

Antimicrobial activity of silver nanoparticles against *proteus mirabilis* isolated from patients with food diabetes ulcer

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ABSTRACT

The aim of this study was to study the antibacterial activity of Silver nanoparticles synthesized by *L. acidophilus* and the biosynthesis of AgNPs from nonpathogenic bacterial isolates. Fifty samples were obtained during the period from December 2020 to March 2021. AgNO₃ was used as a precursor for the synthesis of AgNPs. Biological AgNPs were originally shown by change the color, yellow to reddish-brown. The categorization of AgNPs accomplished by SEM, XRD, AFM, and EDS. SEM exhibited well-dispersed AgNPs, homogenous with a diameter of 40-60 nm, with inconstant shapes, mostly spherical form. XRD detected that the size of AgNPs was 34 nm. AFM also showed the three-dimensional structure of AgNPs and their diameter which was 45.82 nm. EDS exhibited that the AgNPs fabricated was 94.82% silver and 5.18 % oxygen. Silver nanoparticles displayed antibacterial action to MDR of *P. mirabilis*.

Keywords: Biogenic silver nanoparticles, *L. acidophilus*, Antimicrobial activity, *P. mirabilis*.

Article type: Research Article.

INTRODUCTION

Nanotechnology is a recent domain of science that deals with nanoparticle creation and application (NPs). Its size ranges from 1 to 100 nm. Because of their unique physicochemical features, including antibacterial, optical, electronic, and magnetic properties and also catalytic activity, NPs have been widely examined (Kavitha *et al.* 2013; Murugan & Shanmugasundaram 2014; Johari *et al.* 2015, 2016; Bagherzadeh Lakani *et al.* 2016; Ghazanfari *et al.* 2020). One of the most crucial components of nanotechnology is the steady advancement of experimental procedures for the production of nano-sized materials with a wide range of chemical configurations, dimensions, high mono-dispersity, and controlled morphologies. Moreover, the consumption or release of hazardous compounds and large amounts of energy in conventional synthesis procedures encouraging researchers to focus and develop new enabling green technologies in material production (Emsbo *et al.* 2015; Vigneshvar *et al.* 2016). Biosynthesizing AgNPs exhibited antioxidant and anti-inflammatory activities (El-Rafie & Hamed 2014). The melting temperature, magnetic behavior, redox potential, and color of AgNPs may be adjusted by varying their size (1-100 nm) and forms such as spherical, triangular, and rod (Gurunathan *et al.* 2009). Because of their high conductivity, chemical stability, and utility as catalysts, AgNPs have attracted a lot of interest (Emsbo *et al.* 2015). The study is aimed to study the effect of AgNPs on *Proteus mirabilis* and examining their antimicrobial activity. Diabetic foot ulcers are one of the leading causes of death and morbidity among diabetics. It includes a damage to all layers of skin, necrosis, or gangrene on the soles of the feet as a result of peripheral neuropathy or peripheral arterial disease (PAD) in diabetics (Guangquan *et al.* 2012).

It is believed that about 80 % of human foot ulcer infection involve bacterial biofilms. Biofilms form when bacteria attach to a surface or aggregates and form micro-colonies, embedded in a protective extracellular polysaccharide matrix (Thomas *et al.* 2010).

MATERIALS AND METHODS

Patients and sample collection

This study was conducted in Najaf governorates during the period between December 2020 and march 2021. A total of 50 specimens were collected from out and in patient with diabetic foot ulcer infection (most data obtained from hospital). Specimens were collected with their medical record reviewed for each patient. All specimens were incubated into MacConkey, blood and nutrient agars and incubated at 37 °C for 18-24 hours (El-Rafie & Hamed 2014).

Preparation of cell free supernatant of *Lactobacillus acidophilus*

After 24 h, the culture was subjected to 6000 rpm for 20 min to make supernatant from *L. acidophilus*. After centrifugation, the cells precipitated at the lowest part of the tube were removed and supernatants were taken to use in AgNPs biosynthesis (Chaudhari *et al.* 2012).

Bacterial synthesis of AgNPs

AgNO₃ was substrate for bacterial synthesis of AgNPs by *L. acidophilus*. Four concentrations of AgNO₃ (2, 4, 8 and 10 mM) were added to the *L. acidophilus* supernatant. Reaction condition was in dark room. The pH altered to 8. After incubation at 36 °C, 150 rpm for 18 h, the color change was noticed and the mixture was separated for 20 min at 8000 rpm. The supernatant was replaced using centrifuge for three times and the pellet was dried at 45 °C for 30 h (Chaudhari *et al.* 2012; Sarvamangala *et al.* 2013).

Culture of bacterial isolates

According to the diagnostic producer guidelines, the bacteria were identified by macfaddin (2000) and Mims *et al.* (2008). The identification of bacteria was based on culture and physical characteristics, such as colony form, non-lactose fermenter, “swarming” appearance on culture media, and so on. Urease, oxidase, and indole synthesis tests are examples of morphological and biochemical tests, MR-VP, Simmon citrate test, triple sugar iron agar test, and automated biochemical identification (VITEK and 16srRNA gene sequencing).

The *L. acidophilus* was obtained from a higher student laboratory in the Faculty of Science. BHI was inoculated with *L. acidophilus*, incubated at 37 °C, 24 h. *L. acidophilus* was identified based on biochemical and morphological tests (Holt *et al.* 1994). The second activation was worked from the first activation and incubated aerobically at 37 °C for 24 h (Chaudhari *et al.* 2012).

Molecular identifications of bacterial isolates

Identification to species-level was determined by polymerase chain reaction (PCR) with universal primers. The 16S rDNA was subjected to nucleotide sequencing. Favor Prep Genomic DNA Mini Kit was used to extract total DNA following the manufacturer protocol. The concentration of DNA and the purity of the DNA solution were determined by the spectrophotometer. PCR mixture 2.5 uL (10 μM) 27F AGAGTTTGATCCTGGCTCA, 1492R GGTTACCTTGTTACGACTT, with Pre Mix- Kit, and 8 μL of DNA sample, then completed to 20 uL of DDW. PCR was run at 95 °C for 4 min then 38 cycles of 30 sec. at 95 °C, 60 sec at 55 °C, 120 sec at 73 °C and 5min at 73°C. Electrophoresis was performed on the PCR product by a 1% agarose gel with EB and photographed under UV, followed by sequencing 16S rDNA57. The 16S rRNA gene sequences determined by utilizing the tool, the sequences of type strains retrieved from the GenBank using CLUSTAL X (version 1.82) (Thompson *et al.* 1994).

Characterization of AgNPs

XRD was used for structure and size of AgNPs, the powder of AgNPs was used for test. AFM was achieved for analyzing the AgNPs. The powder of AgNPs was used for the AFM. SEM (Inspect S50. FEI) was characterized to dimension, form and scattering of AgNPs (Natarajan *et al.* 2014).



Antibacterial action of AgNPs

Antibacterial actions of biological AgNPs were carried out with consuming agar well diffusion against *Proteus mirabilis*. The examined bacteria with 1.5×10^8 CFU mL⁻¹ were inoculated onto MHA plates. The each agar well was filled with 100 μ L AgNPs at the concentration of 200 μ g mL⁻¹. One petri dish sub cultured for each pathogenic bacterium, used as control and incubated for 18 h at 37 °C.

RESULTS

Molecular identifications of bacterial isolate

Isolate was detected using 16sRNA sequencing by extraction and amplification of this gene in addition to biochemical detection. DNA samples (clear without fragmentation) were used as PCR templates during 16sRNA gene amplification. The extracted DNA templates were used in the amplification of the 16sRNA gene using 16sRNA gene universal primers. The resulted 16sDNA bands were 1400 bp. Bacteria were identified as *L. acidophilus* with 99% accuracy using the BLAST program's alignment with the GenBank database.

Biosynthesis of AgNPs

Supernatant of *L. acidophilus* demonstrated ability of synthesis AgNPs using AgNO₃ (10 mM) as an initiator for synthesis AgNPs followed by shaking incubation for 18 h, 150 rpm at 37 °C. *L. acidophilus* changed color to reddish brown as pointer with creating AgNPs.

SEM categorization of AgNPs

SEM analysis exhibited well-isolated AgNPs, revealing homogenous and well-dispersed NPs, similar to diameter of 40-60 nm, with inconstant shapes, mostly spherical form (Fig. 1).

XRD categorization of AgNPs

X-ray crystallographic diffraction (XRD) analysis was used to determine average size of the silver nanoparticles. *P. mirabilis* produced silver nanoparticles with an average size of 34 nm (Fig. 2).

AFM analysis of AgNPs

The average diameter and three-dimensional structure of silver nanoparticles were discovered using an Atomic Force Microscope (AFM). *P. mirabilis* produced silver nanoparticles with an average diameter of 45.82 nm (Fig. 3). The color change, XRD, and AFM were used to describe and characterize nanoparticles. As a result, the morphology, size, distribution, and existence of metal composites were investigated.

Energy Dispersive X-Ray Spectroscopy (EDS)

Energy Dispersive-x-ray Spectroscopy (EDS) was used in the quantitative assessment of AgNPs through detecting the peaks of optical absorption by silver metal. The percentage of elemental constituent weight of the AgNPs fabricated was 94.82% silver and 5.18 % oxygen (Fig. 4).

Antibacterial activity

Antibacterial activity of AgNPs were used to estimate their ability in preventing growth of multidrug-resistant bacteria (MDR). To identify the antibacterial activity of nanoparticles using biogenic AgNPs at the concentration of 200 μ g mL⁻¹. After incubation, the inhibition zone was measured in mm of the AgNPs. The results revealed that AgNPs inhibited the examined microorganisms (gram neative bacteria). The major inhibition zone of the biogenic AgNPs displayed large diameter in gram-negative bacteria which was 35 mm for *P. mirabilis*. The diverse antibacterial activity in other bacteria was due to dissimilarity in their sensitivity to AgNPs.

DISCUSSION

L. acidophilus exhibits optimum incubation temperature for the production mixtures prepared (37 °C). Different concentrations of AgNO₃ were added under dark conditions and adjustment of pH to 8.3. The medium color was changed from yellow to reddish-brown. The optimum incubation time for production of AgNPs was 18 h (Hans 2014).

Presence of brown color is a vibrant suggestion of the synthesis of AgNPs as a reduction by the bacterial metabolites (enzymes, proteins, amino acid, polysaccharides, etc.) in the supernatants (Natarajan *et al.* 2014; Sreedevi *et al.* 2015). Bacterial supernatant is the easy way for the size-meticulous creation of AgNPs. The conditions of the supernatant can be effortlessly reformed and sustained than cell components in the cytoplasm in attempting to preserve environment like heat shock proteins and require purity (Kalimuthu *et al.* 2008). Not all organisms are competent for the synthesis of silver nanoparticle. The specific reaction mechanism that causes all species to produce silver nanoparticles has yet to be discovered. The organisms which comprise “silver resistance machinery” can create silver. Extracts from microorganisms may perform as reducing and capping in AgNPs production. The reduction of silver ions occurs by groupings of biomolecules (Thakkar *et al.* 2010).

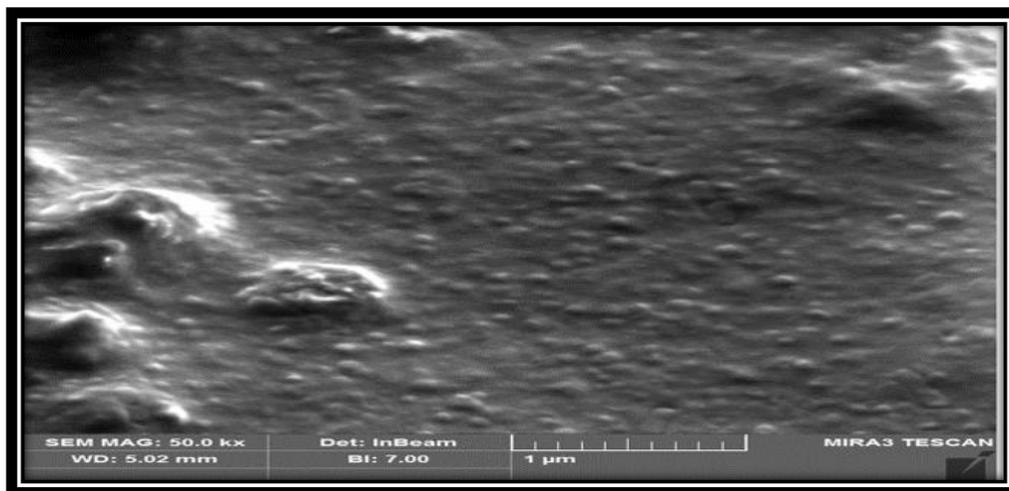


Fig. 1. SEM analysis of synthesized AgNPs from *L. acidophilus*.

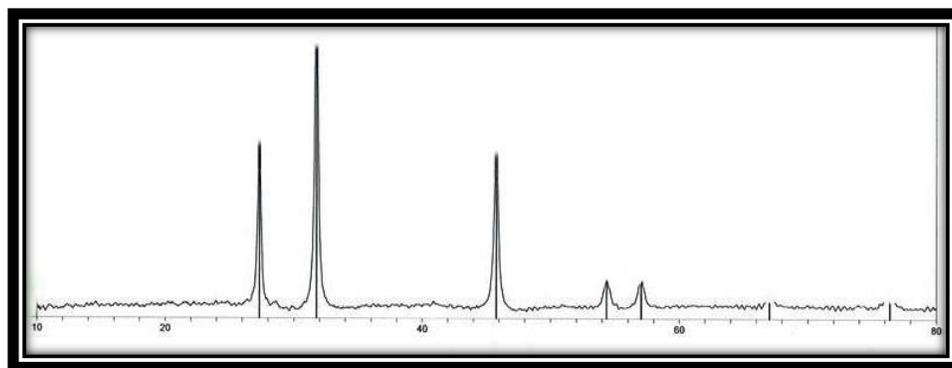


Fig. 2. XRD analysis of Biosynthesized nanoparticles size from *L. acidophilus*.

Characterization of silver nanoparticles

The initial approval of the extracellular biosynthesis of silver nanoparticles were achieved by observing the color change due to the excitation of surface plasmon vibrations of the synthesized AgNPs. The conversion of extracellular medium color clearly indicates the fabrication of AgNPs (Luo *et al.* 2018). The conversion of extracellular moderate color evidently designates that the procedure of development of AgNPs is extracellular (Bhainsa & D'Souza 2006; Shaligram *et al.* 2009). The presence of brown color in a mixture encompassing the biomass is a strong suggestion of creating AgNPs in the reaction mix and is owing to the excitation of surface plasmon vibrations in the nanoparticles (Ahmad *et al.* 2003). XRD noticed that the middling size of AgNPs synthesized from *L. acidophilus* was 22 nm, the others indicated an average diameter of AgNPs from 20-100 nm. AFM analysis detects the 3D structure of AgNPs and the average size of the nanoparticle (34.82 nm) belonged to silver nanoparticle biosynthesized from *L. acidophilus*.

After characterization results (shape, size, and dispersity of nanoparticles), AgNPs were improved in inferiority: lowest size and few polydispersities, with *L. acidophilus*. This could be due to altering in the reduction belonging to extracellular metabolites, biomolecules and subsequently offering their capability of interaction with AgNO₃ (Chaudhari *et al.* 2012) in the culture of microbes.

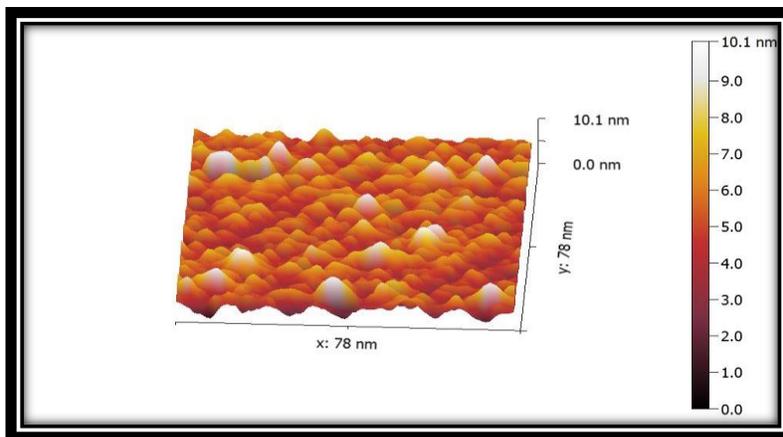


Fig. 3. Topography and granularity of cumulating distribution report of biogenic AgNPs fabricated by *L. acidophilus* 3D characterization. Avg. Diameter: 72.88 nm <= 50% Diameter: 65 nm <= 10% Diameter: 38 nm <= 90% Diameter: 95 nm.

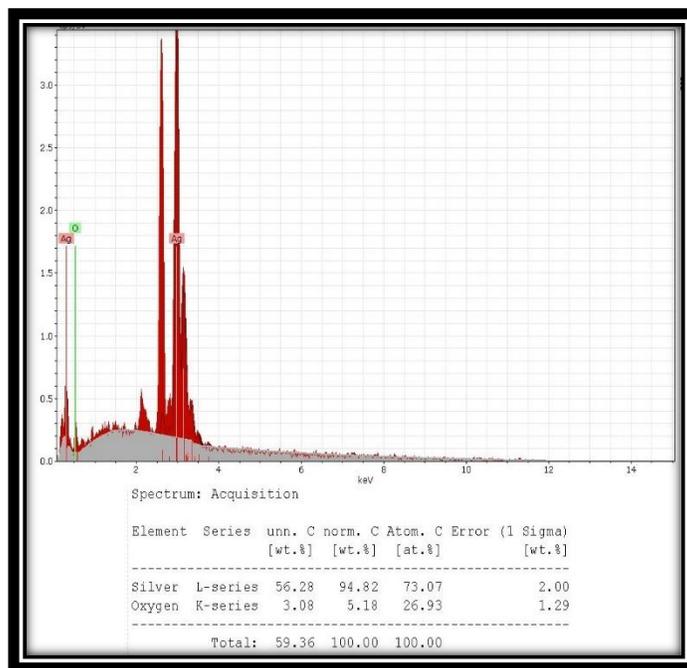


Fig. 4. EDS point analysis spectrum of *L. acidophilus* demonstrating the amount and availability of AgNPs.

Antimicrobial activity of nanoparticles

Because of the emergence and increased number of multiple antibiotic-resistant microorganisms, nanoparticles are now considered alternate to antibiotics and have a great perspective to resolve this problem. AgNPs were mostly good-looking for the making of a novel class of antimicrobials (Pinto *et al.* 2009; Rai *et al.* 2012). The AgNPs were tested to evaluate their antibacterial activity against gram-negative bacteria using the agar well diffusion method. AgNPs seems to be more lethal than Ag⁺ ions against *Escherichia coli* (Soo-Hwan *et al.* 2011).

The positive charge is able to interact with the negative charge of bacteria with electrostatic action. Interaction can overpower other factors that can impact the bacterial death (Holt *et al.* 1994; Sarvamangala *et al.* 2013). Silver ions

could make structural changes in DNA and protein and cell wall. Ag ion interrelates with functional groups. The AgNPs damage membranes and discharges ROS, making free radicals with antibacterial action (Rajeshkumar & Malarkodi 2014). More small nanoparticles demonstrated more activity than big particles, due to a large surface area on microbe (Gurunathan *et al.* 2009). Hydrogen concentration has a characteristic role in the antimicrobial action of AgNPs. The lowest biogenic AgNPs in alkaline hydrogen concentration exhibited more antimicrobial action than the big particles which are manufactured in acidic hydrogen (Kalimuthu *et al.* 2008). The shape of AgNPs is very important in the antibacterial action of AgNPs. Hexagonal AgNPs display the maximum antimicrobial action related to further shape that was ascribed to the facet reactivity as well as particular surface areas (Thakkar *et al.* 2010; Ranganath *et al.* 2012).

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