Biosynthesis, Characterization and Biological Applications of Gold Nanoparticles

ASMA MUMTAZ

Registration # 00000171286

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan

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Biosynthesis, Characterization and Biological Application of Gold Nanoparticles

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By

ASMA MUMTAZ

Registration # 00000171286

Supervised by: Dr. Rumeza Hanif

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan.

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(Supervisor)

Dr. Rumeza Hanif

Assistant Professor, Healthcare Biotechnology

ASAB, NUST

Dedicated to

My Beloved Parents

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All grace and gratitude for the One Who doesn't leave me for the blink of an eye, Who listens to all my whims and woes and walks all roads with me; to the Almighty, the creator the Exalted.

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Asma Mumtaz

CONTENTS

LIST OF ACRONYMS

0D	Zero Dimension
1D	One Dimension
2D	Two Dimensions
3D	Three Dimensions
nm	Nanometer
μm	Micrometer
NPs	Nanoparticles
SPR	Surface Plasmon Resonance
Au	Gold
AuNPs	Gold Nanoparticles
$HAuCl_4.3H_2O$	Chloroauric acid
ROS	Reactive Oxygen Species
AgNPs	Silver Nanoparticles
UV/Vis	Ultraviolet/Visible
FTIR	Fourier Transformed Infrared Spectroscopy
XRD	X-Ray Diffraction
SEM	Scanning Electron Microscopy
EDS	Energy Dispersive Spectroscopy
DPPH	2,2-diphenyl-1-picrylhydrazyl hydrate

MTT	3-(4,5-Dimethylthiazole-2yl)-2,5-Diphenyltetrazolium Bromide
UV	Ultraviolet
m^2/g	Square meter per gram
CNT	Carbon Nanotubes
FeNPs	Iron Nanoparticles
Al_2O_3	Aluminum Oxide
CeO_2	Cerium Oxide
Fe_2O_3	Iron Oxide
Fe_3O_4	Magnetite
SiO_2	Silicon Oxide
MNPs	Metal Nanoparticles
MTs	Metallothioneines
VNPs	Virus mediated nanoparticles
POM	Polarized Optical Microscopy
TEM	Transmission Electron Microscopy
XPS	X-Ray Photoelectron Spectroscopy
DLS	Dynamic Light Scattering
HRTEM	High Resolution Transmission Electron Microscopy
CPP	Cell Penetrating Peptides
siRNA	small interfering RNA
CTAB	Cetyltrimethylammonium bromide
MDR	Multi Drug Resistant
MRS	de Man, Rogosa and Sharpe Media

mg	Milligram
mM	Millimolar
mm	Millilitre
MRI	Magnetic Resonance Imaging
Huh-7	Human Hepatoma Cell Line 7
keV	Kilo Electron Volt
KBr	Potassium Bromide
Kg	Kilogram
Hr	Hour
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
NMR	Nuclear magnetic Resonance
w/v	Weight/Volume
v/v	Volume/Volume
rpm	Revolutions per minute
mA	milliampere
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DI	De-ionized water

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Nanotechnology is an interdisciplinary science that deals with development and manipulation of functional systems at atomic and molecular level. Nanoparticles are objects that range in size from 1 - 100nm and may differ from their bulk material in chemical and physical properties. Among a variety of nanomaterials, metallic nanoparticle such as gold nanoparticles have gained significant importance in past due to their administration in the field of material and manufacturing industry, cosmetics, therapeutic and pharmaceutical industries. Nanoparticles can be synthesized using physic-chemical and biological approaches. Physico-chemical methods provide nanoparticles of definite size but synthesis process is toxic and expensive. Biological methods are preferred because they provide ease of handling, synthesis and no toxic material by products formation. In this study, *Lactobacillus rhamnosus* is used for synthesis of gold nanoparticles. Bacteria provide bioactive oxidoreductase enzymes that act as catalyst in bio-reduction reaction and reduces gold ions in solution to gold nanoparticles. Synthesis of these nanoparticles is evaluated by change in color of reaction mixture. These nanoparticles were further verified by UV/Vis spectrophotometer that shows peak for AuNPs at 540nm. Comparison of Ultraviolet/Visible spectra of bacterial biomass and AuNPs showed characteristic change in absorbance. Their elemental composition, evaluated by Element Disspersive Spectroscopy analysis, indicated presence of 4% gold element in the sample mixture. Crystallinity of nanoparticles were analyzed by X-Ray Diffractogram, whereas Fourier Transformed Infrared spectrum indicated the presence of amine groups depicting that particles are stable in nature. Morphology and diameter were measured by microscopic analysis such as Scanning Electron Microscopy. Newly synthesized AuNPs were spherical with varying size of 50-70nm. Bactericidal activity of nanoparticles was performed against strains of *Salmonella enterica*, *Escherichia coli*, *Pseudomonas nitroreducens* *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Antibacterial results showed that AuNPs can be used as an alternative to antibiotic. DPPH inhibition assay revealed scavenging ability of biogenic AuNPs compared with ascorbic acids at different concentrations. 500μg/ml of biogenic AuNPs show 70% free radical scavenging ability.

Chapter 1

INTRODUCTION

Nanotechnology refers to the field of science that deals with the preparation of nanomaterials. Nanomaterials are smaller than one micrometer at atleast one dimension. These materials are studied at nanoscale to evaluate their atomic and molecular properties (Mansoori, 2005). Nanotechnology is a multidisciplinary field which has applications in biophysics, electronics, bioengineering, and molecular biology. It also provides devices, tools and materials for pharmaceutics, medicine, and other specialized fields. Development of nanotechnology to several levels such as devices, materials and systems is expected in the near future. At present, the most advanced level is nanomaterials, both in commercial and scientific applications (Murray, Kagan, & Bawendi, 2000). Types of nanometerials commercially available include ceramics, quantum dots and semi-conductors (Dubchak, Ogar, Mietelski, & Turnau, 2010).

Nanoparticles (NPs) are building blocks that laid the foundation of nanotechnology. They range in size from 1-100nm and are composed of metal, metal oxides or organic matter (Hasan, 2015).These particles, in amorphous and crystalline morphology, were the center of attention due to their contribution in development of nano-based products (Buzea, Pacheco, & Robbie, 2007; Horton & Khan, 2006; Kawasaki & Player, 2005). These particles possess the ability of exhibiting increased mechanical strength, large surface area to volume ratio, different size and shapes, because of which they have enormous applications in fields of diagnostic probes, catalysis and disease diagnosis i.e. cancer, detection of toxic metals in environment and drug discovery (Balbus et al., 2007). NPs have different dimensions such as zero dimensional like quantum dots in which height, length and width are fixed at one point, one dimensional that possess only one parameter, two dimensional that have length and width or three dimensional that has all parameter (E. J. Cho et al., 2013). Their shapes may vary from spherical to rod, tubular, cylindrical or spiral (Machado, Pacheco, Nouws, Albergaria, & Delerue-Matos, 2015). Previously, NPs were studied due to their physico-chemical properties based on their sizes. Now these

particles have entered an era where they are studied for their magnetic and optical properties (Paull, Wolfe, Hébert, & Sinkula, 2003).

Metallic nanoparticles are the NPs that are synthesized through metals either by constructive or destructive approach. Almost all the metals can be used for the preparation of their NPs (Salavati-Niasari, Davar, & Mir, 2008). Commonly used metals include Silver (Ag), Carbon (C), Gold (Au), Lead (Pb), Copper (Cu), Iron (Fe) and so on. These particles exhibit unique shapes and optical properties. Extinction spectra of these particles are dominated by surface plasmon resonance (SPR) (Dulkeith et al., 2004). These resonances responds to the excitation of electrons present in conduction bands, excited by the electromagnetic field. Free electrons of metallic nanoparticles exhibit coherent oscillations corresponding to positive metallic lattice, in the presence of an oscillating electromagnetic field (Dreaden, Alkilany, Huang, Murphy, & El-Sayed, 2012).

Among all the metal elements found in earth crust, gold (Au) is one of the rarest metal and is mostly found in the form of aqueous solution as Au (0), Au (I), Au (III). Au ions can be converted into gold nanoparticles. Gold nanoparticles (AuNPs) are mostly preferred among metallic nanoparticles in the field of biotechnology and biomedicine because of following features, a). Increased physical and chemical stability, including their biocompatibility with human cells, b) Optoelectronic properties, c) Ease of surface functionalization with other biomolecules (Amendola & Meneghetti, 2009; Mout, Moyano, Rana, & Rotello, 2012), d) Strong binding affinity to disulfides, amines and thiols (Zhou, Wang, Zhu, & Chen, 1999) and e) ease of synthetic manipulation (Yuanchao Zhang et al., 2014). Au is used internally by humans due to its chemical inertness. One of the major characteristic of AuNPs lies in its multi-functionalization. They are highly used in the field of biomedical such as cellular imaging (Austin, Kang, & El-Sayed, 2015; Murphy et al., 2008), sensing, chemical analysis and catalysis (Anker et al., 2010; Jahn et al., 2016), nonlinear optical processes (Hentschel, Metzger, Knabe, Buse, & Giessen, 2016), delivery of drugs and other conjugates (Ghosh, Han, De, Kim, & Rotello, 2008).These particles have the tendency of integration to different kinds of molecules such as ligands, therapeutic agents, imaging labels and other molecules to enhance drug delivery and cellular uptake. Using these functionalities, AuNPs can convert a poorly active drug into an active drug. Thus, they

highly contribute in cancer diagnosis, cancer and HIV therapy (Bowman et al., 2008). It was reported that doxorubicin, an anticancer drug, can be conjugated with AuNPs. After conjugation, it is fairly activated and exhibit increased anti-cancerous activity (Aryal, Grailer, Pilla, Steeber, & Gong, 2009).

With increasing development in nanotechnology, several strategies are devised for synthesizing NPs. Two major approaches that are followed include Top-down and bottom-up approaches. Following Top-down approach, particles are formed by reducing the size, by pyrolysis or attrition, from bulk material into a nanoscale form (Thakkar, Mhatre, & Parikh, 2010). These techniques are not useful as they cannot maintain uniform size distribution and disturbs NPs surface quality that is used for catalytic activity. These techniques require high technology instrumentation facilities and thus, are very expensive. While following bottom-up approach NPs are synthesized from nanoscale level. Various chemical and physical methods are used for synthesizing NPs. Brust-Schiffrin and Turkevish methods are commonly used but these are very costly, time consuming, use of toxic substrates and toxic waste production limit the applications of NPs (Y.-M. Park et al., 2011; Salam et al., 2012). Physical techniques are not preferred due to the consumption of large amount of energy (Choi, Chiu, & Luo, 2010; X. Liu, Wu, Wunsch, Barsotti Jr, & Stellacci, 2006). Therefore, green methods are indispensable for NP synthesis, as compared to physico-chemical methods, that provide safer, economic and provide ease of scaling up (Rajathi, Parthiban, Kumar, & Anantharaman, 2012). Using biological systems i.e. bacteria, plants, fungi, actinomycetes and algae for synthesizing nanostructures of bio-compatible metals is known as biomimetic.

Plant biomass provides an excellent alternative for biological NPs production as compared to chemical and physical method. Plant metabolites are employed as extracts of seeds, fruits, leaves etc. These extracts contain certain metabolites such as phytochemicals that contributes towards reduction and stability of NPs. Much research has been conducted by using plants because of their abundance (Prathna, Chandrasekaran, Raichur, & Mukherjee, 2011). Several studies reported that NPs synthesized from plants are unstable and agglomerate due to the increased concentration of phytochemicals in the aqueous extracts (Song & Kim, 2009).

Microorganisms are used as 'Nano-factories' for developing eco-friendly, safe and cost effective methods of NPs synthesis (X. Li, Xu, Chen, & Chen, 2011). Microbes accepts electrons from their environment and use their enzymes, produced by cellular metabolism, to synthesize metallic NPs and convert metals ions into elemental metal atoms. NPs can be synthesized either outside or inside bacterial cell according to the location of formation. NPs synthesized extracellularly involves the entrapment of ions on bacterial surface followed by their reduction using enzymes. Whereas following intracellular synthesis, ions are transported inside the cell where they are reduced. *Escherichia coli* can precipitate Au (III) from $AuCl_4^-$ solution and transform them into AuNPs (Du, Jiang, Liu, & Wang, 2007; Srivastava, Yamada, Ogino, & Kondo, 2013). Au (III) stress renders the Au ions toxic due to reactive oxygen species (ROS) production (Rea, Zammit, & Reith, 2016; M. Tiwari et al., 2016). Various bacterial strains of *Pseudomonas*, *Bacillus* and *lactobacilli* are used for the production of metallic NPs. Recently, *Bacillus methylotrophicus* and *Bhargavaea indica* have been used for the production of silver NPs and AuNPs (Singh, Kim, Wang, Mathiyalagan, & Yang, 2016; C. Wang et al., 2016). In this study, a probiotic *lactobacilli* is used for the preparation of non-toxic AuNPs.

Lactobacillus belongs to family *Lactobacillaceae*. This family is comprised of non-spore forming, non-motile and gram-positive bacteria (Liang et al., 2011). Various species of this genus are used for the production of metallic NPs either intracellularly or extracellularly (Markus et al., 2016). Various species of this genus are involved in the synthesis of AuNPs however studies on their biological applications and characterization have not been reported (Thiruneelakandan et al., 2013) . *Lactobacillus rhamnosus* is a facultative anaerobe appearing in form of chains. Some strains of *L. rhamnosus* are used as probiotics and are helpful in the treatment of diseases. This study is designed to use *L. rhamnosus* for the preparation of AuNPs.

Biomass of bacteria *L. rhamnosus* obtained after incubation in media is used to react with Au solution for biosynthesis of AuNPs. Further optimization methods yield the optimum physical parameters for maximum yield of AuNPs. Newly synthesized particles are subjected to standard characterization techniques to evaluate different properties. These characterization techniques include Scanning electron microscopy (SEM),

Ultraviolet/visible (UV/Vis) spectrophotometry, X-Ray Diffraction (XRD), Fourier transformed infrared (FTIR) spectroscopy and Energy dispersive spectroscopy (EDS).

After complete characterization, antioxidant activity of newly biosynthesized AuNPs is evaluated by DPPH assay. Furthermore, anti-bacterial activity of these AuNPs was evaluated by disc diffusion assay against different bacterial species including two strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas nitroreducens*, *Bacillus subtilis* and *Salmonella enterica*.

1.1. Hypothesis of the Study

Biosynthesis of AuNPs uses reductase enzymes for the reducing gold solution. Presence of these enzymes in biomass is evaluated if bacteria is able to synthesize AuNPs. These biogenic AuNPs have been reported to be stable in nature. Metallic gold is a renowned antimicrobial agent and AuNPs are also known to exhibit inhibitory effects on some bacterial species. So, these AuNPs should have some bactericidal activity associated with them.

1.2. Objectives of the study

Specific objectives of this research include:

- Biosynthesis of AuNPs from *Lactobacillus rhamnosus*
- Optimization of Physico-chemical parameters for biosynthesis reaction.
- Characterization of newly synthesized AuNPs by SEM, UV/Vis, FTIR, EDS and XRD
- Optimization of antioxidant ability of AuNPs by 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) assay.
- Determination of bactericidal potential of AuNPs against 6 different strains of bacteria.

Chapter 2

Literature Review

2.1. Nanotechnology and Nanoparticles

Nanotechnology is an experimental and theoretical field for applied sciences and technology. It encircles the production of functional systems on an atomic and molecular level. It also exploits the structural contents of matter sized 1-100nm, also known as NPs. Importance of nanotechnology dates back to 2000, when United States laid the foundation of an institution called, National Nanotechnology Initiative (NNI), responsible for progress and research in Nanotechnology (Petcu, Zaharie, Gorgan, Pop, & Tudor, 2007). The word 'Nano' takes its origin for from Greek word 'Nanos' which means extremely small (Russell, 2013). It was mentioned by Richard P. Feynman, a Nobel Laureate, in his prominent lecture known as, 'There's Plenty of Room at the Bottom' (Feynman, 2012). Nanotechnology is often termed as general technology because it impacts almost every industry. It is an evolutionary development in the fields of Science and Engineering and is progressing rapidly (Ramsden, 1990). Modern science, using the unique properties of nature at nano scale, presents a new medium for integration, innovation and discovery of technology (Roco, 2007). It is driven to produce material with unique properties that have application in all domains of life including biological, chemical and physical sciences.

A large number of nanotechnology products are available but still research is being conducted in various research laboratories to develop nano branches that could affect the global market for agriculture, minerals and non-fuel commodities. It provides several probable solutions to problems using nano techniques. Products of everyday use contain nanoscale materials. Nanoscale zinc oxide and titanium oxide are present in sunscreens that reflects ultraviolet (UV) to avoid sunburns. Nanomaterials have been used to manufacture batteries to provide more power, efficacy and dissipate less heat. Nanotechnology also finds its application in the manufacturing of sports goods, vehicles and cosmetics.

The unique properties of NPs are governed by their structural features such as size, composition and shape. They are usually composed of 3 layers: a) a surface layer equipped with different molecules, surfactants and metal ions, b) a shell layer that is different from core of nanoparticle in its chemical composition and c) central portion of NPs is known as core and refers to nanoparticle itself (Shin, Cho, Kannan, Lee, & Kim, 2016).

2.2. Properties of Nanoparticles

2.2.1 Surface and size functionality

The unique characteristic of interest in NPs is their larger surface area to volume ratio. NPs are comprised of 10^6 atoms or even lesser. Most of these atoms are exterior atoms that form fewer bonds with lattice and provide multiple active sites (Wilcoxon & Abrams, 2006). Their interaction with environment can alter the reactivity and solubility of NPs. The surface of NPs is provided in square meter per gram (m^2/g). A good catalyst should have 100-400 m^2/g (Catherine & Olivier, 2017) whereas some NPs provide as high as 6000 m^2/g surface area (Widegren & Finke, 2003). Its surface functionality allow their interactions with lipid bilayer, that can be specific or non-specific (Goodman, McCusker, Yilmaz, & Rotello, 2004). Electrostatic interactions are employed for the translocation of cationic NPs to the anionic surface of cell exterior. Research has shown that, while targeting lipophilic domains hydrophobicity can be an important factor. (Verma & Stellacci, 2010). In one of the previous study, it was showed that the structure and composition of capping ligand molecule is an important factor as it helps in the internalization of the particles. Serum proteins have been reported to help in the movement of NPs (Kam, Liu, & Dai, 2006). Their success also depend on targeting specific cells by internalization (Sperling, Gil, Zhang, Zanella, & Parak, 2008). It was recently reported that size and shape of NPs play an important role in their cellular movements. In a research, it was discovered that 50nm particles provide best internalization results as compared to particles of other sizes. Use of neutrally charged AuNPs enhanced their drug delivering efficiency (Agasti et al., 2009; C. K. Kim et al., 2009).

2.2.2 Geometrical Structure

NPs with same composition can provide different shapes such as spherical, cubic, rod and triangular shaped NPs. Their different shapes provide different banding patterns. Spherical shaped NPs exhibit single peak whereas ally shaped shows multiple peaks on their absorbance profile (Mie, 1908). Therefore, spherical NPs are mostly used because they have an increase uptake than rod shaped particles (Chithrani, Ghazani, & Chan, 2006). Differently shaped NPs have different charge polarization edges and corner atoms and provide different reactivities to the particles. These are used in development of different conductors and optical technologies (Kelly, Coronado, Zhao, & Schatz, 2003; Panda & Deepa, 2011).

2.2.3. Plasmon Resonance:

The term plasmon is defined as the oscillations exhibited by excited free electrons in metal (Maier, 2007). These negatively charged electrons upon irradiation are displaced from equilibrium position and cloud coherently around positively charged lattice of metal ions (Kreibig & Vollmer, 1995; Luk'yanchuk et al., 2010). Irradiation of a spherical NP by light, generates an electric field around it, thus giving it a net dipole. This phenomenon can make NPs amenable to small chemical changes that can be observed using a detector and be used in biosensing industry.

2.3. Classification of Nanoparticles

NPs are categorized into various classes due to their chemical composition, properties, size and morphology. Some of these classes include:

2.3.1 Organic Nanoparticle

Organic nanoparticles or polymers consists of dendrimers, liposomes and ferritin. These are non-toxic and biodegradable, some of these NPs contain a hollow core and are termed as Nanocapsules. These nanocapsules are sensitive to electromagnetic and thermal radiations (D. K. Tiwari, Behari, & Sen, 2008). These NPs have vast applications in biomedical field such as drug delivery carriers, as they provide drug carrying capacity, delivery systems and stability of either entrapped or adsorbed drugs. They are efficient and can be used for targeted drug delivery.

2.3.2. Carbon Based Nanoparticles

NPs composed solely of carbon are known as carbon based NPs. These NPs include carbon nanofibers, fullerenes, carbon nanotubes (CNTs), carbon black and graphene. Fullerenes resemble hollow cage, composed of 28 to 1500 carbon atoms arranged in pentagonal and hexagonal carbon units, forming a spherical shape. Fullerenes are used commercially because of their increased strength, structure, electron affinity, versatility and electrical conductivity (Astefanei, Núñez, & Galceran, 2015). CNTs are tubular, elongated structures, about 1-2nm in diameter (Sharma, Kumar Mehra, Jain, & Jain, 2016). In morphology, they resemble self-rolling graphite sheets. These are synthesized by depositing carbon atoms on metallic NPs. Carbon atoms used are vaporized either by laser or electric arc. CNTs as nanocomposites have several commercial applications such as gas adsorption for environmental remediation (Ngoy, Wagner, Riboldi, & Bolland, 2014), fillers (I. Khan, Saeed, & Khan, 2017) and as support for organic and inorganic catalyst (Mabena, Ray, Mhlanga, & Coville, 2011).

2.3.3. Inorganic Nanoparticles

Inorganic NPs constitutes NPs that are not composed of carbon. These mostly include metal oxide based and metallic NPs.

2.3.3.1 Metal oxide Nanoparticles

Metal oxide based NPs alter the properties of metal based NPs. For example, in the availability of oxygen, iron nanoparticles (FeNPs) instantly oxidize to Fe_2O_3 that enhances its reactivity as compared to FeNPs. These are commonly synthesized due to their enhanced efficiency and reactivity (Tai, Tai, Chang, & Liu, 2007). Commonly used metal oxide based NPs include Cerium oxide (CeO_2), Aluminium oxide (Al_2O_3), Magnetite (Fe_3O_4), Iron oxide (Fe_2O_3) and silicon dioxide (SiO_2). These NPs possess unique properties as compared to their metallic nanoparticles.

2.3.3.2 Metallic Nanoparticles

Metal NPs (MNPs) hold special attention because of their optic, electric, magnetic and catalytic properties that are different from their bulk materials (Atkins & Overton, 2010; Yan Zhang, Cui, Shi, & Deng, 2011). MNPs first synthesis is dated back to 1400-1300

BCE in Egypt that involved the production of opaque red glass with colloidal copper. In 4th century, Lycurgus cup, that changes its color from deep red to green based on source of incoming light, was developed in Rome. These optical properties were exhibited because of Ag (silver) and Au (gold) NPs embedded in glass (Barber & Freestone, 1990). The alchemist Al Razi described the 'Ruby Red Glass' as the molten glass that attracted Ag and Au thereby increasing its weight 1000 times. The production of NPs was emerged in 19th century for the experimental work of Michael Faraday. In 1908, optical properties of colloidal MNPs were studied by and he explained that the particles were smaller in size as compared to wavelength of light. In 1959, a physicist Richard Feynman predicted the criticality of scaling down to nano-level for technological advances (Richard, 1959). Norio Taniguchi first introduced the term 'Nanotechnology' in 1974 (Taniguchi, ARAKAWA, & KOBAYASHI, 1974). NPs are commercially used in various industries including catalysis, telecommunication, optics, food, green energy, space technology, medicine and many other fields.

2.4. Gold Nanoparticles

Among metals, Au is one of the first to have been discovered. Gold nanoparticle (AuNPs) are of particular interest due to their resistance to oxidation, stability and biocompatibility (Corti & Holliday, 2004; Corti, Holliday, & Thompson, 2002). Their easy functionalization provide platform for preparation of nano-biological assemblies with antibodies (Capomaccio et al., 2015), proteins (Bhattacharya & Mukherjee, 2008) and oligonucleotides (T. Zhang et al., 2011). History of AuNPs dates back to 4th century where they received huge importance because of their bright red color (Colomban, 2009). They can be found in various ornaments such as the red color of Lycurgus cup (Freestone, Meeks, Sax, & Higgitt, 2007; Wagner et al., 2000), lustre plates in 15th -16th century (Colomban, 2009) and the purple of Cassius colored with red ruby glasses in middle Ages (Hunt, 1976; Ruivo et al., 2008). Initially, data on the presence of colloidal Au can be dated back to the treaties of Arabians and Chinese as early as 5-6 century BC. At that time, it was used as medicinal solution among other uses. During Middle Age in Egypt, colloidal Au was used and studied in laboratories related to Alchemistry. Paracelsus obtained colloidal Au by reducing gold chloride using vegetable extracts of oils and alcohol. In this study, he

used Au to cure mental diseases such as syphilis. Au also found its application is treatment of other diseases such as leprosy, epilepsy and diarrhea.

Doctor of medicine Francisco Antonii published first book on colloidal au in 1618, describing the phenomenon to obtain it and its medical uses (Dykman & Khlebtsov, 2017). In 1857, Faraday reported the production AuNPs by reducing tetrachloroaurate in carbon disulfide environment by phosphorus (Turkevich, 1951). This report increased attention for synthesis of AuNPs of controlled shapes and sizes. Despite its early applications, the use of AuNPs in biological studies was started in late 20^{th} century. In 1951, Turkevich method of AuNPs preparation using citrate (stabilizing agent) was presented. In this method, tetrachloroaurate and trisodium citrate dehydrate are used for the preparation of 15nm particles (Blatchford, Campbell, & Creighton, 1982). Further improvement was done by Frens to make a broad range of AuNPs from 15-150nm (S. Park, Sinha, & Hamad-Schifferli, 2010). Brust and Schriffin, in 1994, achieved a breakthrough in creating alkanethiole stabilized organic soluble AuNPs. This biphasic reduction protocol employed a reducing agent i.e. sodium borohydride and tetraoctylammoniumbromide as phase transfer reagent (Hostetler et al., 1998; Lévy et al., 2004). British researchers Faulk and Taylor presented the method for conjugation of colloidal Au to antibodies for the visualization of surface markers on salmonellae under electron microscope (Roth, 1996). Many researches have been conducted to understand the functionalization of nanoparticle-conjugates in fields of microbiology, cytology, immunology and morphology.

In modern science, the range of AuNPs application is extremely wide. Although in bulk, Au act as a poor catalyst but AuNPs exhibit strong catalytic activity such as alcohol oxidation (Ide & Davis, 2013), 4-nitrophenol hydrogenation (W. Liu, Yang, & Xie, 2007), low temperature oxidation of carbon monoxide (Valden, Lai, & Goodman, 1998) and in selective epoxidation reactions (Lee et al., 2009; Sinha, Seelan, Tsubota, & Haruta, 2004). Due to the Lewis acid nature of gold, it act as an excellent catalyst for alkyne activation reactions (Takale, Bao, & Yamamoto, 2014). It is used in genomics, immunoanalysis, photothermolysis and detection of microorganisms and cancer cells; used as drug delivery vehicle, monitoring and bio imaging of targeted cells and tissues.

AuNPs are synthesized through different physico-chemical and biological methods. Physical method of AuNPs synthesis include microwave irradiation, ultraviolet radiation, laser ablation, sonochemical method, γ irradiation and photochemical process. γ radiation produces NPs of high purity with a size range of 5-40nm. Microwave radiation uses photochemical reduction or heating for synthesis (Y. Wang et al., 2013). In thermolytic and UV irradiation methods, high temperature and UV radiations act as reducing agents. NPs of various sizes can be achieved by optimizing certain parameters (Singh, Kim, et al., 2016; L. Wang et al., 2010). Photochemical methods are used to produce nanorods, that possess complex self-assembly behavior and physical properties (L. Wang et al., 2010).

Following chemical approach, reactions are conducted in aqueous medium and a reducing environment. Sodium borohydride and citrate are commonly used as reducing agents. Turkevish method is a conventional method for chemical synthesis of AuNPs that produce particles of controlled size, more stable and easy to synthesize (B. Wang et al., 2014). In 1951, Turkevish modified Hauser and Lynn' preparation. In this procedure, sodium citrate was used as a reducing agent. It produced spherical AuNPs with narrow size range. In 1994, Brust-Schiffrin method used interactions among thio-gold to protect particles with thiol ligands (Yeo et al., 2017). In this method, sodium borohydride reduced $AuCl_4^-$ in the presence of alkanethiols, it produced NPs of size ranging from 1-3nm (Wadhwani, Shedbalkar, Singh, Karve, & Chopade, 2014). Biological methods use living systems such as fungi, bacteria, actinomycetes, plants and algae for synthesizing non-toxic and stable AuNPs. Main objective of biosynthesis is to avoid the use of expensive, toxic solvents (Venkatesan et al., 2013).

2.5. Synthesis methods for Nanoparticles

2.5.1. Synthesis by Microbes:

Microorganisms are widely used for the synthesis of NPs due to many factors that include easy handling, safety maintenance, growth on low cost medium and presence of enzymes to reduce metal ions into NPs (K. S. Kumar et al., 2014). Synthesis of particles within microbe can be intracellular or extracellular. Intracellular synthesis requires the transport of positively charged metal ions into the cell wall that is negatively charged. These toxic

metallic ions are converted to non-toxic NPs by the action of enzymes, produced as a result of metabolic processes, within the cell wall. Extracellular mechanism involve enzyme such nitrate reductase, produced by many prokaryotes, for the conversion of metal ions into NPs. For detoxification, microbes use metal binding or vacuole compartmentalization. Under metal-stress situations, microbes perform mechanisms to decrease heavy metal concentration. These mechanisms include the efflux of toxic metal ions through cell membrane, conversion to non-toxic ions and accumulation of ions within the microbe. Heavy metals such as Au enter cells through carrier mediated transport, endocytosis or ion pumps (K. S. Kumar et al., 2014). Molecules such as glutathione (Xu et al., 2014) and Metallothioneines (MTs) are involved in metal detoxification (Gomathy & Sabarinathan, 2010).

2.5.1.1. Synthesis by Viruses

Viruses are being used for the production of NPs as they provide the advantage of size-constricted reaction cage (Slocik, Naik, Stone, & Wright, 2005) and presents functional groups on their capsid surface (Mukherjee et al., 2002). These NPs are generally termed as Virus-mediated nanoparticle (VNP). They find application in field of medicine and material science. VNPs are very small, robust, monodispersed, stable and can be easily prepared in large scale. VNPs provide ease of alteration either by modifying genetic makeup of virus or by chemical bio conjugation methods. Viral material can be used for reduction of environmental contaminants. Production of reduced FeNPs from M13 virus possess ability to convert soluble uranium (U^{6+}) to insoluble form (U^{4+}).

2.5.1.2. Synthesis by Fungi

Few fungal species have been studied for NPs production. Generally, fungal microbiology is much less investigated because of difficulty in characterization, as fungal structure complicates mechanistic process used for characterization of NPs within fungi. Main advantage of using fungi is that they secrete large amount of extracellular proteins with several functions, have ability of bioaccumulation and provide high metal tolerance. NPs synthesis from fungi provide ease of scaling-up, cost-effective, ease of downstream processing and handling biomass. Different size and shapes of NPs can be achieved by active biomolecules from fungi. These biomolecules absorb Au ions and convert them into

AuNPs intracellularly (Mukherjee et al., 2002). These active molecules can either be proteins like glyceraldehyde-3phosphate dehydrogenase, ATPase, 3-glucan binding protein or reducing sugars. Extensive studies on *Verticillium spp*. proposed that not only reduced sugars of cell are responsible for Au ions reduction but they can be reduced by adsorption on cell wall enzymes through electrostatic interactions with lysine groups (Durán et al., 2011). AuNPs synthesized from Penicillin oxalicum reveals the average size of particle to be 4-6nm in diameter.

2.6. Characterization Techniques

Successful synthesis of NPs also require the characterization of different properties of these particles. These techniques are performed to understand the morphology, reactivity and nature of NPs.

2.6.1. Visual color change and UV/Vis Analysis

Characterization of NPs starts by observing change in color of solution. It works on SPR principle. Change in color occur due to an increase in the size of NPs. This change is measured by UV/Vis spectroscopy (X. Zhang et al., 2016). NPs exhibit these optical properties due to the electron oscillations present on their surface. Suspension of bacterial biomass in water produces milky white solution which is changed to pale yellow color of $HAuCl_4$ after the addition of gold salt. After incubation, visual change occur from yellow to red color indicate the synthesis of NPs. For AuNPs, increase in size changes color from deep red to purple (Seo, Kim, Hyun, Kim, & Park, 2015).

2.6.2. Morphological Characterization

Morphological features of NPs are of prime importance since it greatly influences their properties. Different techniques are used for morphological studies, but most important include microscopic techniques that include SEM, TEM (Transmission Electron Microscopy) and polarized optical microscopy (POM). SEM is based on the principle of electron scanning. It is not only used for morphological studies but also explains the dispersion of nanomaterial in matrix. Similarly TEM technique is also based on the principle of electron transmittance. It provides information about NPs from low to high magnification. AuNPs of different morphologies, prepared from different methods, are

studied through this technique (Khlebtsov & Dykman, 2011). TEM also provide information about the presence of two or more layered nanomaterial that include quadrupolar hollow spherical structure of Co_3O_4 NPs.

2.6.3. Structural Characterization

Structural characterization provide useful details about sample composition, bonding of nanomaterial and characteristics of the bulk metal. These structural properties are analyzed through XRD, EDS, Raman and Zieta sieze analyzer techniques. XRD is a useful technique to study the structure of NPs. It tells about the phase and crystallinity of the subject material. Rough idea of size can also be deduced from XRD results using Debye Scherer equation (I. Khan, Ali, Mansha, & Qurashi, 2017). XRD works well for both single and multi-phase NPs identification (Emery, Saal, Kirklin, Hegde, & Wolverton, 2016). NPs with smaller size appear difficult for correct measurements of structure and parameters. EDS technique provides information about the elemental composition of nanomaterial. It provides elements along with their abundance. Whereas X-ray Photoelectron Spectroscopy (XPS) is considered as a very sensitive technique for determining elemental abundance and the presence of bonding among elements in nanomaterial. XPS can be used to provide in depth information about composition and its variation in nanomaterial. It relies on simple spectroscopic principle and XPS spectrum generally consists of Y-axis representing number of electrons and X-axis representing the bonding energy of electrons. Each element possess a specific binding energy and thus show unique peak on XPS spectrum. Characterization of NPs vibration is studied by FTIR and Raman spectroscopies. These techniques provide ease of functionalization and are developed as compared to other elemental analytical techniques. Fingerprint region is the most important region for NPs as it provide signature information regarding materials. FTIR technique is used to identify unique biomolecules that can be used for capping and impart stabilization to AuNPs (Connor, Mwamuka, Gole, Murphy, & Wyatt, 2005). Size of a nanoparticle can be measured using various techniques such SEM, XRD, TEM, AFM and dynamic light scattering (DLS). All these technique give accurate measurement about the particle size, but the size of extremely small NPs can be determined by Zeta potential.

2.7. Applications in Biotechnology

AuNPs have provided new direction and ideas to make better and more effective diagnostic and therapeutic tools for application in medical biotechnology industry. Some of these applications include:

2.7.1. Bioimaging

AuNPs have been used for identifying biological and chemical entities. Their high electron density enables TEM to detect bio-specific interactions. First 3 volumes of colloidal Au application included TEM using AuNPs (Hayat, 2012). Modern application of electron microscopy include the use of systems of digital recording and image processing and high resolution instruments such as HRTEM (high resolution transmission electron microscopy). Major use of electron microscopy in biological studies include the identification of surface markers on causative agents of infectious diseases. Fluorescence microscopy (Phillips, Miranda, You, Rotello, & Bunz, 2008), scanning probe microscopy (Drygin et al., 2009) and SEM (Naja, Hrapovic, Male, Bouvrette, & Luong, 2008) are also used for identification. Visualization method using AuNPs particularly confocal microscopy has gained popularity in biological and medical research. This process use an optical system for the detection of micro-objects, which permits the scanning of objects and their height analysis. 3d images can also be obtained by superposition of scanograms (G. Wang, Stender, Sun, & Fang, 2010). Using AuNPs and AuNPs-antibody conjugate enable the detection of Au penetrating living cells at a mono particle level and total amount of Au (Klein, Petersen, Taylor, Rath, & Barcikowski, 2010). In resonance scattering dark field microscopy, AuNPs are used for detecting microbes and their metabolites (York et al., 2007), investigation of endocytosis (S.-H. Wang, Lee, Chiou, & Wei, 2010), imaging of tumor cells (Hu et al., 2009) and surface receptor detection (Bickford et al., 2008). Other applications of AuNPs in bio-imaging and detection constitutes a name ‘biophotonic methods’. These methods include tomography (C. Li & Wang, 2009), photoacoustic microscopy (Zharov, Galanzha, Shashkov, Khlebtsov, & Tuchin, 2006), fluorescence correlation microscopy (Chen & Irudayaraj, 2009), X-ray and magneto-resonance tomography (D. Kim, Park, Lee, Jeong, & Jon, 2007) and optical coherence tomography (Zagaynova et al., 2008).

2.7.2. Therapeutics

AuNPs play an active role in therapeutics and perform various diagnostic assays. In 1977, successful administration of colloidal Au was reported in a patient suffering from RA (rheumatoid arthritis). In another successful example application of colloidal Au in rats suffering from collagen-induced arthritis was reported (Tsai et al., 2007). These successful results are attributed to anti-angiogenic activity followed by the binding of AuNPs with VEGF (vascular endothelial growth factor) resulting in decreased inflammation and macrophage infiltration. Similarly, successful results were observed in rats with pristan- and collagen- induced arthritis upon the subcutaneous introduction of AuNPs (Brown, Whitehouse, Tiekink, & Bushell, 2008).

Colloidal Au was used to deliver TNF in mice by the researchers of Maryland University (Paciotti et al., 2004).The intravenously injected AuNPs conjugated TNF accumulated in tumor cells of the mice. Accumulation of NPs was verified by observing color change in tumor cells, and it also relates with the maximum tumor-specific TNF activity. As compared to native TNF, the conjugated molecule showed higher efficacy, lower toxicity and maximum antitumor activity was observed on low dose administration of drug. The anti-angiogenic properties of AuNPs, studied both in vivo and in vitro, revealed that these NPs interact with growth factors of cardiac epithelium and fibroblast and heparin-binding glycoprotein –vascular permeability factors. These factors mediate angiogenesis, therefore AuNPs inhibit these factors. Angiogenesis is one of the root problem of tumor growth, so AuNPs can be used for therapeutic purposes. It was also demonstrated that AuNPs can suppress multiple myeloma cells proliferation (Bhattacharya et al., 2007) and increase the apoptosis of lymphocytic leukemia cells .

2.7.3. Drug Delivery Vehicle

AuNPs can be synthesized with varying sizes ranging from 1nm to 150nm which can be easily modified, therefore, AuNPs possess great characteristic to be used as a drug carrier into different cell types (Duncan, Kim, & Rotello, 2010). The most popular targeted delivery objects include antitumor agents and antibiotics. AuNPs surface provide the stable platform enabling further modifications such as the addition of molecules or substances to form a specific monolayer that enhance their dispersion in organic media and prolong

stability and conjugation of targeted drugs or probes (Love, Estroff, Kriebel, Nuzzo, & Whitesides, 2005). These modified AuNPs gain effectiveness to target cancerous cells by either active or passive mechanistic system (Daniel & Astruc, 2004; Gao, Cui, Levenson, Chung, & Nie, 2004). Active targeting use drug or peptide conjugated AuNPs to locate and target tumor cells. Passive targeting use EPR (enhanced permeability and retention) effect to coat tumors with antitumor drugs, common property of nanoparticle conjugated drug delivery to tumors (Maeda, Wu, Sawa, Matsumura, & Hori, 2000). Lipid bilayer of cells act as a non-permeable barrier to molecules. It has been reported that several peptides facilitate the entry of molecules in the cell, these peptides are commonly known as cell penetrating peptides (CPP). CPP are either amphiphilic or cationic and are extensively used in drug delivery and gene therapy (Kichler, Mason, & Bechinger, 2006; Nativo, Prior, & Brust, 2008; Thorén, Persson, Lincoln, & Nordén, 2005).In a study, Tat protein sequence have been reported to facilitate entry of AuNPs in the cells (de la Fuente & Berry, 2005). These particles capped with CPP Tat enter the cytoplasm through the exterior of cells and acted as a functional drug deliver unit. These nanoconjugates can enter cells via different routes. They can either enter directly or through endocytosis and are released by endosomal release. This study suggests the use of a combination of peptides to allow the release of NPs into cellular matrix from endosomes (Nativo et al., 2008). In cancer cells, AuNPs showed great potential in delivering small interfering RNA (siRNA) and antisense oligonucleotides (Rosi et al., 2006), as they provide protection to the molecule from RNAses and ease of targeting (Mukherjee et al., 2007). Thus they played a significant role in down regulation of genes in cancer cells by posttranscriptional gene silencing and blocking gene function. AuNPs were used to cause cellular apoptosis by the release of radicals that affects cancer cells without affecting surrounding healthy cells (S. H. Cho, 2005).

2.8. Cytotoxicity of AuNPs

NPs acquire characteristics that are different from their larger counterparts so their effects and behavior cannot be evaluated from the properties of bulk material. Bulk Au has been studies extensively for its medicinal properties and AuNPs have been thought to possess similar properties. Literature shows that AuNPs lack the ability to cause acute and adverse

toxicity (Connor et al., 2005) and are thus considered as a promising candidate to be used in biomedical applications as biocompatible entities (Shukla et al., 2005). Recent studies shows that there could be more to AuNPs toxicity and its response corresponds to its size (Chen & Irudayaraj, 2009; Pan et al., 2007). Further studies revealed that smaller size AuNPs correlated to increased penetration potential within certain tissues, efficient cell internalization, widespread tissue distribution and increase in toxic effects (Johnston et al., 2010). In case of surface functionality, alteration of AuNPs surface affects its cytotoxicity, uptake by the cell and interaction with cellular constituents (Chithrani et al., 2006; Pan et al., 2009). Several studies showed that exertion of cytotoxicity by AuNPs occur due to the induction of oxidative stress. In a study, exposure of 1.4nm AuNPs to HeLa cells resulted in increased ROS production causing oxidative stress followed by lipid and protein degradation, disrupted mitochondrial function leading to cell death (Pan et al., 2009). Furthermore, mRNA expression analysis of whole genome verified that AuNPs treatment resulted in up-regulation of inflammation- and stress-related genes and a remarkable down-regulation in cell cycle genes expression. Another research conducted human K562 cell line provided that the application of naked 18nm AuNPs did not provide any detectable toxicity even at a concentration of 250µM. Similar results were obtained after conjugation of AuNPs with biotin and cetyltrimethylammonium bromide (CTAB). Despite the quick internalization of NPs, no detrimental effects were observed (Connor et al., 2005). Citrate AuNPs of similar size were further investigated on HeLa cells with a concentration 2nM for 3 hours showed no detectable results or any change in protein folding or gene expression (J. A. Khan, Pillai, Das, Singh, & Maiti, 2007). AuNPs non-immunogenicity, biocompatibility and high membrane permeability without disrupting cellular functionality was described extensively. In this study, borohydride reduced AuNPs were used and showed no toxicity in macrophage cell line even at a high concentration of 100µM (Pernodet et al., 2006). Researcher described the accumulation of nano-aggregates in lysosomes. Above mentioned studies do not describe the toxicity of AuNPs but it is reported that these are very toxic both in vivo and in vitro. Toxicity of citrate AuNPs was deeply investigated and it was reported that 13nm citrate AuNPs can cross membrane and accumulate in specialized vacuoles where they disrupts actin filaments, reduces adhesion, motility and proliferation of primary cells. There effects were reported to be concentration

dependent for both short and long term incubation (Uboldi et al., 2009). In another study on alveolar type-II cell line, application of polydispersed AuNPs in micromolar concentration reduced cell viability upon incubation. Such toxicity occurred due to the presence of citrate salts on NPs surface.

2.9. Antimicrobial activity of AuNPs

Because of their unique physic-chemical properties, AuNPs can be used as antimicrobials (Allaker, Vargas-Reus, & Ren, 2012). Its bactericidal effects depend on the size and shape of nanoparticle. NPs can act as antimicrobial because they can interact with microbes (Dror-Ehre, Mamane, Belenkova, Markovich, & Adin, 2009; Hernández-Sierra et al., 2008). In bacteria, NPs attach to cell surface and cause structural changes, disrupting cellular functions such as reduction of respiratory chain enzymes, permeability, causing pits and eventually cell death (W.-R. Li et al., 2010; M. Rai, Yadav, & Gade, 2009). Their success as antibacterial agent also provide alternative to the use of antibiotics (Podsiadlo et al., 2008). Their antibacterial mechanism depends on the bacteria, permeability, surface modification and intrinsic properties (Bindhu & Umadevi, 2014). AuNPs with smaller size are known to penetrate bacterial cell and cause disruption. Triangular AuNPs show better results against Gram-negative and Gram-positive bacteria as compared to spherical AuNPs (Smitha & Gopchandran, 2013). Irrespective of size and surface functionality, sharp-cornered triangular AuNPs can penetrate endosomal membranes and enter cytoplasm. NPs attach to bacterial membrane through electrostatic interactions and disrupts cellular integrity (P. K. Tiwari & Soo Lee, 2013). They can inhibit tRNA binding to ribosomal subunit thus, affecting translation, alter membrane potential and diminishes ATP level (Cui et al., 2012). AuNPs generate gaps in cell membrane causing leakage of cellular content and binds with DNA causing transcriptional inhibition (A. Rai, Prabhune, & Perry, 2010). AuNPs aggregates in biofilms of bacteria and cause cell wall damage that can be used to reduce side effects of drugs and treatment durations (Yousef & Danial, 2012). Oxidative stress caused by ROS can also lead to death of bacterial cells (Pissuwan, Niidome, & Cortie, 2011). In a research, antibacterial potential of AuNPs in different solution with different concentrations was proved effective against both gram positive and gram negative bacteria. AuNPs exhibited activity against multi drug resistant (MDR) pathogenic bacteria

Pseudomonas aeruginosa, *E.coli* (Gram-negative) and *Bacillus subtilis* (Gram-positive) AuNPs were used in 100µl, 200µl and 300µl concentrations. Maximum activity observed at 300µl concentration was 17mm, 19mm and 16mm against *P.aeruginosa, E.coli* and *B.subtilis*. *E.coli*. AuNPs synthesized by leaf extracts of *Pergularia daemia* showed highest bactericidal potential than any other AuNPs. Thus, it was proved that AuNPs from this plant were more effective against gram-negative bacteria as compared to gram-positive. AuNPs also possess antifungal activity against several fungi (Aljabali et al., 2018). All fungal species possess a cell wall and a cell membrane. Their wall is composed of β-glucan, β-glucan chitin and mannoprtoeins whereas, cell membrane is composed of phospholipids. AuNPs interacts with all these components before reaching ionic channels and integral proteins. AuNPs show antifungal activity by two methods either fungicidal or fungistatic. Antifungal effect is caused by altering cell wall's integrity, reduction in mechanic resistance leading to cell disruption (Letscher-Bru & Herbrecht, 2003) whereas, fungicidal effects is caused by the inhibiting β-glucan synthase and thus cell wall formation (Romero, Cantón, Pemán, & Gobernado, 2005). Antifungal activity of AuNPs was performed on different strains of Candida including *C.glabrata, C.tropicalis* and *C.albicans*. AuNPs showed excellent results against all three species.

Chapter 3

MATERIALS AND METHODS

The research was conducted in Integrative Laboratory of Atta-Ur Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad. This study is focused on biosynthesis, characterization and application of AuNPs. All the methods applied and the materials used during research are mentioned below:

3.1. Chemicals

3.1.1. de Man, Rogosa and Sharpe Media (MRS)

de Man, Rogosa and Sharpe media (LAB, United Kingdom) was used for the growth of bacterial strains, used in the synthesis of AuNPs. This media was prepared by adding 70 grams of powder broth in 1 liter of distilled water. Media was then sterilized by autoclaving at $121^{O}C$.

Table 3.1: Chemical composition and percentage representation of de Man, Rogosa and Sharpe media

Chemicals	Percentage (w/v)/liter, (v/v)/liter
Beef Extract (Merck, Germany)	1%
Yeast Extract (Merck, Germany)	0.4%
Peptone (Merck, Germany)	1%
Glucose (Merck, Germnay)	2.0%
Sodium Acetate Trihydrate (Sigma Aldrich, Germany)	0.5%

Triammonium Citrate (Sigma Aldrich, Germany)	0.2%
Di potassium Hydrogen Phosphate (Merck, Germany)	0.2%
Magnesium Sulphate (Merck, Germany)	0.02%
Manganese Sulphate Tetrahydrate (Merck, Germany)	0.005%
Tween 80	0.1% v/v

3.1.2. Nutrient Media

Nutrient agar (Oxoid, Germany) and nutrient broth (LAB, United Kingdom) media was used for the growth of pathogenic bacteria and for disc diffusion assay to evaluate anti-bacterial property of AuNPs. This media was prepared by adding the following components in 1 liter distilled water followed by autoclaving at 121^{O}C for sterilization.

Table 3.2: Chemical composition and Percentage representation of nutrient media

Chemicals	**Percentage (m/v)/liter**
Sodium Chloride	0.5%
Agar	1.5%
Peptone	0.5%
Beef extract	0.3%

3.2. Biosynthesis of Gold Nanoparticles (AuNPs)

3.2.1. Isolation of Lactobacillus specie

Molecularly identified species of lactobacilli was used for the preparation of AuNPs. These strains were identified on the basis of their 16S rRNA sequencing. The lactobacilli specie, isolated from animal sources, *Lactobacillus rhamnosus* HFI-K2 was used for AuNPs synthesis.

The strain was stored in 25% glycerol stocks at -20^{O}C.

3.2.2. Sample Preparation

Sample for inoculation was prepared by inoculating 20µl of glycerol stocks containing spores of lactobacilli strain on MRS agar plate. These plates were kept in an incubator at 37^{O}C for 24 hours. To identify strains on the basis of morphology, inoculum was spread on MRS agar plates using inoculating loop. Single colonies on plates were picked to inoculate MRS broth. The stocks were further stored at -20^{O}C.

3.2.3. Inoculation and Incubation

Cultures on MRS agar plates were used to inoculate 100ml of MRS broth, maintained in reagent bottles, using inoculating loops. These broth cultures were kept in a shaking incubator at 37^{O}C for 24 hours. After incubation, turbidity of broth was evaluated and 25ml of broth was separately taken in 50ml falcon tubes. These falcons were then centrifuged at 6300 x g for 5min and pellet was obtained. These were washed thoroughly with distilled water and supernatant was removed. Pellet was then dissolved in 25ml of 1mM chloroauric acid solution. Reaction mixtures were then incubated on shaking incubator for 48-72 hours at 37^{O}C and 120 x g in dark. After incubation, red colored pellet settles down with a clear supernatant. Centrifuge this suspension at 2500 x g for 5min. Supernatant was discarded and alternating cycles of sonication and centrifugation were performed on the reaction mixture, now in eppendorfs. Sonication was performed at 40 amplitude, complete cycle for 20 minutes. Sonication led to centrifugation at 2500 x g for 5min. These processes were performed 3 times with a final centrifugation step. AuNPs were collected in pellet form by

centrifugation at maximum speed for 15min. These pellets were washed with 80% methanol and air dried to obtain AuNPs in powder form.

3.3. Optimization of Physico-Chemical Parameters

AuNPs synthesis involved the optimization of various physico-chemical parameters for better yield. These reactions were performed before subjecting AuNPs to characterization techniques. For optimization, different reaction mixtures were set before analysis through UV/Vis spectrophotometer. Absorbance was measured at wavelength 200-800nm but 540nm is the accurate wavelength for AuNPs. Deionized water was used as blank in spectrophotometer. Spectral analysis of reaction mixtures were performed by software attached to instrument and data was plotted on graphs to observe data of different samples simultaneously.

3.3.1. Effect of Concentration of Salt Solution

To evaluate optimal concentration of salt solution required for AuNPs synthesis, bacterial biomass was challenged with different concentrations of salt solution. For optimization, solutions of 1mM, 1.5mM 2mM, 3mM, 4mM and 5mM were prepared. After setting up reaction, color change was observed and spectral analysis were performed.

3.3.2. Effect of Temperature

Reaction mixtures were set up by challenging bacterial biomass to 1mM salt solution concentration and these reaction mixtures were incubated at 20^0C, 30^0C, 40^0C, 50^0C and 60^0C. Color change was observed in these mixtures and were then subjected to spectral analysis.

3.3.3. Effect of Reaction Time

AuNPs biosynthesis was also evaluated at different reaction times. It provided us the time taken by reactants and substrate to synthesize AuNPs. Reactions were set for 24hr, 48hr, 72hr, 96hr and 120hr. These reactions were then subjected to UV/Vis spectral analysis.

3.4. Characterization of AuNPs

3.4.1. UV/Vis Spectrophotometry

Newly synthesized AuNPs were subjected to characterization by Ultraviolet – Visible spectrophotometry (UV/Vis) because these particles interact with light at a specific wavelength which results in specific optical properties of these NPs. Synthesis of AuNPs was further verified by evaluating the absorbance spectrum of reaction mixture in 300-800nm range. Spectrum was obtained using 2ml test volume with 1cm path length quartz cuvette. Deionized water was used as blank for correction of base line of spectrophotometer. UV/Vis spectral analysis software was used to recording, storing and analyzing spectral data. The numerical data obtained was plotted on graphs for comparison.

3.4.2. Fourier Transform Infrared (FTIR) Spectroscopy

This spectroscopy utilized the Perkin-Elmer Spectrum-100 spectrometer (United States) for determining functional groups present on AuNPs. Air-dried sample was mixed with KBr, due to its hygroscopic properties, to remove any water molecule present in sample. Hydraulic press was used to form pellet of sample and KBr. Pellet was then subjected to infrared waves and was scanned at a range of 4000-350 cm^{-1} with 4 cm^{-1} resolution. The spectrum obtained was plotted against transmittance (%) on Y-axis v/s wave number (cm-1) on X-axis. Peaks obtained from this spectrum was compared with standard functional group charts by manual peak picking method. FTIR spectra of AuNPs and biomass, from which bacteria was synthesized, were also compared.

3.4.3. X-Ray Diffraction (XRD)

XRD analysis were performed on D8 Advance instruement of BRUKER company (Germany). Air-dried sample of AuNPs was used for XRD analysis. Sample was loaded on the glass plate of instrument and spectra was recorded. The instrument was ran at a voltage of 40kV and 40mA current was passed with Cu-Kα radiation (λ= 1.58Å). Samples were scanned at 2θ with a range of 20-80^{O} and time an increment of 0.02^{O}/0.1 sec interval. Average diameter of NPs was measured using Debye-Scherrer equation:

$D = 0.9\lambda/\beta\cos\theta$,

D (nm) is the size, λ (nm) is the wavelength of Cu-K radiation, β (radians) is the full width at half maximum (FWHM), and θ (radians) is the half of the Bragg angle.

3.4.4. Scanning Electron Microscopy (SEM)

SEM analysis are performed to evaluate morphology and size of NPs. SEM was conducted in a VEGA3 instrument of TESCAN company (Czech Republic). Accelerating voltage of microscope range was set at 20kV, 11 beam intensity and working distance of 15mm for the sample.

3.4.4.1. Sample Preparation

10µl of colloidal gold nanoparticle solution was added to 10ml deionized water in glass vials. These glass vials were subjected to ultra-sonication (Ultrasonicator, Cole Parmer) for 1 hour at $37^{O}C$. A drop of these homogenized sample was transferred to $1cm^2$ cut glass slides and dried in oven for 15 minutes. Sputtering of sample, with gold palladium, was done in a Sputter coater Model SC7620, running at 18-20mA, 240V to a thickness of 15nm. Sputtering was performed to make sample conductive and was then used for SEM analysis. Images were taken at different resolution and magnification for characterization of AuNPs.

3.4.5. Energy Dispersive X-Ray Spectroscopy (EDS)

EDS describe the elemental composition and their abundance in sample. These compositional analysis were conducted by the EDS attached with LMU SEM.

3.5. Antibacterial Activity

Antibacterial activity is the major property of AuNPs. To evaluate their bactericidal potential, disc diffusion assay was performed against 6 bacterial isolates.

3.5.1. Collection of Bacterial Isolates

Bacterial isolates of *Pseudomonas aeruginosa, Escherichia coli* and *Bacillus subtilis* were kindly provided by Dr. Fazal Adnan ASAB, NUST. Bacterial isolates of *Pseudomonas nitroreducens, Escherichia coli* and *Salmonella enterica*, isolated from animal sources were identified by 16S rRNA. These strains were grown overnight on nutrient agar plates prior to performing disc diffusion assay.

3.5.2. Preparation of Concentration of AuNPs

Pellet of AuNPs were dispersed in 1ml of deionized water by vortexing at high speed, followed by sonication for 1 hour. Different concentrations of AuNPs 60µg/ml, 80µg/ml were prepared in eppendorff tubes. Theses suspension were used for evaluating antibacterial activity.

3.5.3. Preparation of Cell Free Supernatant (CFS)

The lactobacillus specie was inoculated in 10ml MRS broth for 24 hours at $37^{O}C$. Cultured media was centrifuged at 10000 rpm for 10min. Supernatant was syringe filtered using a 0.2µM syringe filter (Corning, USA) and stored at $4^{O}C$.

3.5.4. Preparation of Media

Nutrient agar media was prepared by dissolving 28g in distilled water and sterilized by autoclaving. Liquid media was poured in autoclaved petri plates and allowed to solidify in laminar flow hood to avoid contamination. Plates were then sealed and kept in an incubator at $37^{O}C$ to ensure the sterility of plates before experimentation.

3.5.5. Inoculation of Bacterial Isolates

All 6 bacterial isolates, streaked on nutrient agar plates, were used to inoculate nutrient broth in 15ml falcon tubes using inoculation loops. All 6 falcons were incubated at $37^{O}C$ for 24 hours.

3.5.6. Disc Diffusion Assay

Disc diffusion method was employed to evaluate bactericidal potential of AuNPs. This experiment was performed in laminar flow hood after application of UV light for 15 minutes to avoid contamination. Nutrient agar petri plates were used for this activity. 5 sections were marked on each petri plate, two sections represented different concentrations of AuNPs such as 60µg/ml and 80µg/ml, next section was of cell free extract (CFE) and last two sections represented positive and negative control. These petri plates were cultured using sterilized cotton swabs and dipping them in nutrient broth containing bacterial isolates growth. A broad spectrum antibiotic cefoxitine disc was used as a positive control. Whereas, for negative control, deionized water was used. Incubate these reaction for 24hrs

at 37^{O}C and evaluate inhibition zone on petri plates after incubation. This activity was performed in triplicates.

3.6. Antioxidant activity of AuNPs

Antioxidant potential of fully characterized AuNPs was evaluated by using 2,2-di-phenyl-2-picryl hydrazyl hydrate (DPPH) assay. DPPH assay is popular in determining antioxidant activity of natural products. Protocol used for performing this assay was modified from previously described DPPH assays. This assay works on the principle that a hydrogen donor is known as an antioxidant. DPPH reagent have the ability to interact with the antioxidant that causes its reduction. DPPH is a calorimetric substance and its reduction can be monitored by observing visual change from purple to light yellow color or by performing spectral analysis using UV/Vis spectrophotometer at 517nm. To perform this assay, fresh 1mM DPPH solution was prepared in methanol and different concentration of AuNPs (50µg/ml, 100µg/ml, 250µg/ml and 500µg/ml) were prepared and added to this solution. These reaction mixtures were incubated at 37^{O}C for 30 minutes in dark, before measuring absorbance at 517nm. % Free radical scavenging potential of AuNPs was deduced using following formula:

$$\% \text{ scavenging radical} = [\text{Absorbance of Control} - \text{Absorbance of Test Sample} / \text{Absorbance of Control}] \times 100$$

Control samples are untreated DPPH solution in methanol whereas, test samples are AuNPs treated with methanolic DPPH solution. This experiment was performed in triplicates and obtained results were plotted on graph to compare antioxidant property of AuNPs at different concentrations.

Chapter 4

RESULTS

4.1. Morphological analysis of *Lactobacillus rhamnosus* HF1-K2

Previously sequenced bacterial strain of *Lactobacillus rhamnosus* HF1-K2 was taken from glycerol stock and inoculated on MRS agar plates to identify it on the basis of morphology. Their morphology was also evaluated by observing the growth pattern of biomass in culture flasks.

L.rhamnosus was selected for the preparation of AuNPs due to excessive production of its enzymes that can convert Au ions into NPs. Figure 4.1 shows the morphology of a typical *L.rhamnosus* strain on MRS plate.

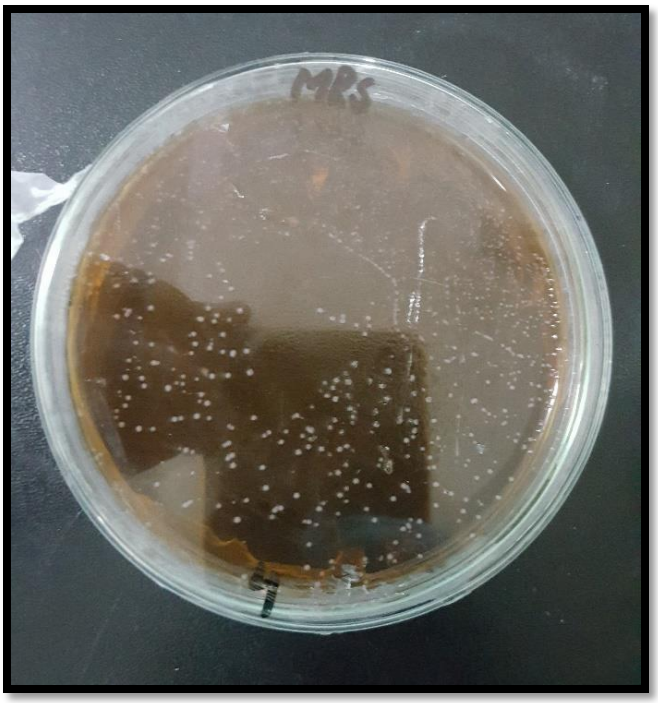


Figure 4.1: Pure culture of molecularly identified *Lactobacillus rhamnosus* on petri dish containing MRS agar

4.2. Observation of color change as initial confirmation of AuNPs synthesis

As the reaction between bacterial biomass and chloroauric acid proceeds, color change of reaction mixture confirms AuNPs synthesis. Color of reaction mixture was compared with the original color of chloroauric acid solution. As the reaction proceeds further, the color of reaction mixture changes from yellowish-white to dark violet. After 48 hours of incubation, solution mixture is dark red in color, whereas after 72 hours supernatant appeared colorless and a violet colored pellet settled down. This color change was attributed to the reduction of Au ions into gold metal due to oxidoreductase enzymes present in bacterial biomass. Observing color change is believed to be the first step in confirmation of synthesis of AuNPs by this bio-reduction reaction.

a) b)

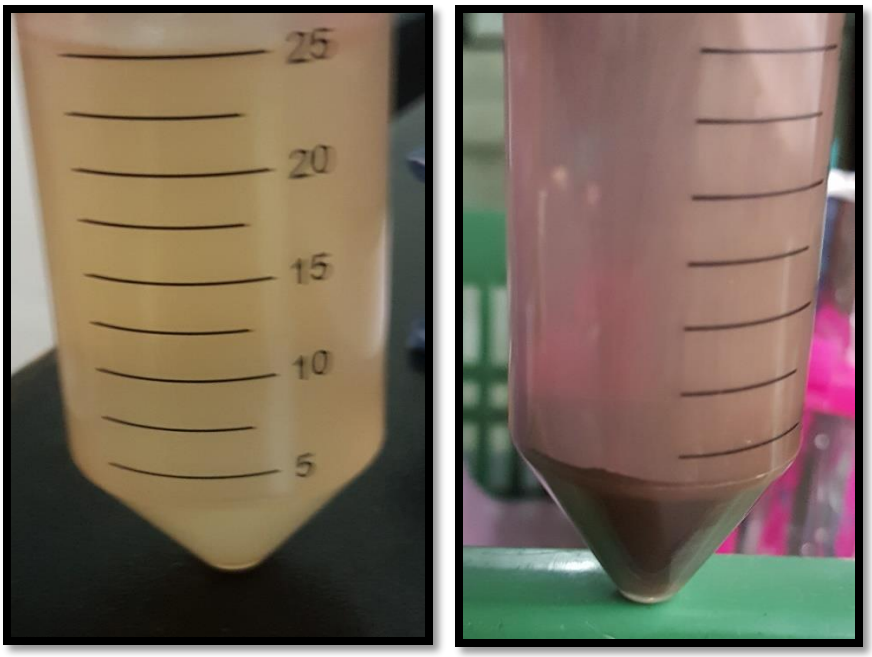


Figure 4.2: Comparison of Color change between a) Gold salt solutions [after 24hrs] b) Reaction Mixture (bacterial biomass and salt solution) [after 48hrs].

4.3. Confirmation by UV/Vis Spectral Analysis

Spectral analysis of reaction mixture was performed through Ultra-Violet/Visible (UV/Vis) spectrophotometer that confirmed synthesis of AuNPs as absorbance peak was observed in the range of 300-800nm, as was reported in literature. Figure 4.3 shows the UV/Vis spectra of bacterial biomass in the absence of gold salt solution, here it is observed that it lacks the peak indicating the synthesis of AuNPs. Whereas Figure 4.4 shows the UV/Vis spectrum of the reaction mixture containing bacterial biomass and salt solution. It can be clearly observed that the absorbance peak obtained around 540 nm are unique to confirm the synthesis of AuNPs in the reaction mixture. Spectral analysis between 200-300 nm are not presented as there were certain proteins that have shown absorbance in this region which tend to make noise. Following figures of spectral analysis shows the synthesis of AuNPs as a function of interaction between enzymes in bacterial biomass and salt solution.

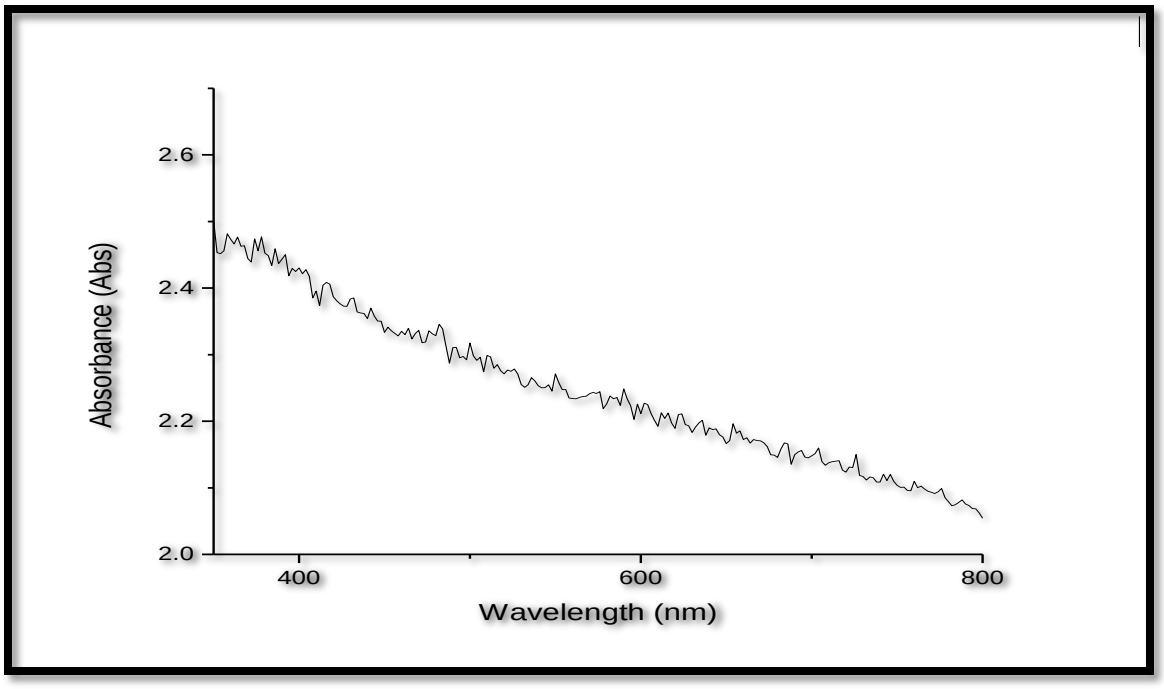


Figure 4.3: UV/Vis spectrum of bacterial biomass indicating absence of peak

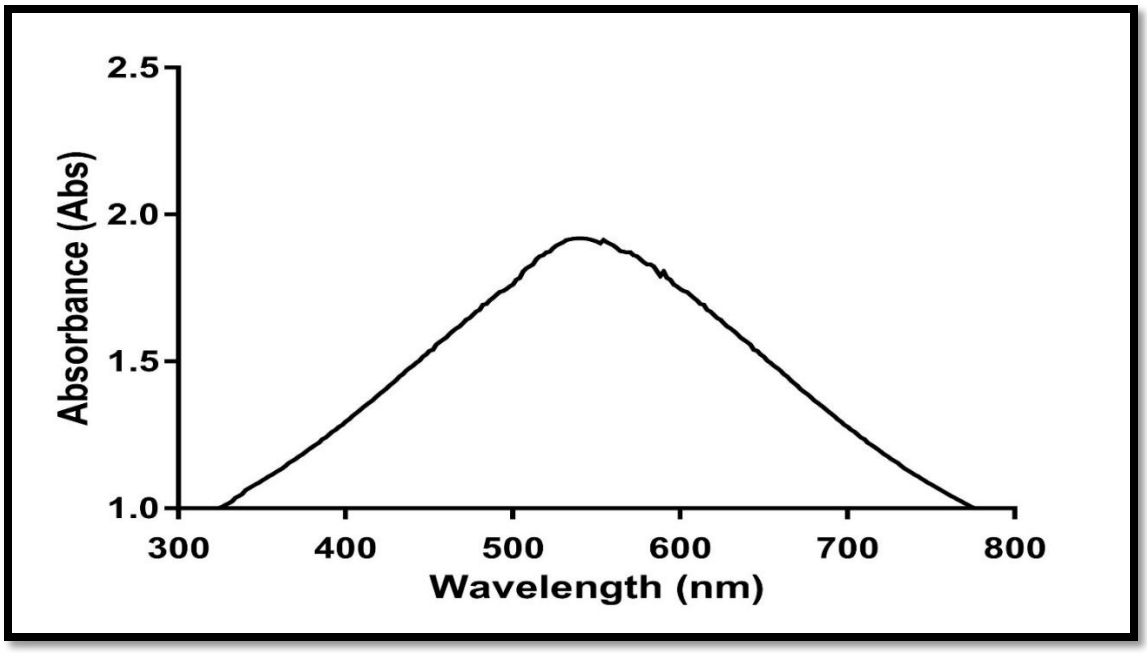


Figure 4.4: UV/Vis spectrum of Reaction Mixture indicating peak at 540nm.

4.4. Optimization of Physio-chemical Parameters for AuNPs Biosynthesis

Different reaction mixtures were set at different conditions to evaluate optimal parameter for AuNPs biosynthesis. All these reaction mixtures were then subjected to UV/Vis spectral analysis and absorbance was obtained.

4.4.1. Effect of Gold Salt Solution

Gold solution act as a substrate for enzyme present in bacterial biomass. Optimal concentration of gold solution is necessary for AuNPs biosynthesis reaction. So, in order to know maximum concentration of substrate required to provide sufficient yield of gold nanoparticles, different reactions were set up. In these reactions, different concentrations of gold solution were subjected to bacterial biomass. It was observed that as the molarity of solution is increased, absorbance of nanoparticles also increased but up to 5mM concentration of salt solution. With further increase in concentration, there was decrease

in the synthesis of AuNPs. It was evaluated that at 10mM concentration, metal ion concentration increases causing toxicity and hinders AuNPs synthesis.

4.4.2. Effect of Temperature

Temperature acts as a crucial factor in the catalytic activity of enzymes. For AuNPs biosynthesis, Au ions are reduced to AuNPs by the activity of enzymes so reaction mixtures were set up at different temperatures and their absorbance values were evaluated. Spectral analysis of these reactions indicated $30^{O}C$ as optimum temperature for AuNPs biosynthesis by *Lactobacillus rhamnosus*. No significant difference was recorded between values at different temperatures but absorbance was very low at higher temperatures as enzymes in biomass cannot work at that temperature

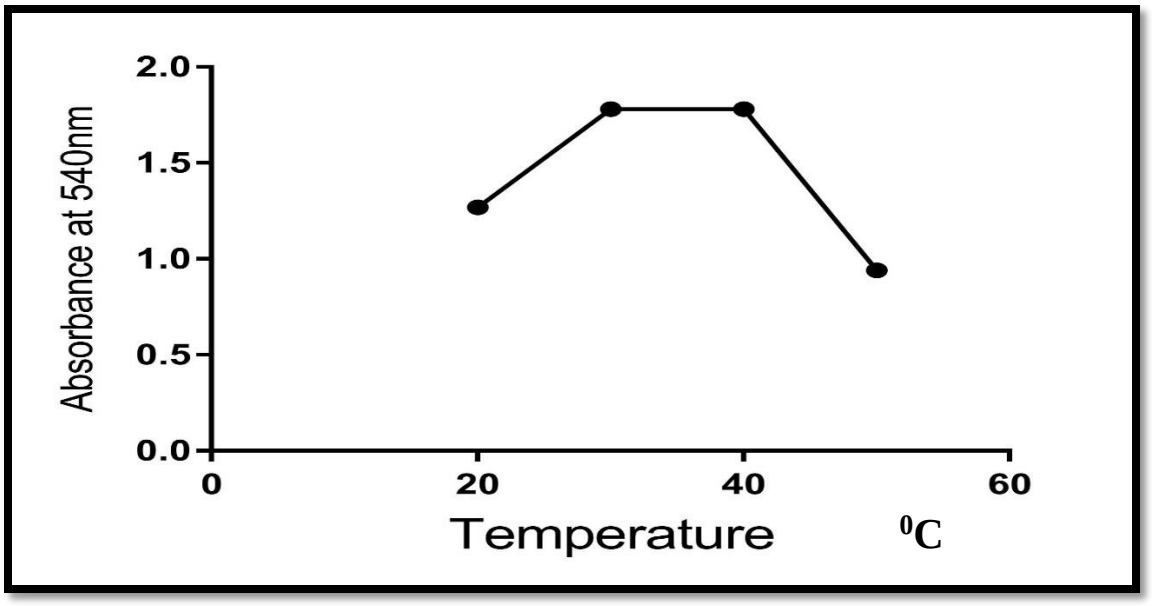


Figure 4.5: Comparison of absorbance value of reaction mixtures at different temperatures.

4.4.3. Effect of Reaction Time

Reaction time or incubation time plays an important role in biosynthesis of AuNPs. Certain amount of time is required by enzymes to reduce metal ions into NPs. It was observed that AuNPs were synthesized in 72 hours. So it was concluded that reactivity of enzymes in

biomass increased with the passage of time and was stabilized after 72 hours. Spectral analysis at 96 hours revealed that AuNPs remained stable in reaction.

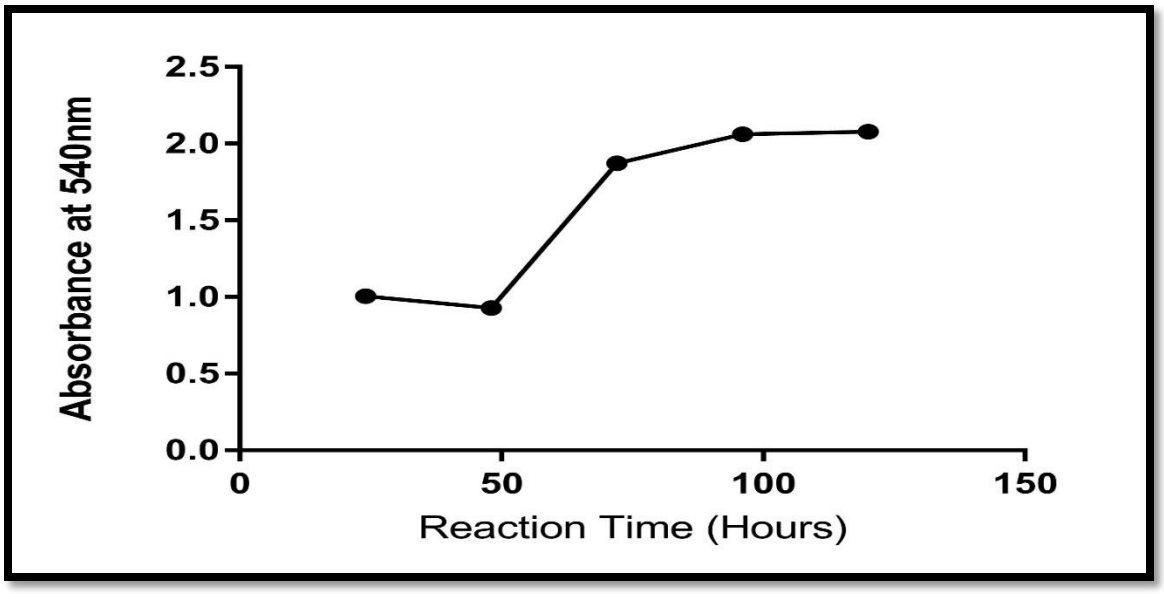


Figure 4.6: Comparison of absorbance value of reaction mixtures set up at different reaction times.

4.5. Characterization of AuNPs

4.5.1. X-ray Diffraction Analysis

XRD analysis was used to verify the phase and crystallinity of AuNPs. The diffracted intensity was recorded from 0^O to 80^O 2 (θ) as shown in Figure 4.7. Characteristic peaks of AuNPs were indexed to (111), (200), (220) and (311) lattice plane of Bragg's reflection. The sharpest peak is observed at 38.29^0 and it corresponds to (111) plane. Three other peaks observed at (200), (220) and (311) planes corresponds to 44.32^O, 64.54^O and 77.59^O respectively. Intensity observed at these lattice planes were much lower than intensity at (111) index plane, it signifies that all nanoparticles are in (111) orientation. XRD results implied that these biogenic AuNPs are face-centered cubic and crystalline.

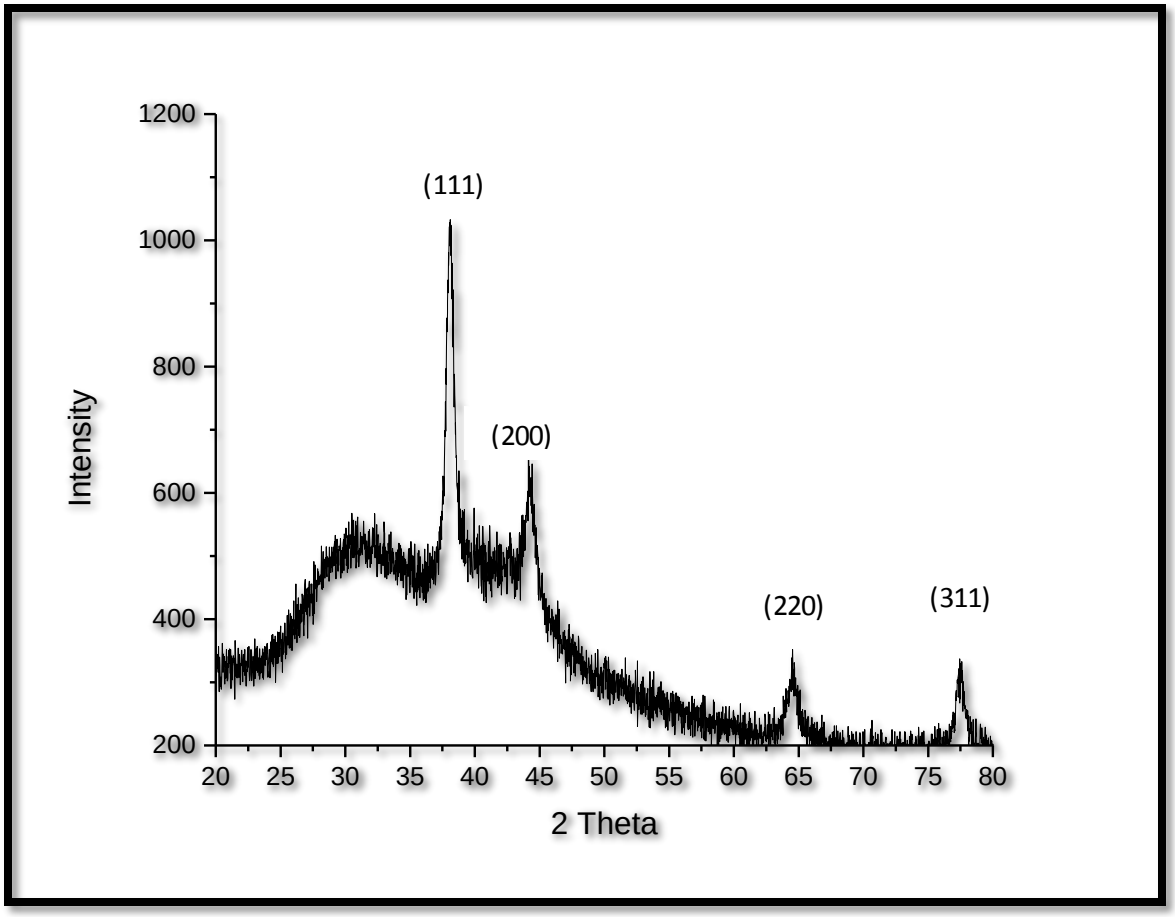


Figure 4.7: X-Ray diffraction pattern of purified AuNPs synthesized from *Lactobacillus rhamnosus*

4.5.2 Fourier Transformed Infrared (FTIR) Analysis

FTIR analysis were conducted to evaluate functional group forming property of the sample. Activity of functional groups, present in chemical and enzymes, is required for biosynthesis of NPs because these functional groups act as stabilizing and capping agents on NPs. Perkin-Elmer Spectrum-100 FTIR spectrophotometer was used for FTIR analysis of sample and following graphs were obtained.

FTIR spectrum of AuNPs was compared with spectrum of biomass of bacteria, Figure 4.8 represents FTIR spectrum of biomass whereas, Figure 4.9 represents FTIR spectrum of reaction mixture. It was evident that FTIR spectrum of AuNPs possess peaks that are not present in spectrum of biomass. Peaks shown at 3331 cm^{-1} is due to aliphatic primary amine

group (N-H) stretching vibration, that shows medium bonding and band appeared is broad. Band at 1638 cm^{-1} represents amide bond (C=O) stretching. Whereas, peak at 1243 cm^{-1} is due to amine (N-H) stretching vibration. Bands for primary alcohol (C-H) appear at 1078 cm^{-1}. C=O represents carbonyl group in amides linkage in proteins. These proteins and amino acid residues (tryptophan, cysteine and tyrosine) have been reported to provide stability to biosynthesized AuNPs. Microbial mediated AuNPs possess binding affinity to amides and thiols, present in proteins. Presence of these functional groups show that free amino groups can form capping layer on AuNPs thus, preventing them from agglomeration.

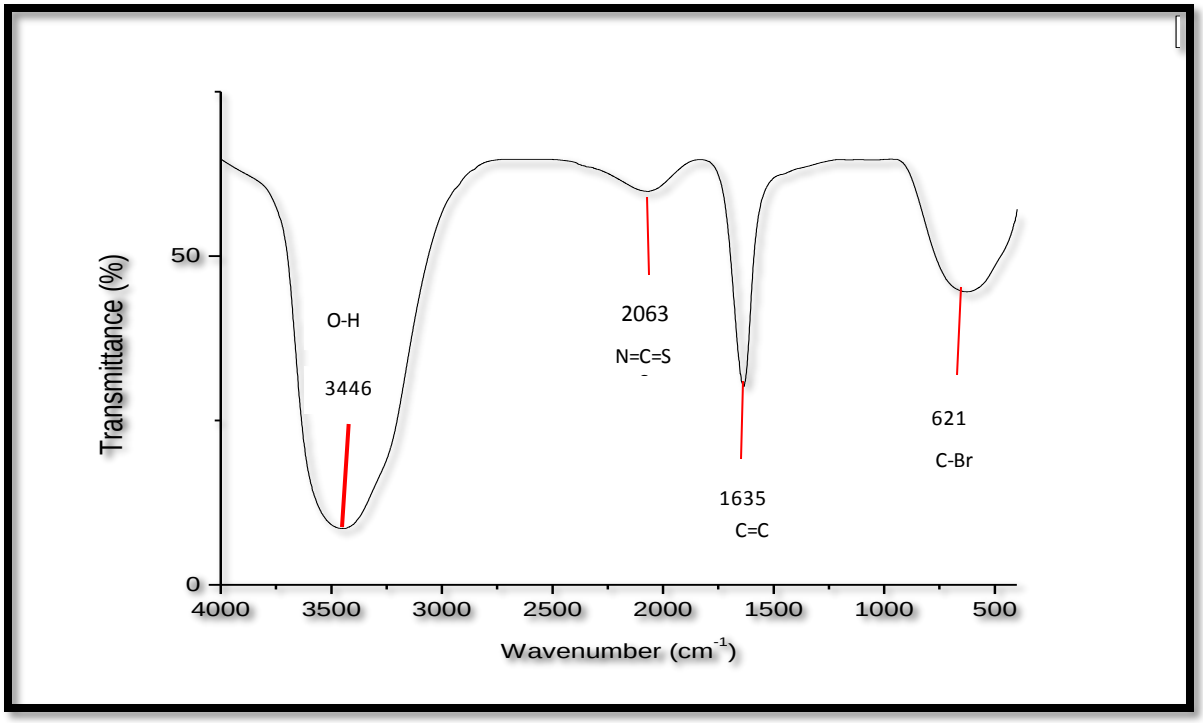


Figure 4.8: Infrared spectrum of bacterial biomass.

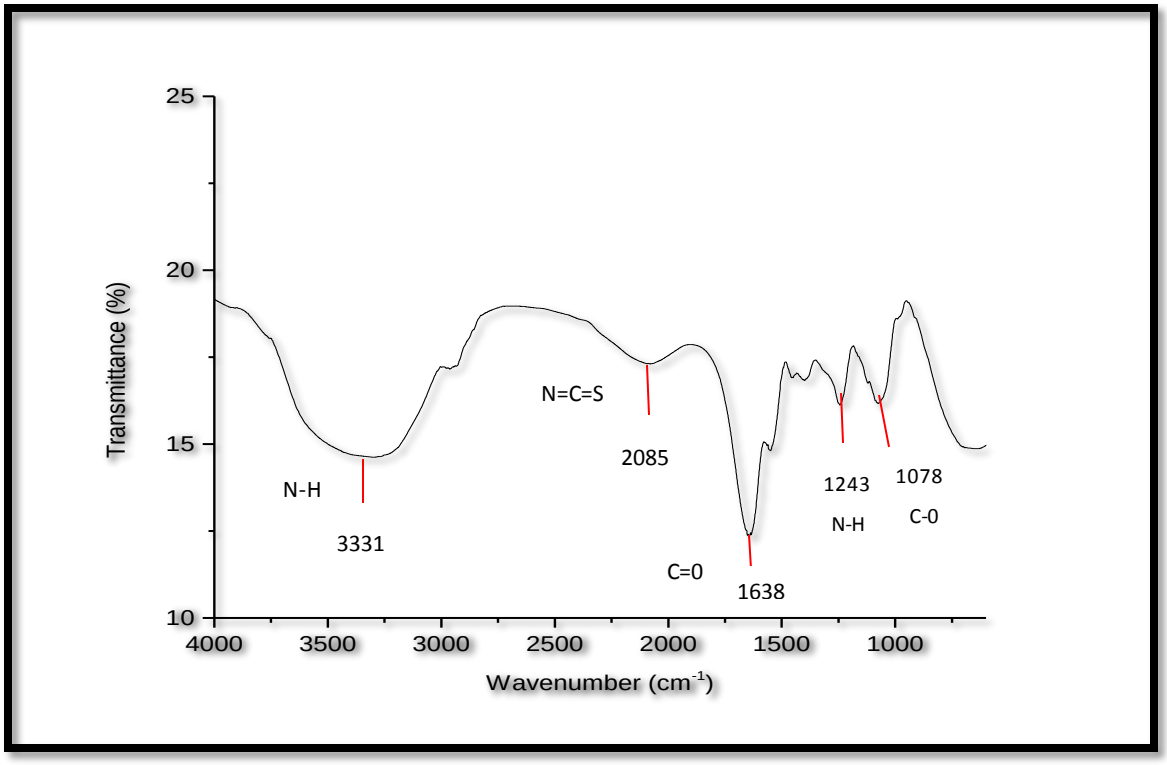


Figure 4.9: Infrared spectrum of reaction mixture containing biogenic AuNPs.

4.5.3. Scanning Electron Microscopy (SEM) Analysis

SEM images of AuNPs were taken at different resolutions to determine average particles size and morphology of these NPs. These images reveal that the newly synthesized AuNPs are spherical and were distributed uniformly in aqueous colloidal solution. SEM images also showed the agglomeration of AuNPs and the biosynthesis of nanorods. These results occurred, due to the delay between sonication process and SEM analysis. Average particles size was 50-70nm according to SEM analysis.

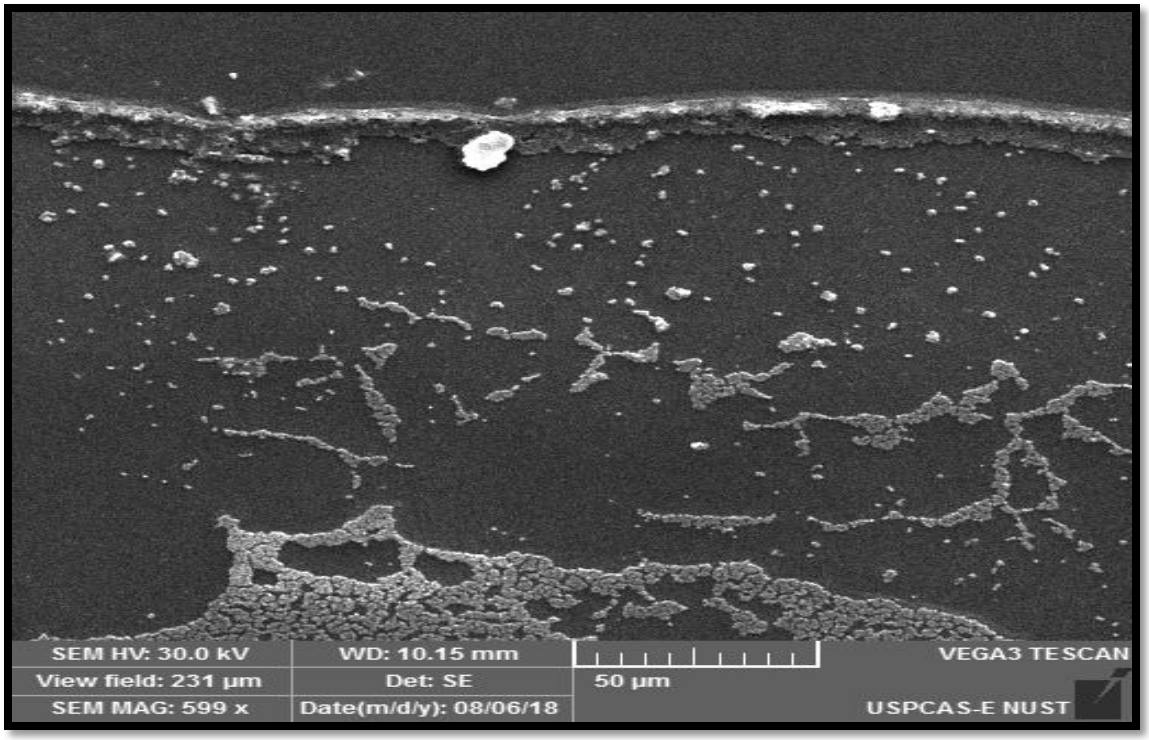


Figure 4.10: SEM image of AuNPs at 50µm resolution.

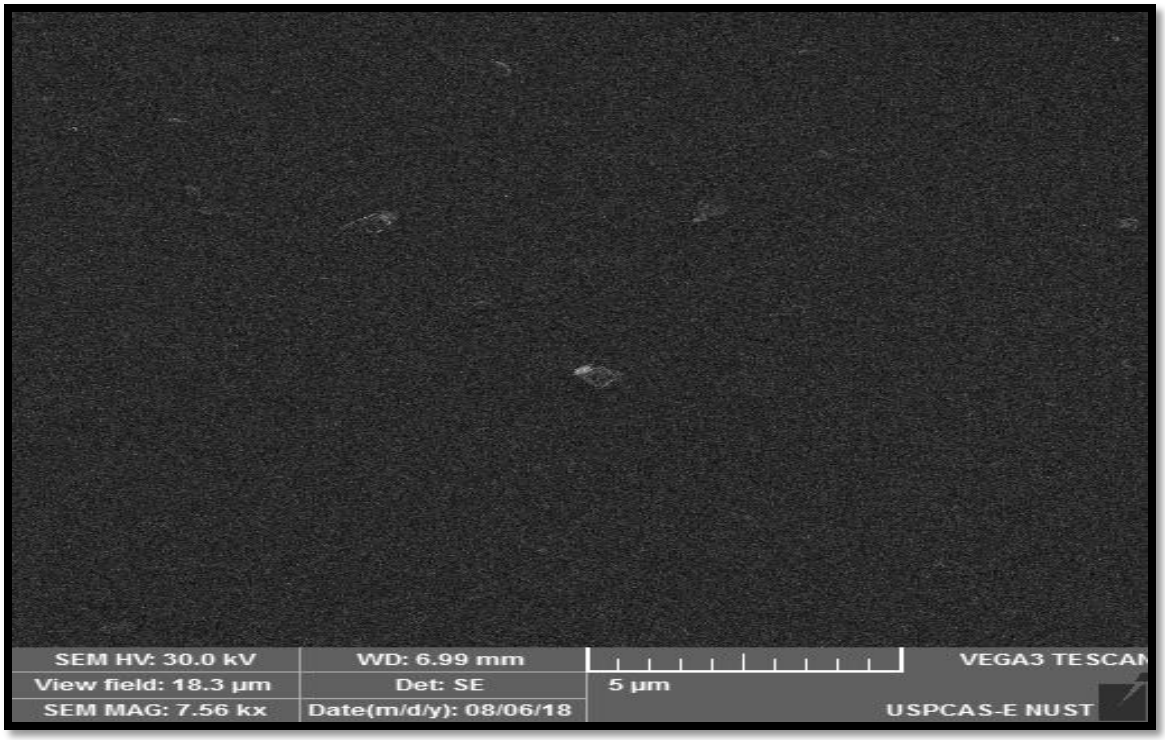


Figure 4.11: SEM image of AuNPs at 5µm resolution.

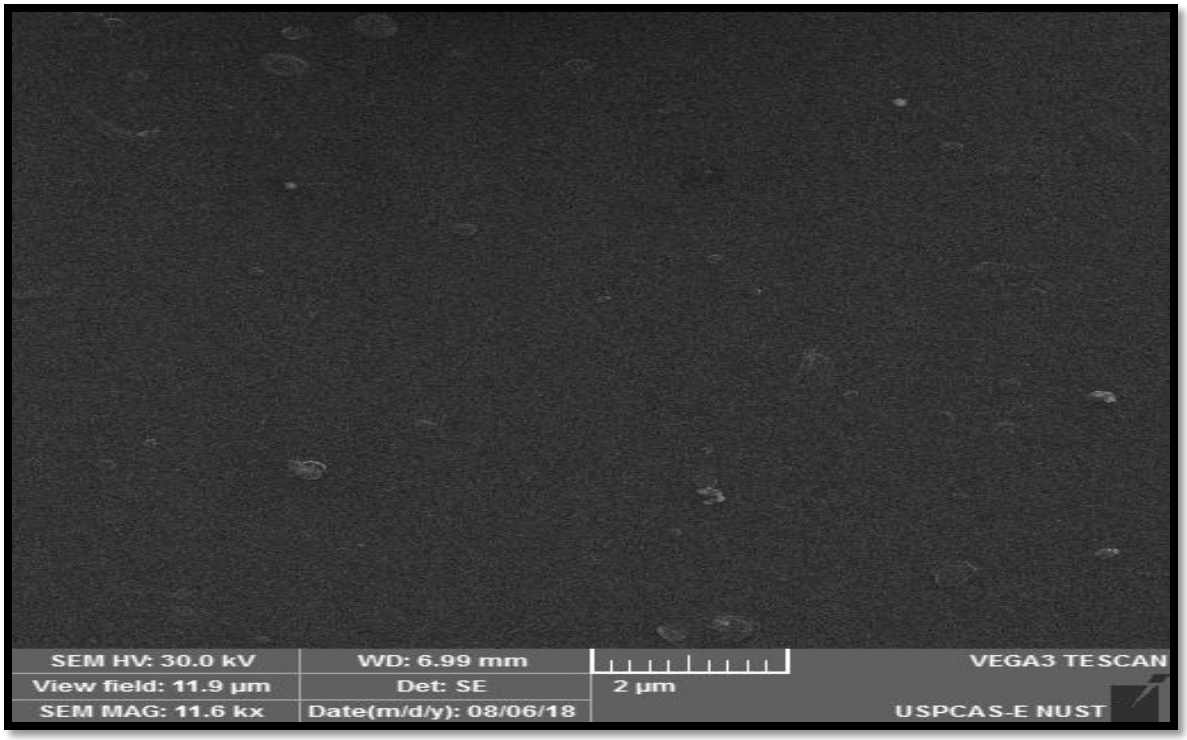


Figure 4.12: SEM image of AuNPs at 2µm resolution.

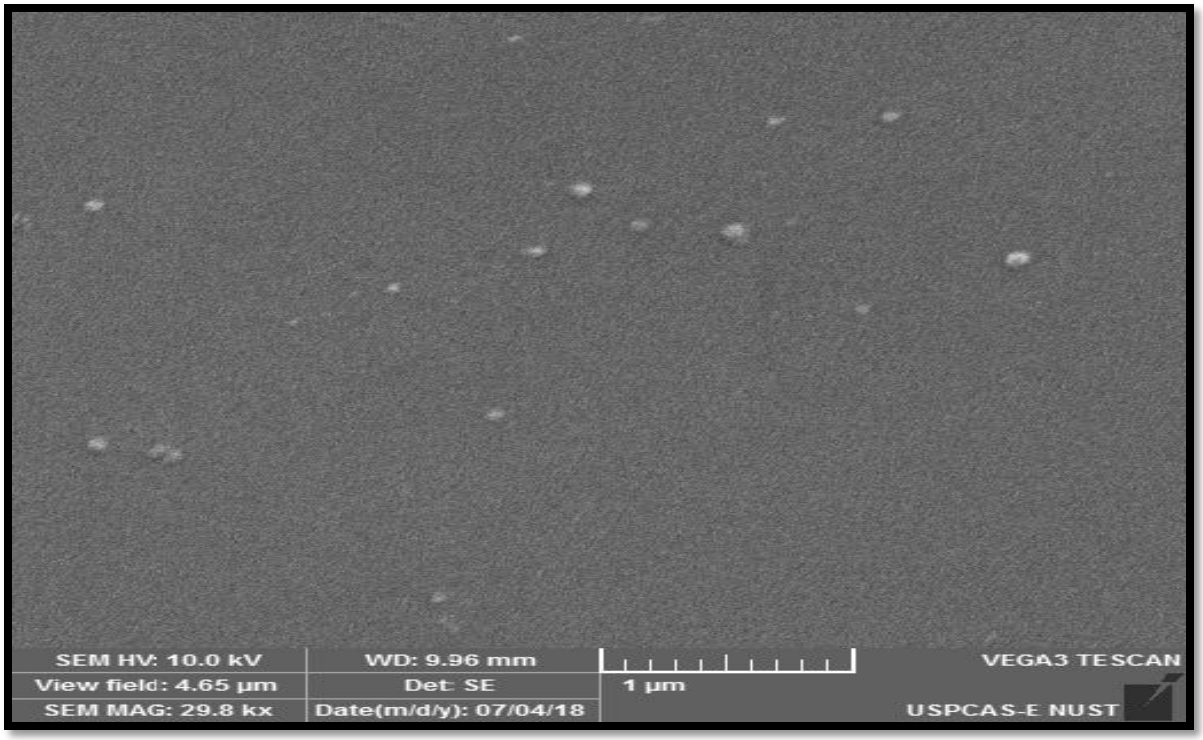


Figure 4.13: SEM image of AuNPs at 1µm resolution.

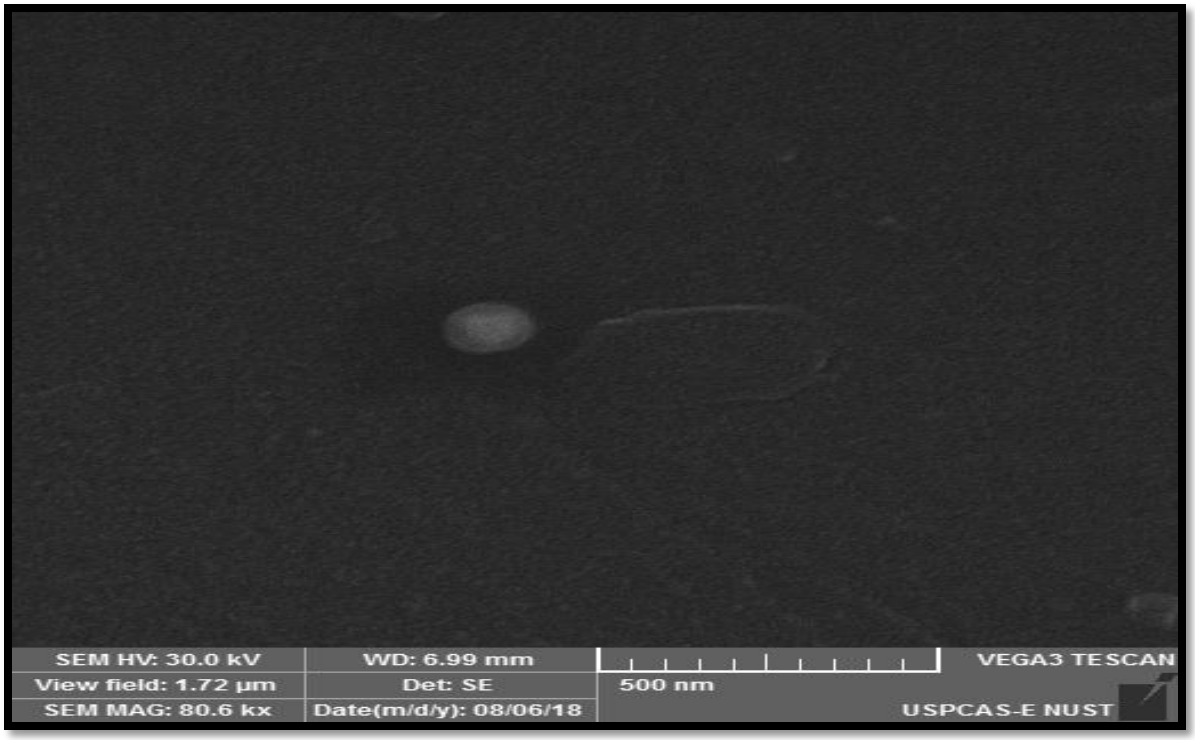


Figure 4.14: SEM image of AuNPs at 500nm resolution.

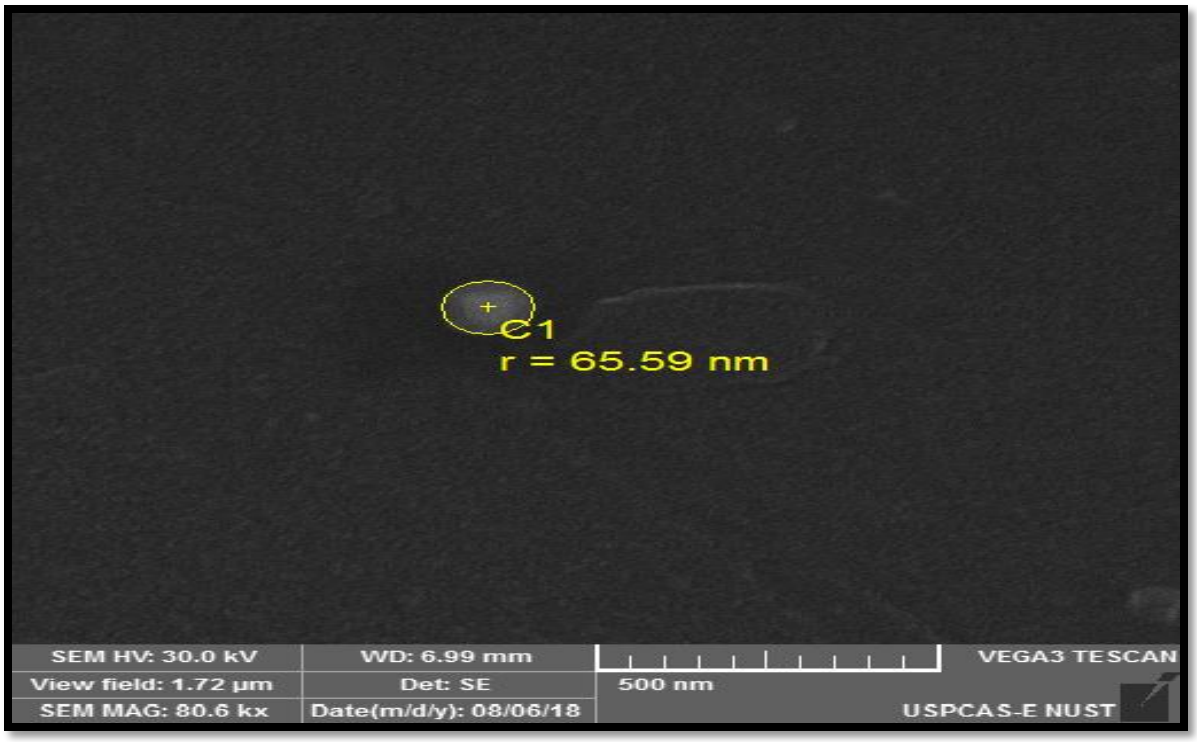


Figure 4.15: SEM image of AuNPs at 500nm resolution with particle identification.

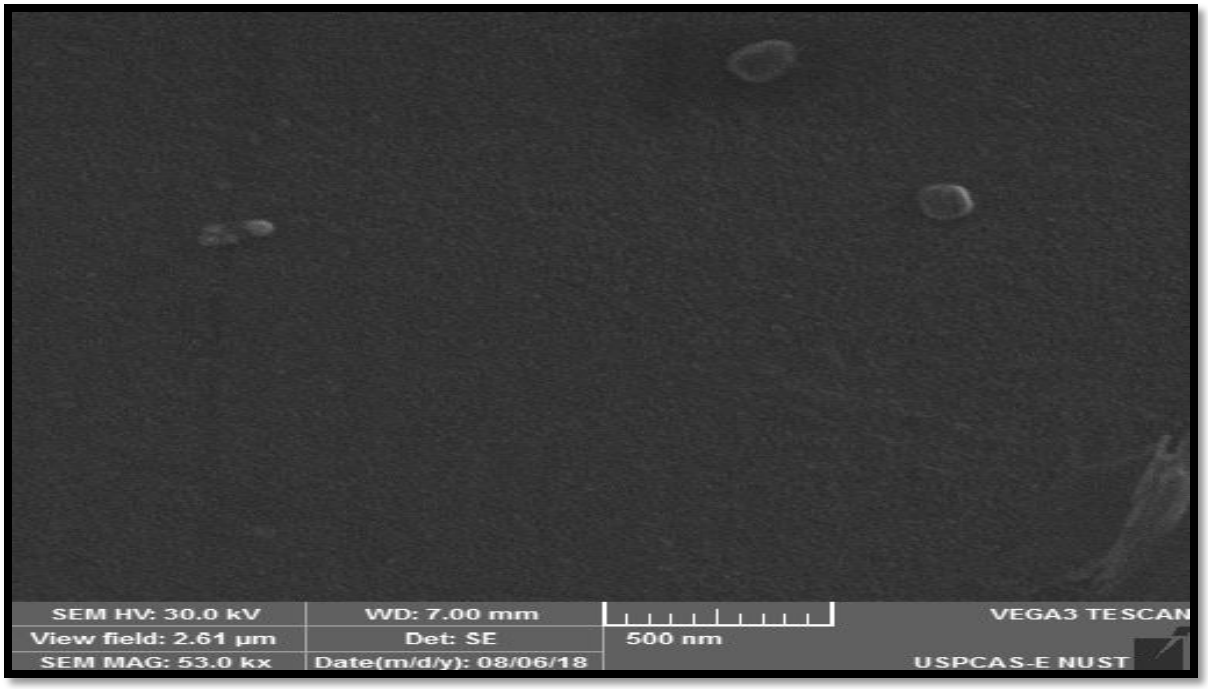


Figure 4.16: SEM image of AuNPs at 50µm resolution.

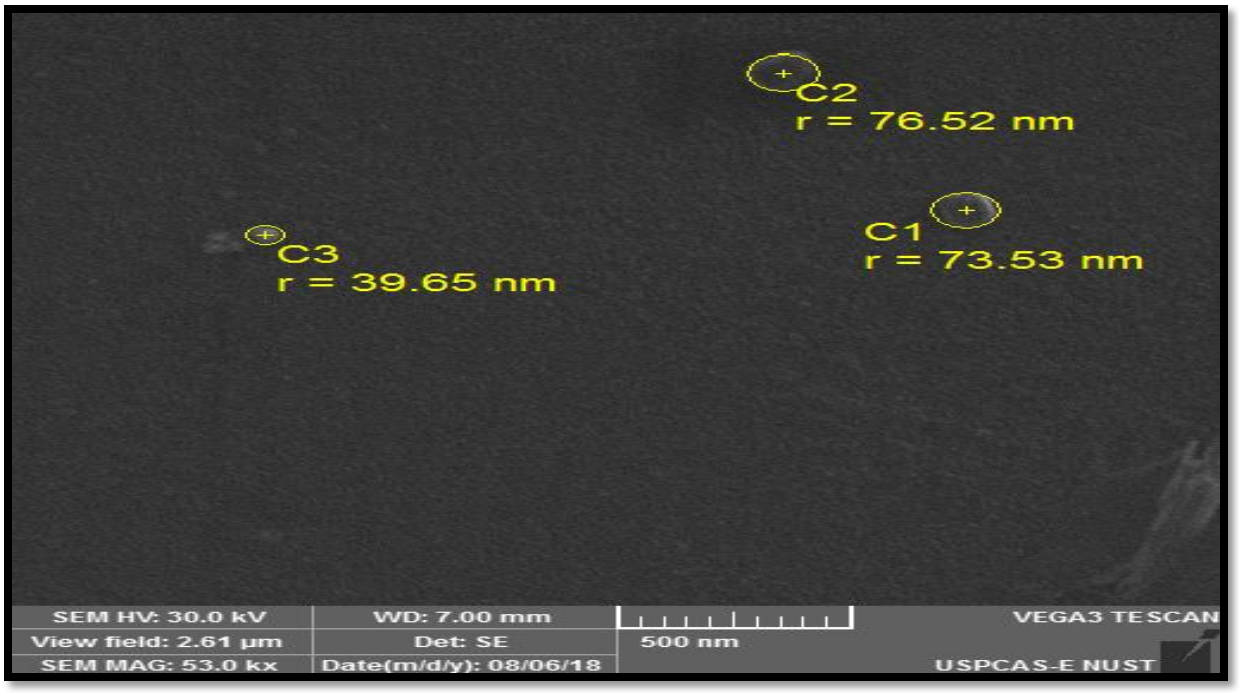
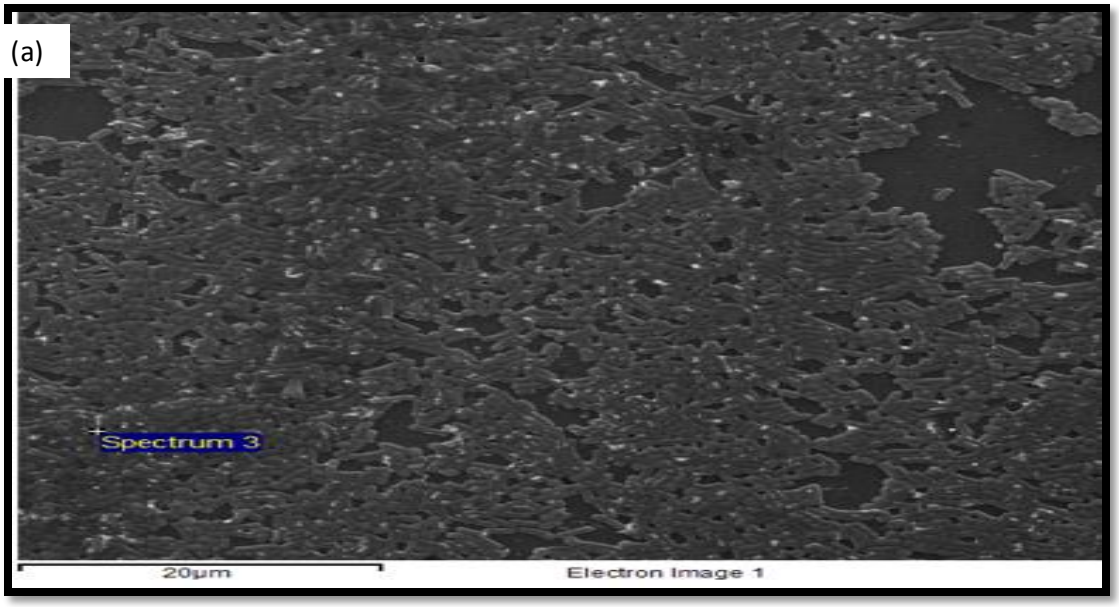
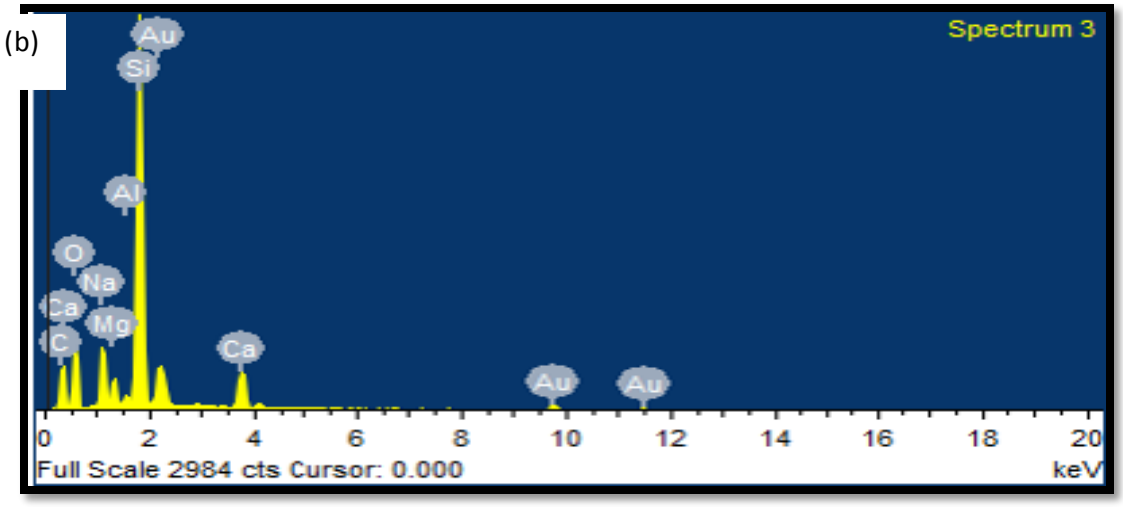


Figure 4.17: SEM image of AuNPs at 500nm resolution with particle identification.

4.5.4 Energy Dispersive X-Ray Spectroscopy (EDS) Analysis

EDS analysis of sample indicated the presence of AuNPs. Their presence was confirmed by the presence of sharp peaks in EDS graph shown below. EDS analysis was done by the instrument linked to SEM and used SEM sample as shown in Figure 4.18. EDS spectrum showed highest peak at 2.3keV which is characteristic peak of metallic Au. Table shows the elemental composition of metal in sample. Au accounts for 4.91% of complete sample.





(c)

Element	App. Conc.	Intensity	Weight %	Atomic
C K	7.99	0.3597	30.47	46.54
O K	10.64	0.5376	27.13	31.11
Na K	3.40	0.9665	4.82	3.85
Mg K	0.94	0.7994	1.61	1.21
Al K	0.25	0.8814	0.39	0.27
Si K	15.31	0.9562	21.95	14.34
Ca K	2.62	0.9331	3.86	1.77
Au M	4.91	0.6880	9.78	0.91
Total		100		

Figure 4.18: EDS graph of AuNPs a) SEM image region from which EDS data was taken. b) EDS analysis graph. c) Elemental composition by EDS.

4.6. Anti-bacterial Assay by Disc Diffusion

AuNPs exhibit significant anti-bacterial activity so various reactions were set up at different concentrations against 6 bacteria by using disc diffusion assay. Two concentrations of AuNPs used were 60µg/ml and 80µg/ml. Cell free extract of bacterial culture was also used to evaluate its bactericidal activity. Results indicate that bactericidal activity of AuNPs is dose-dependent and increases with an increase in the concentration. AuNPs were effective against all bacterial strains such as *Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Pseudomonas nitroreducens* and *Salmonella enterica*. Maximum zone of inhibition was measured to be 18mm thus, signifying the effectiveness of AuNPs against bacterial isolates. Cell free extract of

bacterial strain also exhibited bactericidal properties against all strains. For positive and negative controls antibiotic cefoxitin and deionized water were used respectively. Negative control did not show any zone of inhibition. Thus, it was concluded that zone of inhibition around discs appeared due to presence of AuNPs.

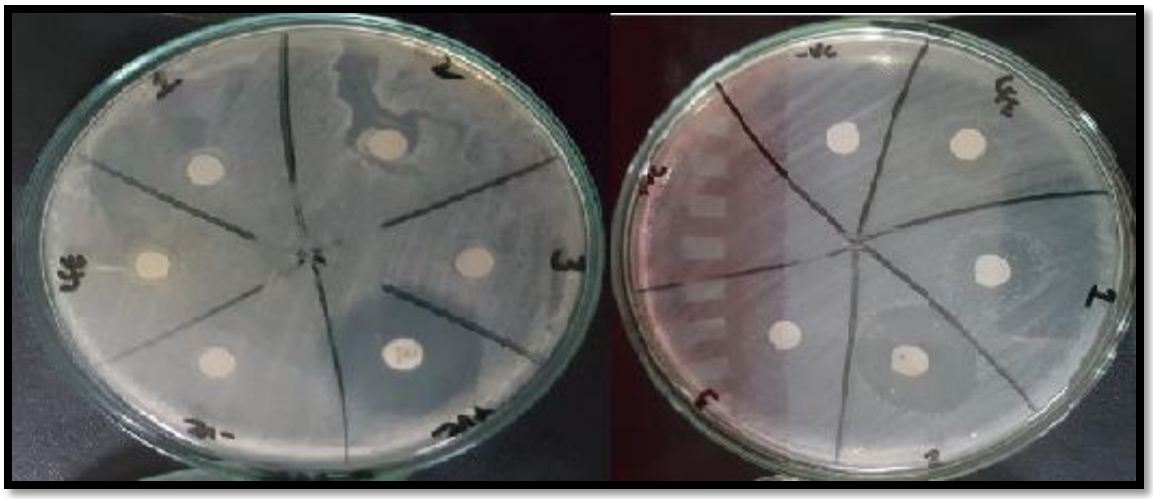


Figure 4.19: Zone of inhibition by biogenic AuNPs and positive control Cefoxitine

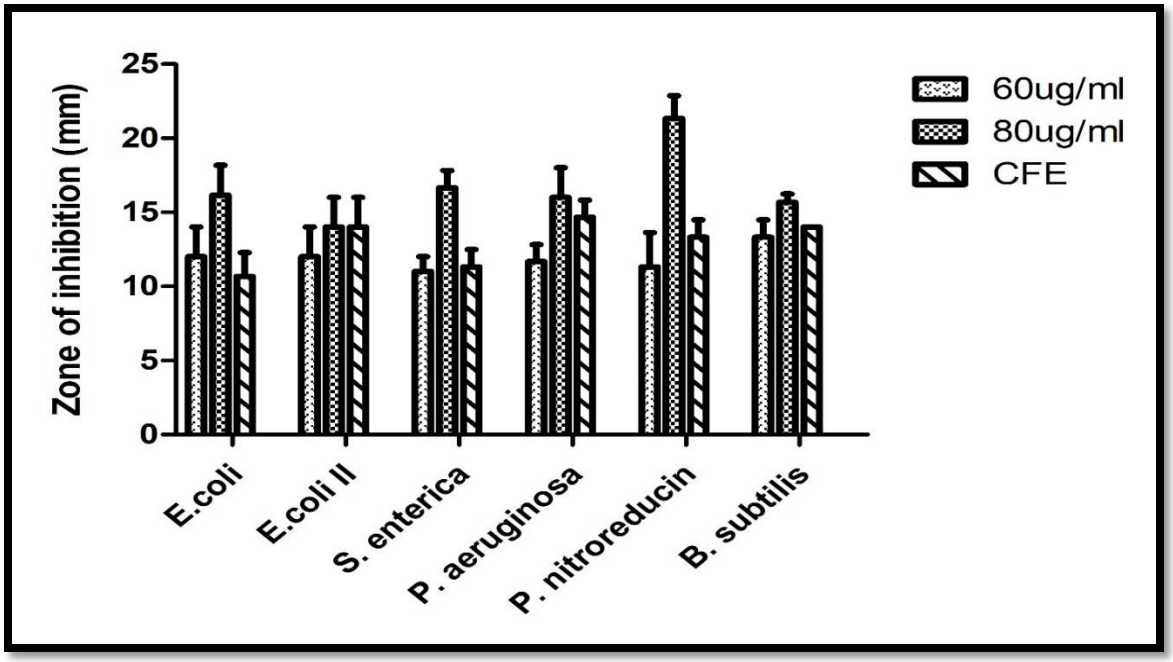


Figure 4.20: Comparison of zone of inhibition of bacterial isolates after application of AuNPs of different concentrations. P value 0.001**

4.7. Antioxidant potential by DPPH assay

Antioxidant potential of biogenic AuNPs was assessed by DPPH assay. Results of DPPH revealed that scavenging ability of AuNPs increase with an increase in concentration. For this assay, percentage inhibition of DPPH was calculated and compared with a reference. Ascorbic acid, a natural antioxidant, is used as positive control. It shows 89% of scavenging ability at 500μg/ml. However, maximum scavenging ability showed by AuNPs was 67% at similar concentrations. These results show that there occur a significant difference between scavenging ability of ascorbic acid and AuNPs. AuNPs possess significant antioxidant ability but it is not comparable with natural antioxidants. Following graph shows free radical scavenging ability of ascorbic acid and AuNPs.

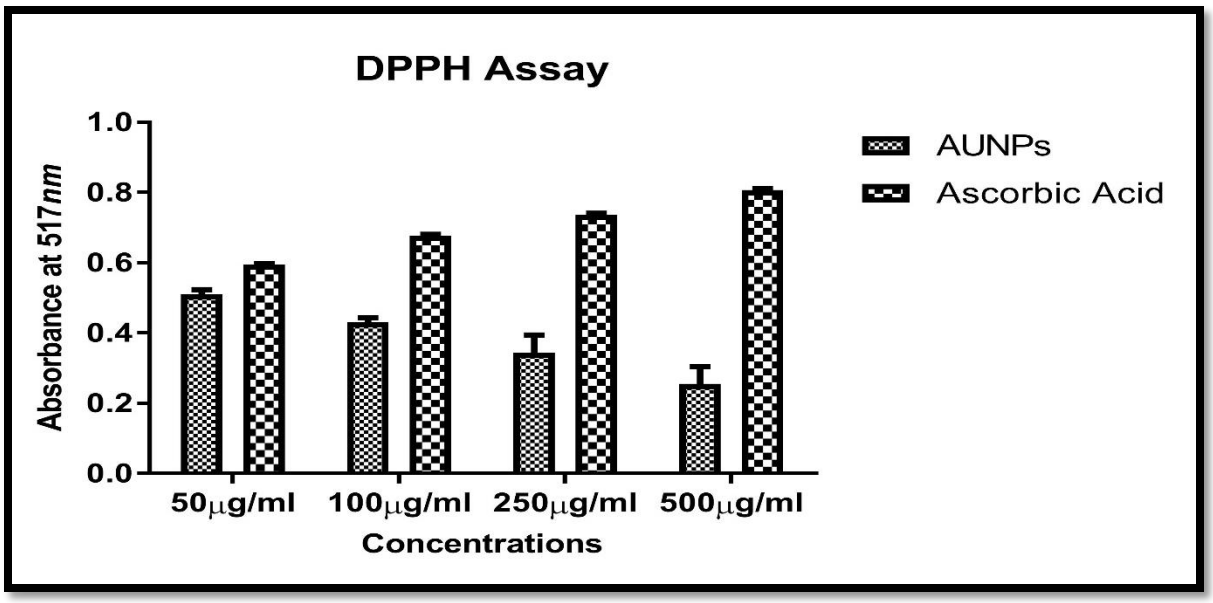


Figure 4.21: Comparison of absorbance values of different concentrations of Ascorbic acid (Positive Control) and AuNPs (Test sample) through DPPH assay P value < 0.001***

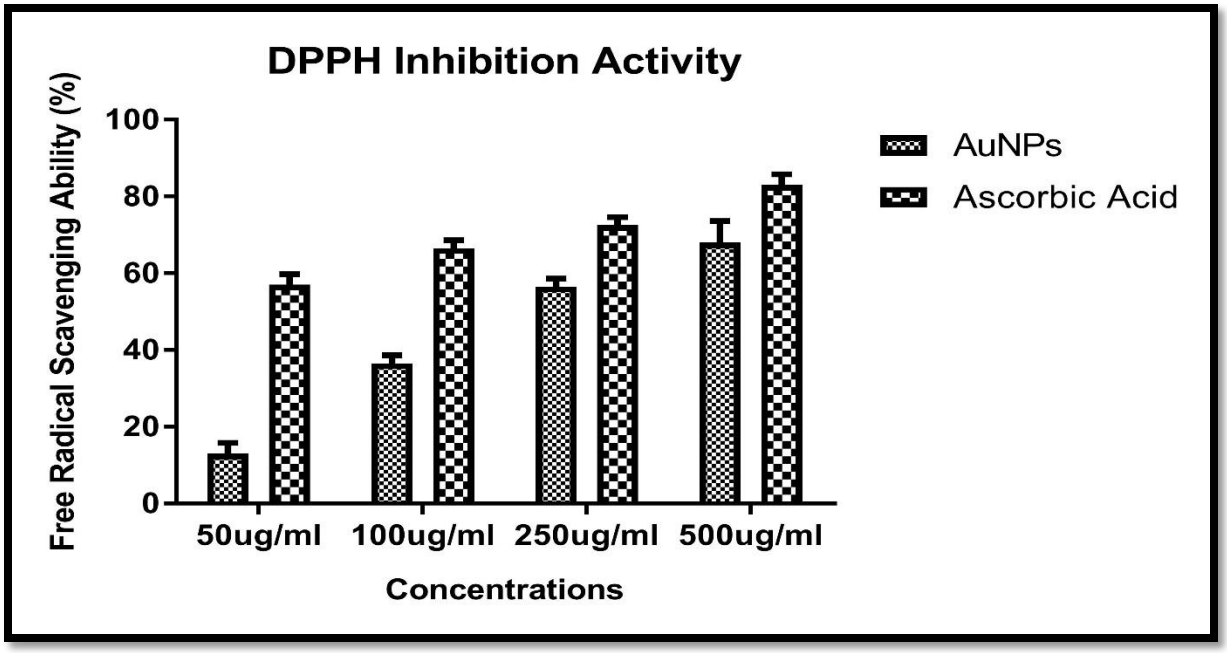


Figure 4.22: Free radical scavenging ability of AuNPs compared with ascorbic acid at different concentration by DPPH assay P value 0.0004***

Chapter 5

DISCUSSION

Nanomedicine deals with synthesis of new drugs and therapeutic tools that overcome the side effects caused by conventional modes of treatment. Using nanotechnology in medicine will revolutionize the way we treat and observe diseases in humans. The term nanotechnology is defined as the manipulation of matter to provide tools for humans. Use of nanomedicines promises to develop products better than conventional methods and bright future for high quality research (Murray et al., 2000).

Among metallic nanoparticles, AuNPs are extensively used in bionanotechnology. AuNPs have attracted great interest due to their ease of synthetic manipulation, optical, magnetic, catalytic and electronic properties. AuNPs have many biotechnology-based applications such as sensory probes, drug delivery vehicle and therapeutic techniques. These particles can be excited by light which allows them to be contrast agents for phototherapeutic and diagnostics application such Raman scattering, light scattering imaging and photo-thermal therapy. Moreover, by changing particle size, aggregation state or surface functionality, electronic and optical properties can be adjusted to use in different fields (Hasan, 2015).

Various methods can be employed for synthesizing NPs. These include several physic-chemical and biological methods. Physico-chemical methods are not preferred today because of the use of toxic chemicals which increases production cost. These processes require heavy instrumentation and extremely capable staff therefore, biological methods or green synthesis is preferred. Biological methods refers to the use of biological system such as algae, viruses, bacteria, fungi, fungi for the production of NPs. These biological system provide ease of biomass production, easy functionality, does not require specific chemicals or media and very cost-effective. These methods also provide the ease of scaling up. Each of these systems have their advantages and disadvantages.

In this study, bacteria *Lactobacillus rhamnosus* HF1-K2 is used to synthesize AuNPs. Several studies have been conducted to produce AuNPs using lactobacillus species but this

bacteria has not been used previously. AuNPs have been synthesized earler using *Lactobacillus kimchicus* and *Lactobacillus casei*. Bacteria are used due to the presence of oxidoreductase enzymes that catalyze the bio-reduction reaction of Au ions into AuNPs. Synthesis of AuNPs was first optimized by setting up different reaction of gold solution and bacterial biomass. Gold solution act as a source of gold ions that can be used by enzymes.

Whole cells of *Lactobacillus rhamnosus* were used as catalysts for AuNPs synthesis. Use of intracellular enzymes largely eliminate costs for downstream processing of nanoparticles purification but coenzymes are required to sustain bioreduction reaction (Korbekandi, Iravani, & Abbasi, 2012). Use of whole cells provide the advantage of coenzymes recovery through metabolic pathways thus, replenishment of expensive coenzymes can be avoided (Iravani, 2014). Moreover, it was reported by Pradeep and Nair that small nanocluster formed outside bacterial cells have tendency to aggregate (Nair & Pradeep, 2002). Therefore, NPs formed inside cells are more stable than synthesized from intracellular enzymes.

Initial AuNPs synthesis confirmation was done by observing color change of the reaction mixture as it turned from pale yellow color to red which is a unique feature of AuNPs due to SPR phenomenon. Synthesis parameters of AuNPs by *L.rhamnosus* were optimized by setting up different reactions at varying temperature, molarity and time. These reactions showed the optimal parameters for biosynthesis to be 72 hours with 1mM gold solution at 30^{O}C. after incubation, violet colored pellet settles down with a clear colorless supernatant. Growth media did not show any color change upon challenging with gold solution, after the removal of biomass. These results clearly indicate that synthesis of AuNPs occurred intracellularly. AuNPs were harvested for biomass by subjecting pellets to alternating cycles of centrifugation and ultra-sonication to disrupt cells. Complete mechanism of AuNPs synthesis has not yet been fully elucidated but it has been reported that Lactobacilli species synthesized AuNPs intracellularly (Nair & Pradeep, 2002). It has been reported NADH-dependent enzymes by microbes are responsible for conversion of gold ions into AuNPs whereas amino acid residues and proteins provide stabilization (Banerjee, 2013).

Synthesis was further verified by UV/Vis spectral analysis that shows characteristic AuNPs peak at 540nm. Absorbance of AuNPs at wavelength of 540nm was recorded to be 1.9. In another study, AuNPs synthesized using *Lactobacillus kimchicus* DCY51 showed an absorbance of 0.8 with average size of particles being 14nm (Markus et al., 2016). Current study shows high absorbance value due to large size of particles synthesized by *L.rhamnosus* i.e. 50-70nm. Absorbance value obtained after 2hr of AuNPs synthesis was recorded to be 1.9 whereas, after 4hr it was 1.5. No further change was observed in absorbance values of these particles.

Crystallinity of AuNPs was confirmed by their XRD profile analysis. Figure 4.6 represents XRd diffractogram of biosynthesized AuNPs. Four peaks were indexed to (111), (200), (220) and (311) planes according to Bragg's equation. Sharpest peak was measured at 38.16^{O}that corresponds to (111) plane. Similar results of XRD analysis were also reported in a study that synthesized flower shaped AuNPs using microbes (Singh, Kim, et al., 2016).

FTIR spectrum of biosynthesized AuNPs provide information about presence of functional groups. AuNPs spectrum was compared with FTIR spectrum of biomass. It is evident from Figure 4.7 and 4.8 that AuNPs spectrum contain bands that are not present in biomass spectrum. Bands shown in spectrum corresponds to stretching of amide, amine and alcohol bonds. Carbonyl groups in amide bonds corresponds to proteins. Proteins and amino acid residues have been reported to provide stabilization to newly synthesized AuNPs (Kanchi et al., 2014). Several studies reported binding affinity of AuNPs to amino residues through electrostatic interactions (C. S. Kumar, Raja, Sundar, Antoniraj, & Ruckmani, 2015). Proteins and amino acids have been studied to provide capping molecules to these AuNPs for protection (Shedbalkar, Singh, Wadhwani, Gaidhani, & Chopade, 2014)

AuNPs were further analyzed by SEM. Figure 4.10-4.16 showed AuNPs to be spherical and ranging in size from 50-70nm. Images of SEM analysis reveals that AuNPs are spherical in shape and did not lose their shape even after purification and disruption of cells. These particles were formed on bacterial cell membrane and retained their shape through process of centrifugation and ultra-sonication (Markus et al., 2016). EDS analysis indicated elemental composition and abundance of these elements in sample. EDS

spectrum showed highest absorbance peak for Au at 2.3keV. This is characteristic absorbance peak for metallic gold as reported by (Singh, Singh, et al., 2016).

Bactericidal property of biosynthesized AuNPs have been studied against various bacteria to minimize the use of antibiotics. In this study, we evaluated antibacterial potential of AuNPs against *P.aeruginosa, E.coli, P.nitroreducens, B.subtilis, and S.enterica*. Different concentration of AuNPs were used and effect of cell free extract of *L.rhamnosus* was also tested. Increase in concentration of AuNPs increased its bactericidal property. In a study by Senthilkumar et al, bactericidal potential of AuNps synthesized from plant was evaluated. Zone of inhibition for 300µl/ml against *P.aeruginosa*, *E.coli*, and *B.subtilis* was 19mm, 17mm and 16mm. Whereas, in the current study, zone of inhibition at 80µg/ml against *P.aeruginosa, E.coli,* and *B.subtilis* was measured to be 17mm, 16mm and 17mm respectively.

% Free radical scavenging activity of biosynthesized AuNPs was evaluated by DPPH assay. AuNPs can reduce DPPH and it can be observed by measuring absorbance value at 517nm using UV/Vis spectroscopy. This assay showed that % free radical scavenging ability of AuNPs increase with increase in concentration of AuNPs. Scavenging ability recorded for the lowest concentration of 50µg/ml of AuNPs was 14%. Whereas, for highest concentration of 500µg/ml scavenging ability was recorded to be 64%. Increase in scavenging activity of AuNPs can correspond to increase antioxidant potential of probiotic bacteria. These results show that AuNPs possess significant radical scavenging ability but it cannot be compared with natural antioxidants.

CONCLUSION AND FUTURE PERSPECTIVES

Current study was focused on synthesizing AuNPs using bacteria *Lactobacillus rhamnosus* as a potential intracellular system. Various physico-chemical parameters such as temperature, molarity and reaction time were optimized for biosynthesis. Optimal salt molarity, recation time and temperature were found to be 1mM for 72 hours at 30^{O}C. Newly synthesized AuNPs were then subjected to characterization techniques such as SEM, XRD, FTIR and EDS. Characterization of AuNPs revealed that these particles are spherical and range from 50-70nm. Amide bonds present in AuNPs help in their conjugation to other molecules that provide protection. AuNPs bactericidal properties against various pathogenic bacteria provides alternative to the use of antibiotics. AuNPs possess significant free radical scavenging ability but it cannot be compared with natural antioxidants such as

After complete analysis of results presented in this study, it is recommended to further characterize these nanoparticles using more sensitive techniques such as zeta potential, TEM and AFM. Most of the pathways of nanoparticles synthesis, their bioactivity and cytotoxicity are still unknown. These underlying pathways needs to be investigated using different methods such as in silico approaches can be used. Interaction of AuNPs with microbial and human components needs to be modeled. Information on cell cycle genes and proteins can provide useful information about their molecular pathways and functioning that leads to cytotoxicity of AuNPs. AuNPs, have been used in many studies, as a drug delivery vehicle so its conjugation with other biomolecules can be performed to enhance its drug carrying and delivering capacity. This research should be taken to animal models, by evaluating the toxicity of these nanoparticles on mouse.

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