization by fungal biomolecules. (B) High-magnification image displaying individual nanoparticles with diameters ranging from 29 to 34 nm, confirming their nanoscale dimensions and consistent morphology.

4.7.6 Scanning Electron Microscopy (SEM)

The surface morphology of the biosynthesized AgNPs derived from the fungal isolate S1I4 was examined using SEM. The micrograph (Figure 4.26) revealed that the nanoparticles were predominantly spherical in shape and moderately distributed across the surface, with regions showing slight clustering. Importantly, these clusters were not amorphous aggregates but retained the identity of individual particles, suggesting ordered nanoparticle assembly. This phenomenon is frequently reported in biogenic nanoparticle systems, where organic molecules from fungal extracts act as capping agents, stabilizing the nanoparticles while allowing limited particle–particle interactions. At high magnification (100,000 $\times$), the particles appeared with smooth surfaces and well-defined outlines, consistent with uniform nucleation during the biosynthesis process. No evidence of irregular crystallite growth or sharp faceted morphologies was observed, further supporting the conclusion that fungal metabolites facilitated controlled particle formation. Such morphological control is typically attributed to proteins, phenolics, and polysaccharides secreted by fungi, which serve

dual roles in reducing Ag⁺ ions and stabilizing the formed nanoparticles (Bagur et al., 2022). The SEM findings are in close agreement with TEM analysis, which revealed nanoparticles of ~29–34 nm average size with spherical geometry. These morphological features are also consistent with reports of other fungal-mediated AgNPs, where particles generally range from 20–70 nm with similar spherical forms (Pandiarajan et al., 2011; Pooja et al., 2020). Together, these results confirm that the biosynthesis pathway employed by S1I4 produced spherical, nanoscale, and structurally stable silver nanoparticles, characteristics that are critical for consistent performance in biomedical and antimicrobial applications.

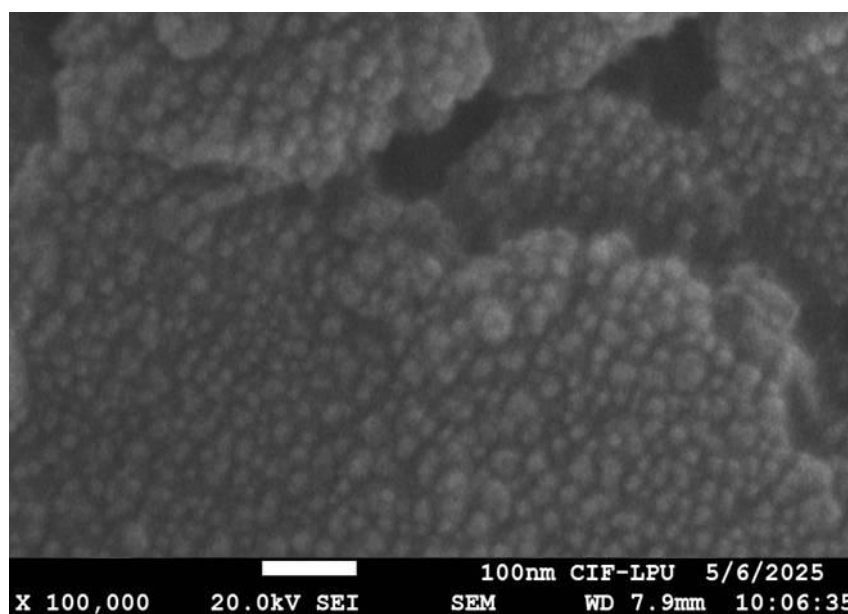


Figure 4.26: Scanning Electron Microscopy (SEM) image of silver nanoparticles synthesized by fungal isolate S1I4. The micrograph, captured at 100,000× magnification, shows nanoparticles that are predominantly spherical with smooth surfaces and moderate clustering. This morphology suggests stabilization by fungal biomolecules, while the ordered particulate assemblies confirm successful biogenic synthesis and preservation of structural integrity.

4.7.7 Energy Dispersive X-ray (EDX) Spectroscopy

EDx analysis provided elemental confirmation of the biosynthesized AgNPs. The EDx spectrum (Figure 4.27) exhibited a strong and characteristic silver (Ag) signal at ~3

keV, confirming the presence of metallic silver (Ag^0). Quantitative analysis indicated that silver constituted approximately 15–18 wt.% of the total composition, validating successful reduction of Ag^+ ions during fungal-mediated synthesis. This strong Ag signal is consistent with reports of mycogenic AgNPs where silver typically represents 10–30% of the total detected weight percentage, depending on the extent of biomolecular capping (Barabadi et al., 2025; El-Aziz, 2014). In addition to silver, major peaks of oxygen (45–50 wt.%) and carbon (25–30 wt.%) were observed, indicative of organic biomolecules on the nanoparticle surface. These elements are attributable to proteins, polysaccharides, and phenolics from the fungal extract that functioned as both reducing and capping agents. Their presence aligns with FTIR data, which confirmed hydroxyl, amine, and carbonyl functional groups on the nanoparticle surface. Such biomolecular capping not only prevents agglomeration but also enhances colloidal stability and bioactivity (Singh et al., 2018).

Trace elements including sodium (5–7 wt.%), calcium, potassium, and magnesium (1–2 wt.% each) were also detected, most likely derived from fungal metabolites or the growth medium. Their detection is consistent with earlier reports of biogenic AgNPs, where such elements co-exist as residual stabilizing agents (Vanlalveni et al., 2018). Overall, the EDX analysis confirms that the nanoparticles consist primarily of metallic silver, capped and stabilized by an organic matrix rich in oxygen- and carbon-containing biomolecules. This dual composition underscores the role of fungal metabolites in achieving both reduction of silver ions and long-term nanoparticle stabilization, features that are critical for their biomedical applications.

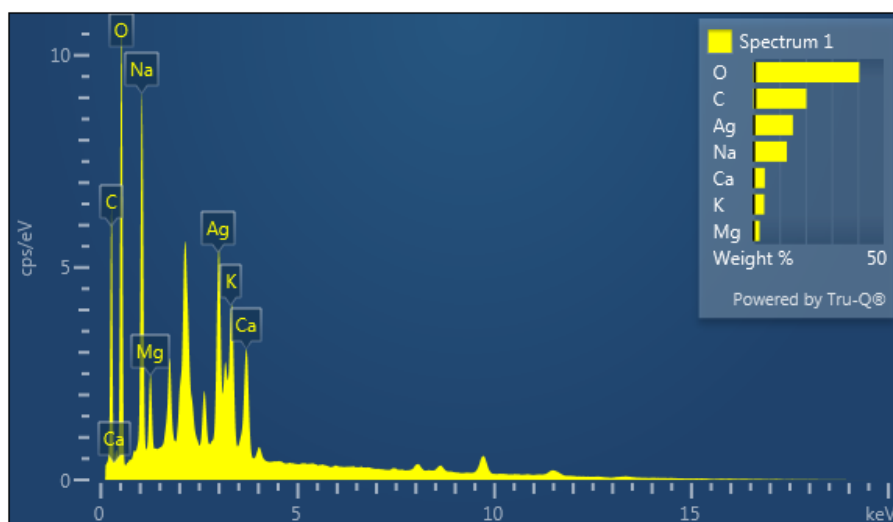


Figure 4.27: Energy Dispersive X-ray (EDX) spectrum of silver nanoparticles synthesized by fungal isolate S1I4. The spectrum confirms the presence of metallic silver along with trace elements attributed to fungal metabolites and medium components involved in nanoparticle capping and stabilization.

4.8. Morphological and Molecular Characterization of the Fungal Isolate

4.8.1 Morphological Characterization

The morphological characteristics of the fungal isolate S1I4 are summarized in Table 4.10. Morphological identification remains a fundamental step in fungal taxonomy, providing the first line of evidence for genus-level classification before molecular confirmation. The isolate S1I4 displayed distinct macroscopic and microscopic features that were consistent with species of the genus *Aspergillus*, particularly the *Aspergillus niger* complex. On PDA, the colonies of S1I4 exhibited rapid growth, attaining a considerable diameter within 5–7 days of incubation at 28 ± 2 °C. Colonies were initially white, becoming progressively dark brown to black due to profuse sporulation (Figure 4.28 A). The colony surface appeared cottony, with dense sporulating mycelia forming a concentric growth pattern, characterized by a central dark zone surrounded by a lighter periphery. Radiating folds were distinctly visible, producing a furrowed texture across the colony (Figure 4.28 B). The reverse of the colonies was pale yellow, a feature frequently associated with members of the *A. niger* group. Such macroscopic characteristics, including rapid growth rate, heavy sporulation, and dark pigmented

colonies, have been widely reported in *A. niger* isolates from diverse environments (Daly et al., 2020).

Microscopic observations using lactophenol cotton blue (LPCB) staining further confirmed the identity of the isolate. The hyphae were hyaline, septate, and smooth-walled, giving rise to erect, unbranched conidiophores originating from specialized foot cells. The conidiophores terminated in large, globose to sub-globose vesicles, which were entirely covered by a biserial arrangement of phialides radiating over the vesicle surface (Figure 4.28 C). The conidia were observed as rough-walled, globose to sub-globose structures, produced in compact chains. The dense black spore masses observed microscopically corresponded with the heavy sporulation recorded macroscopically. These morphological features are strongly diagnostic of the *Aspergillus niger* complex. In particular, the presence of biserial phialides covering the entire vesicle surface, rough-walled globose conidia, and dark pigmented colonies are considered hallmark characteristics for this group. Similar observations have been reported in earlier studies describing *A. niger* strains isolated from soil and plant-based environments, where colony pigmentation, conidial morphology, and sporulation pattern were used as key taxonomic criteria (Pandiarajan et al., 2011). Although morphological characterization provides reliable preliminary evidence for fungal identification, it is often insufficient for precise species-level resolution within the *Aspergillus* genus due to overlapping phenotypic traits. Therefore, molecular characterization using internal transcribed spacer (ITS) sequencing was undertaken to confirm the taxonomic placement of isolate S1I4 at the species level.

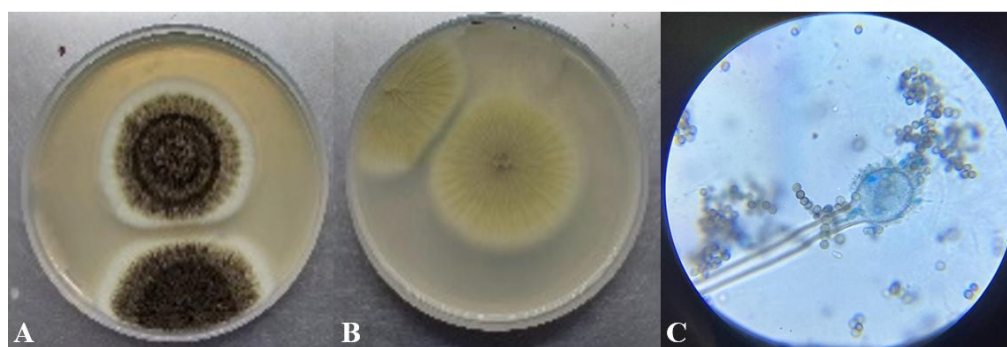


Figure 4.28: Morphological and microscopic features of the fungal isolate. (A) and (B) Colony morphology on PDA medium showing fast-growing, radial

colonies that were initially white and later turned black due to profuse sporulation. The colony surface appeared cottony to granular with a dense black central zone and a white peripheral margin. (C) Lactophenol cotton blue (LPCB) mount depicting biseriate phialides radiating from a globose vesicle, along with rough-walled conidia arranged in characteristic heads, confirming the isolate's identity as *Aspergillus niger*.

Table 4.10: Morphological characteristics of the fungal isolate S1I4 based on colony and microscopic observations.

Characteristic	Observation
Colony Morphology	Colonies on PDA were fast-growing, radial, initially white, turning black due to dense conidial heads. Reverse pigmentation appeared pale yellow, with moderate to heavy sporulation.
Texture	Cottony to granular surface texture with densely sporulating mycelium.
Growth Pattern	Colonies exhibited a central dense black zone surrounded by a white periphery; distinct radiating folds were visible.
Conidiophores	Hyaline, smooth-walled, unbranched; arising from foot cells; erect and stout.
Vesicles	Globose to sub-globose; biseriate arrangement with phialides radiating across the entire vesicle surface.
Phialides	Biseriate; covering the entire surface of the vesicle.
Conidia (spores)	Rough-walled, globose to sub-globose; brownish-black in mass; measuring ~3–5 µm in diameter.
LPCB Microscopy	Clear visualization of conidial heads, phialides, and vesicles; abundant black spore masses observed.

4.8.2 Molecular Identification via ITS Sequencing

Molecular identification of the fungal isolate S1I4 was performed through partial sequencing of the ITS gene, which is widely used as a taxonomic marker for fungal systematics due to its conserved nature interspersed with variable regions that allow discrimination at the genus and species levels. The obtained nucleotide sequence was

subjected to BLAST analysis using the NCBI GenBank database, which revealed a >99% sequence identity with multiple strains of *Aspergillus niger*. This high degree of similarity strongly supports the placement of isolate S1I4 within the *A. niger* complex. To further validate the taxonomic assignment, a phylogenetic tree was constructed using the neighbor-joining method (Figure 4.29). The sequence of isolate *Aspergillus* HG3 (GenBank accession number: PV203216.1) clustered firmly with reference *A. niger* strains, including isolates BSH-5 (OQ236103.1), FTR002 (OP231019.1), SRW11 (KY593543.1), and F3 (MK828713.1), with a bootstrap support value of 81%, indicating robust evolutionary relatedness. The close clustering of S1I4 with authenticated *A. niger* strains provides strong molecular confirmation of its identity. Importantly, the isolate was clearly separated from other closely related species such as *Aspergillus chevalieri* (OR502380.1) and *Aspergillus fumigatus* (PP211447.1), further ruling out misidentification. The integration of morphological and molecular data provides a comprehensive taxonomic resolution of isolate S1I4. While morphological traits such as biserial phialides, rough-walled globose conidia, and darkly pigmented colonies strongly indicated its affinity to the *A. niger* group, molecular identification via ITS sequencing conclusively confirmed its placement within the species. This combined approach follows established protocols in fungal systematics, where morphological classification is strengthened by DNA-based phylogeny to ensure accuracy. Thus, based on both morphological and molecular evidence, isolate S1I4 was definitively identified as *Aspergillus niger*. This identification provides a reliable basis for its subsequent use in the biosynthesis of silver nanoparticles, as several studies have reported *A. niger* to be an efficient biocatalyst in green nanotechnology applications (Zulkifli, 2015).

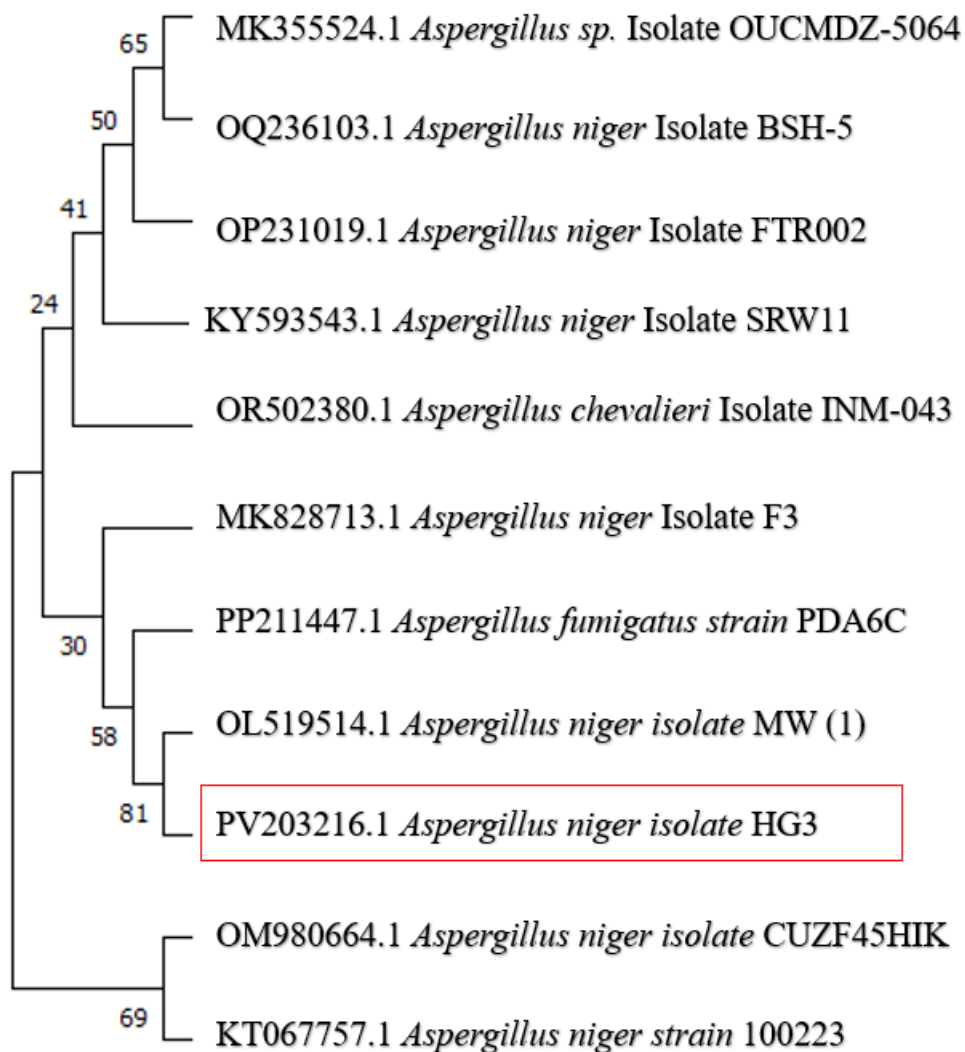


Figure 4.29: Neighbor-joining phylogenetic tree of the ITS gene sequence of fungal isolate S1I4. The phylogenetic tree, constructed using ITS region sequences, depicts the evolutionary relationship of isolate S1I4 (GenBank accession no. PV203216.1) with reference *Aspergillus* species retrieved from NCBI GenBank.

Chapter 5

5.1 Summary

Among these, carbapenem-resistant Enterobacteriaceae (CRE), particularly *Escherichia coli* and *Klebsiella pneumoniae*, are major clinical and public health concerns due to therapeutic failure, prolonged hospitalization, increased treatment costs, and higher mortality. Recognizing this threat, the World Health Organization (WHO) has classified CRE within the “Critical Priority Pathogen” group, underscoring the urgent need for novel, sustainable antimicrobial alternatives.

In this context, the present investigation was designed to develop a biologically inspired, eco-friendly strategy for synthesizing AgNPs using soil-derived fungi and to evaluate their antimicrobial efficacy against carbapenem-resistant *E. coli* and *K. pneumoniae*. Agricultural soil samples collected from Phagwara, Punjab, served as the microbial reservoir for isolation of potential nanoparticle-producing fungi. Twenty-five distinct fungal isolates were obtained, and both extracellular and intracellular extracts were tested for their ability to reduce silver ions, generating fifty biosynthesis reactions. Visible color changes from pale yellow to dark brown, followed by confirmatory UV–Visible spectroscopy (surface plasmon resonance between 391–450 nm), indicated successful nanoparticle synthesis. Preliminary antimicrobial screening identified one highly potent fungal isolate, designated S114, exhibiting the largest inhibition zones against *E. coli* (24.33 ± 1.52 mm) and *K. pneumoniae* (25.33 ± 2.08 mm). Subsequent MIC and MBC analyses revealed strong bactericidal activity even at low concentrations ($5/50 \mu\text{g mL}^{-1}$ for *E. coli* and $25/75 \mu\text{g mL}^{-1}$ for *K. pneumoniae*). Mechanistic investigations confirmed multi-target antibacterial action: AgNP-treated bacteria exhibited delayed growth kinetics, membrane permeability loss, nucleotide leakage, and genomic DNA degradation. Field Emission Scanning Electron Microscopy (FE-SEM) further revealed membrane blebbing, cell wall collapse, and structural deformation, confirming physical disruption as a key killing mechanism.

Physicochemical characterization of the most active AgNP formulation revealed nanosized, monodisperse, and stable particles. DLS recorded an average diameter of 24.55 nm with a low polydispersity index (<0.3), while Zeta potential analysis (-23.66

mV) confirmed good electrostatic and steric stability. FTIR spectroscopy identified functional groups such as hydroxyl, amide, and carbonyl, suggesting that fungal metabolites acted as both reducing and capping agents. XRD confirmed crystalline face-centered cubic silver, while TEM/SEM revealed predominantly spherical particles ranging from 29–34 nm. Energy-Dispersive X-ray (EDX) spectroscopy confirmed elemental silver purity with supporting peaks for carbon and oxygen, indicative of organic capping. The active fungal isolate was characterized morphologically as a fast-growing, dark brown-black species producing profuse sporulation. Microscopic examination showed biserial phialides and rough-walled globose conidia. Molecular identification using ITS1 and ITS4 primers confirmed the isolate as *Aspergillus niger* with >99% similarity to reference sequences (GenBank Accession No. PV203216.1). Cytotoxicity evaluation through MTT assays on NIH-3T3 fibroblast cells demonstrated limited toxicity, with an IC_{50} value of $100.06 \mu\text{g mL}^{-1}$, confirming biocompatibility at therapeutic concentrations. Collectively, the study establishes a robust fungal-mediated system for producing small, stable, and biologically active silver nanoparticles capable of inhibiting multidrug-resistant Gram-negative pathogens. The integration of microbiology, nanotechnology, and toxicology in this work provides a green alternative to conventional antimicrobial synthesis routes, reducing both environmental impact and production cost. The outcomes of this research contribute significantly to the growing field of antimicrobial nanobiotechnology. The findings demonstrate that *A. niger*-derived AgNPs exhibit potent bactericidal effects through multi-site mechanisms and possess excellent physicochemical stability and biosafety. Future research should prioritize in vivo validation, followed by formulation development and industrial scale-up of fungal-mediated nanoparticle synthesis. These advances may support the development of sustainable nanotherapeutics against antimicrobial resistance.

5.2 Conclusion

The present investigation establishes a sustainable, biologically driven strategy for the synthesis of AgNPs using soil-derived fungal isolates and highlights their strong antimicrobial potential against carbapenem-resistant *E. coli* and *K. pneumoniae*. In the backdrop of the escalating global burden of carbapenem-resistant Enterobacteriaceae, this study provides experimentally validated evidence supporting biogenic nanotechnology as a credible alternative to increasingly ineffective conventional antibiotics. Among the twenty-five fungal isolates recovered from agricultural soils, *Aspergillus niger* isolate S1I4 emerged as the most efficient nanofactory, mediating the extracellular synthesis of stable, crystalline, and uniformly sized AgNPs. The biosynthesized AgNPs exhibited pronounced bactericidal activity against both carbapenem-resistant pathogens, outperforming meropenem and demonstrating low MIC and MBC values. Importantly, the antibacterial effect was not restricted to growth inhibition but involved multi-target mechanisms, including disruption of bacterial membrane integrity, leakage of intracellular contents, genotoxic damage, and severe ultrastructural alterations. These effects were consistently supported by growth kinetic studies, nucleotide leakage assays, DNA profiling, and FE-SEM analysis. Such multi-modal antibacterial action is particularly advantageous, as it minimizes the likelihood of rapid resistance development, a major drawback associated with monotherapeutic antibiotics. Comprehensive physicochemical characterization further confirmed the biomedical suitability of the synthesized AgNPs. The nanoparticles were predominantly small, spherical, monodispersed, and colloidally stable, with surface-bound organic functional groups acting as natural reducing and capping agents. These biofunctional moieties not only contributed to nanoparticle stability but also likely facilitated effective interactions with bacterial cells. Notably, cytotoxicity evaluation using 3T3 mouse fibroblast cell lines revealed that the effective antibacterial concentrations of AgNPs were substantially below cytotoxic thresholds, indicating a favorable therapeutic index and acceptable preliminary biosafety. Collectively, the findings of this study demonstrate that *Aspergillus niger*-derived silver nanoparticles represent an efficient, eco-friendly, and biologically compatible antimicrobial system against carbapenem-resistant *E. coli* and *K. pneumoniae*. By integrating green synthesis, mechanistic insights, advanced physicochemical characterization, and

toxicity assessment, this work provides a robust scientific foundation for the development of next-generation nanotechnology-based antimicrobial interventions. Despite these promising outcomes, further investigations are necessary to translate the present findings into clinical and real-world applications. Despite these promising outcomes, further investigations are necessary to translate the present findings into clinical and real-world applications. Future research should primarily focus on in vivo efficacy validation using suitable animal models to assess antibacterial performance, pharmacokinetics, and biodistribution under physiological conditions. Further emphasis should be placed on comprehensive biosafety evaluation, including long-term toxicity, immunotoxicity, and environmental impact. Additionally, exploring synergistic interactions between AgNPs and existing antibiotics may enable dose optimization and restore the effectiveness of conventional therapeutics. Finally, optimization and scale-up of fungal-mediated synthesis under controlled bioreactor conditions, along with integration into antimicrobial coatings, wound dressings, medical devices, and drug-delivery systems, will be essential to enhance industrial feasibility and translational potential. In conclusion, this work addresses a critical antimicrobial resistance challenge and advances sustainable nanotechnology-driven therapeutic strategies, positioning fungal-mediated silver nanoparticles as a promising tool in the ongoing fight against carbapenem-resistant bacteria.

APPENDICES

APPENDIX- I: List of items used during the research

1. CHEMICALS

S/N	Name of Chemical / Reagent	Purpose / Application	Brand / Source
1	Silver nitrate (AgNO_3)	Precursor for AgNP biosynthesis	Merck, India
2	Cetyltrimethylammonium bromide (CTAB)	Fungal DNA isolation buffer (lysis agent)	Loba Chemie Pvt. Ltd.
3	Phenol:Chloroform:Isoamyl alcohol (25:24:1)	DNA purification	Loba Chemie Pvt. Ltd.
4	Chloroform	Solvent for extraction and purification	Loba Chemie Pvt. Ltd.
5	Boric acid	Buffer component for electrophoresis	Loba Chemie Pvt. Ltd.
6	Ethylenediaminetetraacetic acid (EDTA)	Chelating agent in DNA isolation	Loba Chemie Pvt. Ltd.
7	Sodium dodecyl sulfate (SDS)	Cell lysis and protein denaturation	Loba Chemie Pvt. Ltd.
8	Sodium chloride (NaCl)	Ionic strength adjustment, media component	HiMedia Laboratories
9	Potassium dihydrogen phosphate (KH_2PO_4)	Buffer component	HiMedia Laboratories
10	Disodium hydrogen phosphate (Na_2HPO_4)	Buffer component	HiMedia Laboratories
11	Tris-HCl	pH buffer for DNA extraction	SRL Chemicals
12	Glucose	Carbon source for fungal culture	HiMedia Laboratories

13	Peptone	Protein/nitrogen source	HiMedia Laboratories
14	Yeast extract	Growth supplement	HiMedia Laboratories
15	Agar-agar	Solidifying agent for culture media	HiMedia Laboratories
16	Glutaraldehyde	Fixative for SEM sample preparation	Loba Chemie Pvt. Ltd.
17	Hexamethyldisilazane (HMDS)	Dehydration agent for SEM sample coating	Loba Chemie Pvt. Ltd.
18	Ferric chloride (FeCl ₃)	Qualitative biochemical reagent	Loba Chemie Pvt. Ltd.
19	Lead acetate	Biochemical detection reagent	Loba Chemie Pvt. Ltd.
20	Ninhydrin	Amino acid detection reagent	Loba Chemie Pvt. Ltd.
21	Methanol	Solvent and nanoparticle washing agent	Loba Chemie Pvt. Ltd.
22	Ethanol	Dehydration, sterilization, DNA precipitation	Loba Chemie Pvt. Ltd.
23	Petroleum ether	Solvent for pigment/metabolite extraction	Loba Chemie Pvt. Ltd.
24	Hydrochloric acid (HCl)	pH adjustment	Loba Chemie Pvt. Ltd.
25	Sodium hydroxide (NaOH)	pH adjustment	Merck India
26	MTT reagent [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]	Cytotoxicity assay	HiMedia Laboratories Pvt. Ltd.

27	Dimethyl sulfoxide (DMSO)	Solvent for MTT assay	Loba Chemie Pvt. Ltd.
28	Ethidium bromide	DNA visualization stain	Loba Chemie Pvt. Ltd.
29	Agarose	Gel electrophoresis matrix	HiMedia Laboratories
30	Tris–Borate–EDTA (TBE) buffer	DNA electrophoresis running buffer	SRL Chemicals
31	Distilled / Deionized water	Solution and media preparation	In-house laboratory
32	Potassium bromide (KBr)	FTIR pellet preparation	Merck India
33	Ferric nitrate ($\text{Fe}(\text{NO}_3)_3$)	Analytical biochemical reagent	Loba Chemie Pvt. Ltd.
34	Ethyl acetate	Fungal metabolite extraction	SRL Chemicals
35	Acetone	Cleaning and sample preparation	SRL Chemicals
36	Ethanol (Absolute, 99.9%)	DNA precipitation and sterilization	Loba Chemie Pvt. Ltd.
37	70% Ethanol	Surface sterilization	Loba Chemie Pvt. Ltd.
38	Hydrogen peroxide (H_2O_2)	Surface sterilization and biochemical assays	Merck India
39	Distilled water	Media preparation and rinsing	In-house glass-distilled

2. MEDIA AND BUFFERS

S/N	Name of Medium / Buffer	Purpose / Application	Brand / Source
1	Potato Dextrose Agar (PDA)	Isolation and maintenance of fungal isolates	HiMedia Laboratories Pvt. India
2	Potato Dextrose Broth (PDB)	Production of fungal biomass for AgNP biosynthesis	HiMedia Laboratories Pvt. India
3	Nutrient Agar (NA)	Preliminary bacterial culture maintenance	HiMedia Laboratories Pvt. India
4	Mueller–Hinton Agar (MHA)	Antibacterial susceptibility testing (agar well diffusion)	HiMedia Laboratories Pvt. India
5	Mueller–Hinton Broth (MHB)	MIC/MBC determination and liquid culture assays	HiMedia Laboratories Pvt. India
6	Eosin Methylene Blue (EMB) Agar	Isolation and differentiation of E. coli	HiMedia Laboratories Pvt. India
7	MacConkey Agar	Selective medium for Enterobacteriaceae	HiMedia Laboratories Pvt. India
8	Tryptic Soy Broth (TSB)	Preparation of bacterial inocula	HiMedia Laboratories Pvt. India
9	Luria–Bertani (LB) Broth	Routine bacterial growth	HiMedia Laboratories Pvt. India
10	Phosphate Buffered Saline (PBS, 0.1 M, pH 7.2)	Washing and dilution buffer for cell preparation	HiMedia Laboratories Pvt. India
11	Tris–EDTA (TE) Buffer (pH 8.0)	DNA dissolution and storage	SRL Chemicals India

12	Tris–Borate–EDTA (TBE 1×) Buffer	DNA gel electrophoresis running buffer	SRL Chemicals India
13	Agarose Gel Matrix	DNA electrophoresis medium	Loba Chemie Pvt. Ltd.
14	Eagle’s Minimum Essential Medium (EMEM)	3T3 fibroblast cell culture	Sigma–Aldrich India
15	MTT Assay Reagent Medium	Cell viability assessment	HiMedia Laboratories Pvt. India
16	Phosphate Citrate Buffer	pH stabilization during enzyme or metabolite tests	HiMedia Laboratories
17	Tris–HCl Buffer (10 mM, pH 8.0)	DNA extraction and washing steps	SRL Chemicals
18	Sodium acetate buffer (3 M, pH 5.2)	DNA precipitation step	Loba Chemie Pvt. Ltd.

Key: Media were prepared according to manufacturer’s instruction

3. ANTIBIOTICS

SN	Names of antibiotics	Formulation Used	Disk Potency
1.	Meropenem	Powder	Not applicable
2	Imipenem	Disk	10 µg
3	Meropenem	Disk	10 µg

4. INSTRUMENTS

S/N	Instrument Name	Brand / Manufacturer	Model / Specification	Purpose / Application
1	Autoclave	Scienocraft	STI-118 Delux	Sterilization of media and glassware
2	Biosafety Cabinet (Class II)	FAB India S.S 304	BSC 100 FPB	Aseptic handling during microbial and nanoparticle work
3	Laminar Air Flow	—	—	Aseptic transfer and inoculation
4	Incubator	Vevor	—	Bacterial/fungal culture incubation
5	Hot Air Oven	Oven Universal	NSW-143	Dry sterilization of glassware
6	Cooling Centrifuge	Plasto Crafts	Rota 4R- V/FM	Biomass separation and nanoparticle collection
7	Compound Microscope	Magnus	MS21i	Microscopic examination of fungal isolates
8	Phase Contrast Microscope	Olympus / Nikon	CKX53 (typical)	Observation of 3T3 fibroblast cytotoxicity morphology
9	UV-VIS Double Beam Spectrophotometer	Lasany	LI-2800 Ex	UV-Vis spectral analysis of AgNPs

10	Microprocessor Visible Spectrophotometer	Lasany	LI-721	Routine absorbance readings
11	NanoDrop Spectrophotometer	Thermo Scientific	ND-2000 / ND-One	Quantification of DNA purity (A260/A280)
12	Electrophoresis Unit (Gel Tank & Power Pack)	RIDDE Varipack	—	DNA agarose gel electrophoresis
13	Gel Documentation System	NUgenius	G-1.1 / F-07-GEN	Visualization of DNA bands
14	PCR Thermocycler	Eppendorf Mastercycler	Nexus Gradient	ITS gene amplification
15	Refrigerated Micro-Centrifuge	REMI	C-24 BL	High-speed DNA and nanoparticle pellet centrifugation
16	Field Emission Scanning Electron Microscope (FE-SEM)	JEOL	JSM-74190 UEC	Surface morphology and cell-interaction imaging
17	Gold Sputter Coater	JEOL	—	Sample coating for FE-SEM
18	Transmission Electron Microscope (TEM)	JEOL	JEM-2100	Internal morphology and size determination
19	Energy-Dispersive X-Ray Analyzer (EDX)	JEOL	Integrated with SEM	Elemental composition of AgNPs
20	X-Ray Diffractometer (XRD)	PANalytical X'Pert PRO /	—	Crystalline phase and lattice structure

		Rigaku MiniFlex		
21	Fourier Transform Infrared Spectrophotometer (FTIR)	Perkin Elmer	Spectrum Two	Functional-group identification of AgNPs
22	Dynamic Light Scattering & Zeta Potential Analyzer	Malvern	Zetasizer Nano ZS	Particle size and surface charge measurement
23	Rotary Evaporator	Rotavapor	Horizontal	Solvent removal and concentration
24	Sonicator Bath	PCI Analytical / Citizen	—	Dispersion of nanoparticles
25	Soxhlet Apparatus	Borosilicate	—	Extraction of fungal metabolites
26	Vortex Shaker	REMI	CM-101 Plus	Mixing of samples
27	Water Bath	Presto	—	Controlled heating of samples
28	Heating Mantle	Moglix U-Tech	SSI-178	Reflux and heating reactions

APPENDIX–II: Buffers and Reagents Prepared

A. Reagents

1. 1 M Tris–HCl (pH 8.0) 121.14 g Tris base was dissolved in 800 mL distilled water, pH adjusted to 8.0 using concentrated HCl, and volume made up to 1 L. The solution was sterilized by autoclaving at 121 °C for 15 min and stored at room temperature.

2. 0.5 M EDTA (pH 8.0) 146.12 g EDTA disodium salt was dissolved in 500 mL water with gentle heating and stirring; NaOH pellets were added to raise pH to 8.0 until fully dissolved. Volume made up to 1 L, autoclaved, and stored at room temperature.

3. 5 M Sodium Chloride (NaCl) 292.2 g NaCl was dissolved in 500 mL water, stirred until clear, and volume made up to 1 L. The solution was autoclaved and stored at room temperature.

4. 10 % Cetyltrimethylammonium Bromide (CTAB) 100 g CTAB was dissolved in 500 mL sterile water with gentle heating. The final volume was adjusted to 1 L and stored at room temperature.

5. CTAB–NaCl (1:1) Equal volumes (250 mL each) of 10 % CTAB and 5 M NaCl were mixed thoroughly to form a viscous extraction buffer, used freshly during fungal DNA isolation.

6. RNase A Solution (10 mg mL⁻¹) 100 mg RNase A was dissolved in 10 mL sterile water, heated at 90 °C for 10 min to inactivate DNase, and stored at –20 °C.

7. Proteinase K Solution (20 mg mL⁻¹) 200 mg Proteinase K was dissolved in 10 mL sterile water, mixed gently, and stored at –20 °C in a screw-capped vial.

8. 10 % Sodium Dodecyl Sulphate (SDS) 10 g SDS was dissolved in 50 mL sterile water, warmed slightly to aid dissolution, and volume made up to 100 mL. The solution was stored at room temperature.

9. Phenol : Chloroform : Isoamyl Alcohol (25 : 24 : 1) Prepared by mixing 25 mL liquefied phenol, 24 mL chloroform, and 1 mL isoamyl alcohol. The mixture was equilibrated with 10 mM Tris–HCl (pH 8.0) and stored in an amber bottle at 4 °C.

10. Ethidium Bromide Stock (10 mg mL⁻¹) 200 mg ethidium bromide was dissolved in 20 mL sterile water under a fume hood using a magnetic stirrer. The solution was protected from light with aluminum foil and stored at room temperature.

11. MTT Reagent (5 mg mL⁻¹) Prepared by dissolving 5 mg MTT in 1 mL sterile PBS, filter sterilized (0.22 µm), aliquoted, and stored at –20 °C for cytotoxicity assays.

12. Dimethyl Sulfoxide (DMSO) Used directly from stock reagent as solubilization solution for MTT-formazan crystals; pre-warmed to 37 °C before use.

13. Glutaraldehyde Fixative (2.5 %) Prepared by diluting 25 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) to obtain a 2.5 % working solution for SEM sample fixation.

14. Hexamethyldisilazane (HMDS) Used as a final dehydrating agent after ethanol series during SEM specimen preparation.

15. Lactophenol Cotton Blue Stain Composed of phenol crystals (20 g), lactic acid (20 mL), glycerol (40 mL), cotton blue (0.05 g), and distilled water (20 mL); mixed gently and stored in dark glass bottle for fungal microscopic observation.

16. Silver Nitrate (AgNO₃) Solution (1 mM) Prepared by dissolving 0.017 g AgNO₃ in 100 mL double-distilled water and stored in amber bottle in dark conditions for nanoparticle synthesis.

17. 0.5 McFarland Standard

Prepared by mixing 0.05 mL of 1.175 % (w/v) barium chloride dihydrate (BaCl₂·2H₂O) with 9.95 mL of 1 % (v/v) sulfuric acid (H₂SO₄) to obtain a turbid suspension equivalent to ~1.5 × 10⁸ CFU mL⁻¹. The suspension was mixed thoroughly, dispensed into screw-capped tubes, sealed to prevent evaporation, and stored at room temperature in the dark.

Prior to use, the standard was vortexed gently and visually compared against bacterial suspensions to adjust inoculum density.

B. Buffers

1. Tris–EDTA (TE) Buffer (pH 8.0; 10 mM Tris, 1 mM EDTA)

10 mL of 1 M Tris–HCl and 2 mL of 0.5 M EDTA were mixed in 988 mL sterile water to make 1 L of buffer. The solution was autoclaved and stored at room temperature.

2. Tris–Acetate–EDTA (TAE) Buffer (50× Stock)

242 g Tris base was dissolved in ~800 mL water; 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0) were added. The volume was adjusted to 1 L. A 1× working solution was prepared by 1:50 dilution of the stock.

3. Tris–Borate–EDTA (TBE) Buffer (10× Stock)

108 g Tris base and 55 g boric acid were dissolved in 900 mL water; 40 mL 0.5 M EDTA was added, and volume made up to 1 L. The working solution (1×) was prepared by 1:10 dilution before electrophoresis.

4. DNA Loading Buffer (6×)

3 mL glycerol and 25 mg bromophenol blue were mixed and the volume made up to 10 mL with sterile water. The buffer contained 30 % glycerol and 0.25 % bromophenol blue, aliquoted and stored at –20 °C.

5. Phosphate-Buffered Saline (PBS, 0.1 M, pH 7.2)

NaCl 8.5 g, KCl 0.2 g, Na₂HPO₄ 1.44 g, KH₂PO₄ 0.24 g were dissolved in 900 mL water, pH adjusted to 7.2, volume made to 1 L, autoclaved, and stored at 4 °C.

6. Tris–HCl Buffer (10 mM, pH 8.0)

Prepared from 1 M stock by dilution (10 mL per L). pH adjusted to 8.0 and used in DNA extraction and washing steps.

7. Sodium Acetate Buffer (3 M, pH 5.2)

Prepared by dissolving 246 g sodium acetate trihydrate in distilled water, adjusting pH with acetic acid, and making up to 1 L. Used for DNA precipitation.

8. Phosphate–Citrate Buffer (0.1 M, pH 5.0)

Mixed 71 mL 0.1 M Na₂HPO₄ with 29 mL 0.1 M citric acid to achieve pH 5.0; used for enzyme and metabolite assays.

APPENDIX–III: Publications and conference

Publication

1. **Himanshu Jangid**, Deepak Kumar, Gaurav Kumar, Raj Kumar, and Narsimha Mamidi (2023): *Bibliometric Examination of Global Scientific Research about Carbapenem-Resistant Acinetobacter baumannii (CRAB)*. **Antibiotics**, 12(11): 1593. (MDPI, Q1 Journal, SJR = 1.14, Scopus & Web of Science Indexed)
2. **Himanshu Jangid**, Anuprita Ray, and Gaurav Kumar (2025): *Biosynthesis of Silver Nanoparticles by Soil-Derived Rhizopus arrhizus HGS2I2: Antibacterial Potential against Carbapenem-Resistant Escherichia coli and Klebsiella pneumoniae*. **Rasayan Journal of Chemistry**, 18(3): 1717–1724. (Q3 Journal, SJR = 0.32, Scopus Indexed)
3. **Himanshu Jangid**, Anuprita Ray, Suresh Kumar Rajamani Sekar, and Gaurav Kumar (2025). *In vitro and in silico antimicrobial activity of Aspergillus niger HG3 against carbapenem-resistant Escherichia coli and Klebsiella pneumoniae and their antioxidant potential*. **Discover Applied Sciences**. (Q1 Journal; SJR 2024 = 0.543; Scopus & Web of Science Indexed).

Conference

- ❖ Oral presentation on “Antimicrobial activity of silver nanoparticle synthesized from soil fungi and their antimicrobial activity against *E. coli* and *Klebsiella pneumoniae*” in International Conference on environmental, chemical and synthetic biology towards sustainable development goals (2024)
- ❖ Oral presentation on “Antimicrobial activity of silver nanoparticle synthesized from *Rhizopus arrhizus* and their antimicrobial activity against *E. coli* and *Klebsiella pneumoniae*” in Emerging Trends Innovations in Basic Science (2024)

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