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BIOSYNTHESIS OF SILVER NANOPARTICLES WITH POTENT ANTIMICROBIAL ACTIVITY USING LACTIC ACID BACTERIA

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ABSTRACT
In this study, Lactic Acid Bacteria isolated from traditional fermented foods were screened for the synthesis of silver nanoparticles. Antimicrobial activity of the synthesized nanoparticles was assayed against several multi-drug resistant clinical human pathogenic bacteria. Among the bacterial isolates that were tested, K1.16 and MM17 had 99% and 98% relatedness with *Lactobacillus herbarum* strain TCF032 E4 and *Lactobacillus paraplantarum* strain DSM 10667 respectively, and were able to reduce silver efficiently into silver nanoparticles. The Ultraviolet–Visible spectrum showed a plasmon peak at ~ 410 nm confirming the presence of silver nanoparticles. Energy dispersive X-ray spectrum revealed a strong signal in the silver region confirming the formation of silver nanoparticles as well as a optical absorption peak at approximately 3 KeV due to surface plasmon resonance. Diffraction peaks were observed at 38.1°, 44.2°, 64.4° and 77.4° in the 2θ range. The peaks were indexed to (111), (200), (220) and (311) which can be indexed according to the facets of a face centered cubic crystal structure of silver. Scanning transmission electron microscope micrographs recorded from the coated grid of the synthesized nanoparticles showed a spherical shape of silver nanoparticles with the size range of 11-71 nm. Silver nanoparticles produced by *Lactobacillus plantarum* (K1.16) were the most potent as indicated by the lowest minimum inhibitory concentrations across all pathogenic bacterial isolates tested in this study, which included; *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella* Typhimurium, *Bacillus cereus* and *Enterobacter* spp. These findings can be pertinent in the development of novel topical ointments against pathogenic microorganisms.

KEYWORDS: Lactic acid bacteria (LAB), silver nanoparticles (AgNPs), antimicrobial, Energy dispersive x-ray (EDX), Scanning transmission electron microscope (STEM).

INTRODUCTION
Over recent years, the rise of antimicrobial resistance among most medically important bacterial species has led to the urgent need for the development of new strategies to combat these organisms. Among them, nanoparticles have emerged as important candidates in the fight against infectious agents as well as many organisms that easily develop resistance to conventional antimicrobial substances. Nanoparticles are defined as particulate dispersions of solid particles with at least one dimension at a size range of 10-100 nm.[1] These nano sized particles have unique properties which give them the ability to be applied to many areas especially the medical field, the food industry and in waste water treatment. The most important feature of nanoparticles is their surface area to volume aspect ratio, resulting in appearance of new mechanical, chemical, electrical, optical, magnetic, electro-optical, and magneto-optical properties that are different from their bulk properties, thus allowing them to interact with other particles easier.[2]

Silver nanoparticles (AgNPs) are one of the most studied among metallic nanoparticles as they have been proven to be effective antibacterial agents and possess a strong antimicrobial activity against bacteria, viruses and fungi.[3] This is because silver ions and silver-based compounds are highly toxic to a wide range of microorganisms including major species of drug resistant bacteria. Hence this aspect of silver makes it an excellent choice for multiple roles in the medical field. These nanoparticles of silver have been studied as a medium for antibiotic delivery[4] and to synthesize composites for use as disinfecting filters and coating materials.[5]

In addition to their applications in the medical field, silver nanoparticles are vastly used in the food industry. Because of their powerful bactericidal effects, silver nanoparticles are used in food storage, packaging and processing. Many new consumer products containing silver nanoparticles have been launched in the market and are beginning to impact on food associated industries.[6] The World Health Organization includes silver in a colloidal state produced by electrolysis of silver electrodes in water, and colloidal silver in water filters to a number of water disinfection methods specified to provide safe drinking water in developing countries. Such techniques can be adopted in Botswana to increase the quality of drinking water and to prevent water-borne diseases. The synthesis of the silver nanoparticles is therefore very important given their vast array of applications. It can be carried out through various physical and chemical methods. Some of the methods used to synthesize these nanoparticles include laser ablation, photo induction, electrochemical, chemical reduction, microwave assisted, inert gas condensation and solvothermal methods.[7] These methods are however associated with a number of setbacks as they are expensive as well as inefficient in material and energy use, they also produce toxic chemicals which can affect the environment and human health.

There is much concern for environmental contaminations due to the fact that the synthesis of nanoparticles in this manner results in a large amount of hazardous by products. There is therefore, a need for alternative nontoxic and eco-friendly methods of silver nanoparticle synthesis. The ability of bacteria, fungi (actinomycetes and yeasts), algae and plants to accumulate gold and silver ions from solution has been reported and the synthesis of these nanoparticles has been successfully demonstrated in a range of organisms including *Bacillus* sp., fungal species such as *Verticillium* and *Fusarium*, actinomycetes such as *Rhodococcus*, as well as lactic acid bacteria.[8] Such methods present many advantages as they are time saving, cost-efficient and most importantly they are eco-friendly as they do not result in the production toxic compounds during mass production of nanoparticles. The aim of this study was to screen Lactic Acid Bacteria (LAB) strains isolated from traditionally fermented foods in Botswana for biosynthesis of silver nanoparticles. The nanoparticles thus produced were evaluated for antimicrobial properties against pathogenic bacteria.

MATERIALS AND METHODS

Pathogenic bacteria and culture conditions

The following pathogenic bacteria were used in this study; *Escherichia coli*; *Staphylococcus aureus*; *Klebsiella pneumoniae*; *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium); *Bacillus cereus* and *Enterobacter* spp. The pathogenic bacteria were obtained from a culture collection at the National Health Laboratory, Gaborone, Botswana. The cultures were streaked on nutrient agar (MERCK, Darmstadt, Germany) and incubated at 37 ºC for 24-48 hours. The isolates were maintained in a solution containing 80% tryptone soya broth and 20% glycerol and kept at -20˚C until they were utilized to evaluate the antimicrobial properties of silver nanoparticles.

Isolation of lactic acid bacteria: Lactic acid bacteria were isolated from traditionally fermented foods such as *ting* (sorghum mash), *mokuru* (traditional sorghum brew), *khadi* (fermented berries brew), *madila* (fermented sour milk) and *mageu* (fermented mealie meal) obtained from local vendors. Primary isolation of LAB was performed by serial dilutions on MRS (De Man, Rogosa, Sharpe) agar (MERCK, Darmstadt, Germany) supplemented with bromocresol purple and incubated at 37ºC for 24-48 hours. Typical LAB were observed for growth cultural characteristics on MRS agar and were also observed microscopically. Tentative identification of the LAB was achieved by procedures described in Bergey's Manual of Systematic Bacteriology.[9] Only Gram-postive and catalase-negative rods and cocci were then subsequently screened for the synthesis of silver nanoparticles.

Primary screening of LAB for silver nanoparticle synthesis: To test whether the isolated cultures sythesized silver nanoparticles, the method described by[10] was used. Briefly, 20 mL of the supernatant was mixed with silver nitrate ($AgNO_3$) (MERCK, Darmstadt, Germany) solution (10 mM) and another reaction mixture without this solution was used as a control. The prepared solutions were incubated at 37°C for 24 h. All solutions were kept in the dark to avoid any photochemical reactions during the experiment. After 24 hrs the solution turned dark brown from a golden brown coloured solution showing silver was reduced. The silver nanoparticles (AgNPs) were collected by centrifugation at 10,000 rpm for 15 min and washed twice with distilled water, then stored at -20^{o}C for further characterization. Out of 196 isolates, only 5 LAB showed good biosynthetic potential of silver nanoparticles (K1.16, MM17, MAN TNT, T49 and S8). They were thus the ones that were used in the steps that follow below.

Identification of the LAB isolates: The five isolates that synthesized silver nanoparticles were furthermore identified using 16S rRNA sequencing. First, DNA extraction from pure cultures was performed using a Zymo Fungal/ Bacterial DNA miniprep kit (ZYMO RESEARCH, Irvine, CA, USA), followed by amplification of the 16S rRNA gene using universal primers (S-D-BACT-1494-A-S 20 and L-D-BACT-0035-A-A-15),, in a Techne thermocycler (COLE-PARMER, Staffordshire, UK). PCR amplification was performed in a 25 µL reaction mix containing 12.5 µL of 2X Master Mix (NEW ENGLAND BIOLABS, Ipswich, MA, USA), 20 ng total DNA, 0.5 µM of each of reverse and forward primers and the mixture was made up to 25µl with sterile nuclease-free water. Amplification

conditions consisted of a denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min. A final extension step consisting of 7 min at 72 °C was included. The PCR amplification fragments were resolved on 1.2 % agarose gel (Sigma Aldrich, Missouri, USA) at 75 V for 1 h 30 min. The gels were visualized on a gel documentation system (BIO-RAD, Carlifornia, USA). The PCR products were purified and sequenced in both directions using an automated ABI 3500XL sequencer (APPLIED BIOSYSTEMS, Waltham, Massachusetts, USA). The sequence data of 16S rDNA was further aligned using BioEdit program and sequence similarity searches were done using the BLAST program that is available from the National Centre for Biotechnology Information (NCBI). A phylogenetic tree was then constructed by using the Neighbor-Joining method, which produced a unique final tree under the principle of minimum evolution using the MEGA 6 program.[11]

Characterization of silver nanoparticles

Nanoparticle synthesizing bacterial isolates were confirmed by a dark brown coloration in the broth medium showing that efficient reduction of silver ions. For this study the absorbance peak of the synthesized nanoparticles was first determined by optical density (OD) readings which were performed using ultraviolet-visible spectroscopy (UV-VIS) (SHIMADZU, Australasia, Sydney). The absorbance was measured in the range 300-800 nm, which includes the Plasmon absorbance peak of the silver nanoparticles centred at ~400 nm.[12] This method provides a mechanism to monitor how the synthesized nanoparticles change over time in order to determine if any nanoparticle aggregation has taken place.

X-ray diffraction (XRD) studies on thin films of the nanoparticle were carried out using a PANalytical brand θ–2θ configuration (generator-detector) X-ray tube copper λ = 1.54 Å and EMPYREAN diffractometer.[13] A high resolution Carl Zeiss Gemini SEM500 Transmission Electron Microscope (ZEISS, Stockholm, Sweden) capable of operating both in transmission and scanning modes, was employed for assessment of the shape and size of the obtained AgNPs. Elemental composition was assessed through the use of an energy dispersive spectrometer integrated into the instrument.

Assays of minimum inhibitory concentration of silver nanoparticles against pathogenic bacteria

For the minimum inhibitory concentrations (MICs) tests, 96 well micro titre plates were used. To each well 100 µL of sterile Müeller-Hinton broth (MERCK, Darmstadt, Germany) was added. A volume of 100 µL of the AgNP suspensions (stock concentrations of 10 mg/ mL) was pipetted into the first row of the plate; row A well 1-8. The AgNPs were run in duplicates. Serial dilutions were performed using a multichannel pipette and 100 µL from the last well was discarded such that each well contained 100µL of the AgNP suspension in serially descending concentrations. To each well 10 µL of resazurin (MERCK, Darmstadt, Germany) indicator solution (0.03%) was added. Finally 100 µL of the standardized bacterial suspension was added to each well and the plates were incubated at 37C for 18-24 hours. The test organisms were; *Escherichia coli*; *Staphylococcus aureus*; *Klebsiella pneumoniae*; *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium); *Bacillus cereus* and *Enterobacter* spp. Colour change was then assessed visually and the lowest concentration showing inhibition of growth was be considered the MIC of the organism in each set of experiment.

RESULTS

Biosynthesis of silver nanoparticles by lactic acid bacteria: Silver nanoparticles were successfully synthesized using biogenic reduction of 10 mM silver nitrate by Lactic Acid Bacteria. A total of 5 LAB isolates amongst those that were tested were positive for the ability to accumulate and subsequently reduce Ag+ from $AgNO_3$ solution, based on visual inspection of colour change of the challenged solution from light brown to dark brown after a 24 hr incubation period. The colour of the solution further darkened in 72hrs. However, the bacterial strain treated with deionized water retained its original colour.

Identification of the LAB Strains

To identify the nanoparticle synthesizing LAB at molecular level, the 16S rRNA gene region was amplified. Genomic DNA was first extracted from the isolates followed by PCR amplification of 16S rRNA gene region. Further sequencing of the PCR amplified 16S rRNA product was conducted. The sequences of the 16S rRNA gene was aligned against other recorded sequences in the Gen Bank and closely related sequences were selected followed by their analysis using molecular evolutionary computing software MEGA6. The phylogenetic tree constructed indicated that the isolates; K1.16, MM17, MAN TNT, T49 and S8 gave a 99% homology to sequences of *Lactobacillus herbarum* strain TCF032 E4, *Lactobacillus paraplantarum* strain DSM 10667, *Lactobacillus pentosus strain* 124 2, *Enterococcus thailandicus* strain NBRC 101867 and *Enterococcus hirae* strain LMG 6399 respectively (Figure 1).

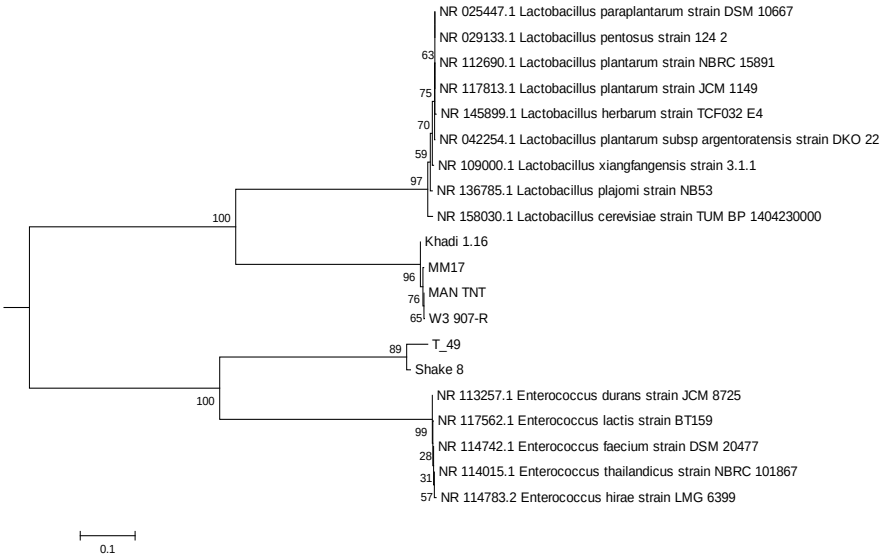

Figure 1: The phylogenetic analysis of the 16S rDNA sequence of the bacterial isolates obtained in the study along with other similar selected sequences from NCBI database. The analysis was conducted using Neighbour-Joining method in MEGA6.

UV-VIS spectroscopy: To further confirm AgNPs synthesis UV-visible spectrophotometry was performed. The UV-VIS spectra of the reaction mixture containing $AgNO_3$ and a culture supernatant of the 5 isolates after incubation showed a typical peak at ~ 410 nm that is specific surface plasmon resonance (SPR) of the AgNPs (Figure 2). Based on the UV-Vis spectra of these isolates, the best producers of the AgNPS were K1.16, and MM17 which were identified as *Lactobacillus herbarum* strain TCF032 E4 and *Lactobacillus paraplantarum* strain DSM 10667. The AgNPs synthesized by these isolates were further characterised using Energy dispersive X-ray, X-ray diffraction and Scanning Transmission Electron Microscopy.

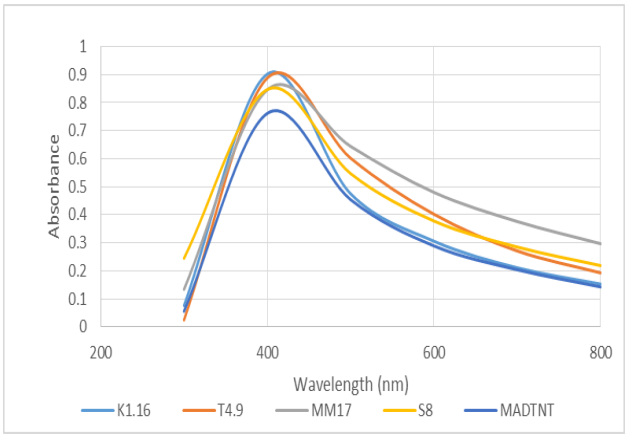

Figure. 2: UV-VIS absorbance maxima of AgNPs after 24h of incubation.

Energy dispersive X-ray Analysis: Further analysis using Energy Dispersive X-ray Analysis (EDX) was carried out which showed that *L. herbarum* (K1.16) and *L. paraplantarum* (MM17) were able to reduce silver as indicated by their EDX spectra showing the strongest signal in the silver region(Figure 3(a) and (b)). Both spectra showed abundance of the silver element which generally has a typical optical absorption peak at approximately 3 keV and 2keV respectively. This confirmed the formation of AgNPs. Other peaks of note from both spectra observed were for oxygen, magnesium and silicon.

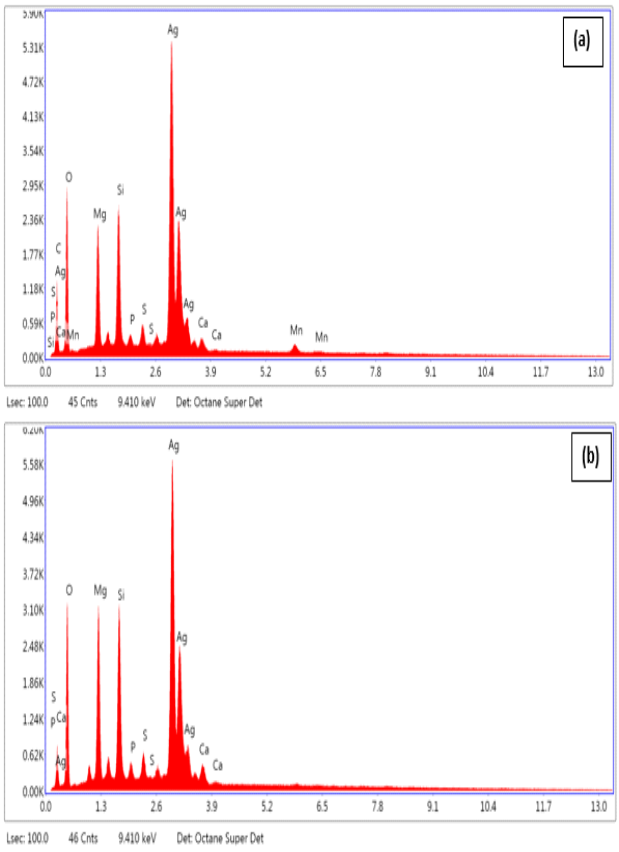

Figure. 3: EDX spectra of AgNPs obtained from (a) *Lactobacillus herbarum* (K1.16). (b) *Lactobacillus paraplantarum* (MM17).

X-ray diffraction analysis: Figure 4(a) and (b) shows the XRD pattern of AgNPs obtained from *Lactobacillus herbarum* (K1.16) and *Lactobacillus paraplantarum* (MM17). There were 4 intense diffraction peaks observed at 38.1°, 44.2°, 64.4° and 77.4° in the 2θ range as shown in Figure 4(a) and (b) which match a typical diffractogram pattern of silver nanocrystals where peaks are usually observed at 2θ= 38°, 45°, 64° and 77°.

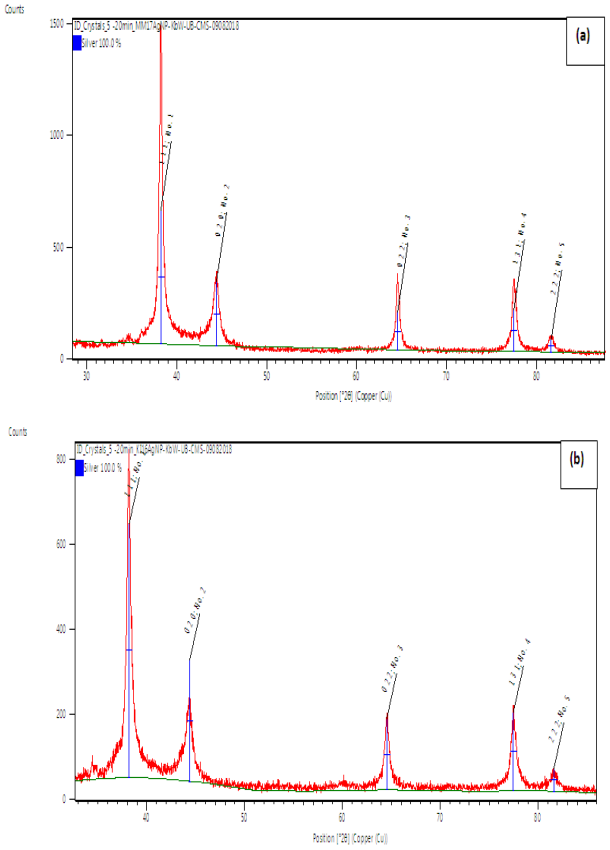

Figure. 4: XRD pattern for AgNPs obtained from (a) *Lactobacillus paraplantarum* (MM17) and (b) from *Lactobacillus herbarum* (K1.16).

Scanning Transmission electron microscopy

Figure 5 below shows the micrographs obtained following analysis of AgNPs obtained from *L. herbarum* (K1.16) and *L. paraplantarum* (MM17). The obtained AgNPs had sizes ranging from 11 – 98 nm (Figure 5(a)) and 13– 35 nm (Figure 5(b)). Both *L. paraplantarum* (MM17) and *L. herbarum* (K1.16) produced AgNPs that were spherical in nature. A larger particle size range was observed when using *Lactobacillus herbarum* (K1.16) as compared to *Lactobacillus paraplantarum* (MM17).

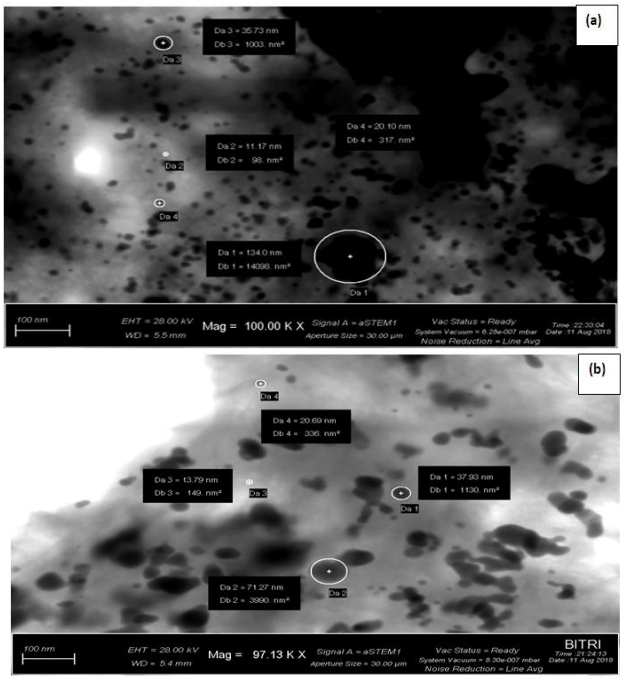

Figure 5: STEM images of synthesized AgNPs obtained from (a) *Lactobacillus herbarum* (K1.16) and (b) *Lactobacillus paraplantarum* (MM17).

Antimicrobial activity of silver nanoparticles (Minimum inhibitory concentrations)

The antimicrobial activity of the silver nanoparticles was studied against Gram-positive and Gram- negative pathogenic bacteria followed by determination of their Minimum Inhibitory Concentrations (MICs) (Table 1). From the results silver nanoparticles possess very potent antibacterial properties with MICs ranging from 0.0175 mg/mL to 2.5 mg/mL. Silver nanoparticles produced by *Lactobacillus herbarum* (K1.16) were the most potent antibacterial agents as indicated by the lowest MICs across all bacterial species. In contrast those synthesized to *Enterococcus hirae* (S8) which was least effective. Generally bactericidal action of AgNPs increases as the particle size decreases. *S. aureus* showed the most resistance to AgNPs produced across all isolates as indicated by much higher MICs while *P. aeruginosa* on the other hand was the most susceptible.

Table. 1: The minimum inhibitory concentrations of the silver nanoparticles on selected pathogenic bacteria.

	Minimum Inhibitory Concentrations of the AgNPs (mg/ mL)				
Test organism	**Khadi1.16**	**MM17**	**MAN TNT**	**T49**	**Shake8**
S.aureus	0.625	1.25	1.25	2.5	2.5
B. cereus	0.075	0.155	0.075	0.3125	2.5
K. pneumoniae	0.075	0.3125	0.3125	0.625	2.5
P.aeruginosa	0.0175	0.037	0.037	0.0175	0.037
Salmonella enterica	0.075	0.155	0.0175	0.075	1.25
Enterobacter spp	0.037	0.155	0.3125	0.3125	2.5
E.coli	0.0175	0.155	0.3125	0.155	0.3125

DISCUSSION

In the current study, lactic acid bacteria isolated from tradionally fermented food products were screened for their ability to synthesize silver nanoparticles. Among the bacterial strains that were tested, 5 isolates; K1.16, MM17, MAN TNT, T49 and S8 were chosen as the most efficient in mediating AgNP synthesis from $AgNO_3$ based on colour changes observed during and after incubation of the reaction mixture. The colour change is due to excitation of surface plasmon vibrations in the AgNPs,[14] similar observations of colour changes were also reported in their study. The LAB isolates that were selected for this study were identified by molecular techniques and were from *Lactobacillus* and *Enterococcus* genus. Fermented food products have been reported to be a good source of LAB with *Lactobacillus* being the predominant species.[15][16][17]

Figure 2 shows the UV-VIS spectra of AgNPs synthesized by the selected isolates with a typical peak at ~ 410 nm which is within the region of specific surface plasmon resonance (SPR) of the AgNPs. According to[18] the region between 400 – 450 nm has been vastly shown to be the characteristic region at which silver plasmons resonate, giving strong absorbance peaks in that region. Similar studies have reported LAB isolates to mediate the biosynthesis of silver nanoparticles from silver nitrate and exhibit absorbance in the range of 400-450 nm in UV- VIS spectra.[10][19]

Further characterization of the AgNPs was carried by EDX, XRD and STEM in order to select LAB isolates that were most efficient producers of AgNPs. The results of EDX presented in Figure 3(a) and (b) as well XRD (Figure 4a & b) were very useful in identifying *L. herbarum* (K1.16) and *L. paraplantarum* (MM17) as very efficient bio-reducers of $AgNO_3$ to AgNPs. EDX analysis demonstrated that there was abundant silver metal produced by these isolates. Silver nanopartilces typically show an optical absorption peak approximately at 3KeV due to surface plasmon resonance.[20] Silver (Ag) was the major constituent element compared to other elements (oxygen, magnesium and silicon) which were found in the tested supernatant. Other elements may have come about as a result of the reduction process of silver, resulting in some the components being left adsorbed onto the surface of the AgNPs. The diffraction peaks revealed by XRD also correspond with the face-centred cubic crystal structure of metallic silver which confirms the synthesis of AgNPs by *L. herbarum* (K1.16) and *L. paraplantarum* (MM17). Similar diffractogram patterns were reported using *Lactobacillus fermentum*.[10]

Evaluation of the size and shape of the AgNPs using STEM analysis showed that both the *L. herbarum* (K1.16) and *L. paraplantarum* (MM17) were spherical in nature but of different sizes. It was observed that *L. herbarum* (K1.16) produced slightly smaller AgNPs than *L. paraplantarum* (MM17). Thus, the ability to reduce the silver ions was strain-specific. The size ranges of AgNPs produced by both isolates falls closer to the size of silver nanoparticles produced by other LAB. The AgNPs produced by *L. fermentum* as reported by[10] had a diameter of 11.2 nm which is close to those of *L. herbarum* (K1.16) reported in this study of MM17 (13-71 nm). However *L. fermentum* had the much narrow range between 10.3 and 12.1 nm.

Silver nanoparticles produced by *L. herbarum* (K1.16) were the most potent antibacterial agents as indicated by the lowest MICs across all bacterial species. In contrast those synthesized by *E. hirae* (S8) were not very effective as they were required in much higher concentration than the others to inhibit bacterial growth. Generally bactericidal action of AgNPs increases as the particle size decreases therefore differences in diameter and shapes of the NPs produced by the different LAB species might explain any differences in MICs.[21] *S. aureus* showed the most resistance to AgNPs as only higher concentration were able to inhibit its growth on both agar and microtitre plates while *P.aeruginosa* on the other hand was the most susceptible. The antimicrobial potential of AgNPs is not only influenced by their size and shape but is also influenced by the thickness and composition of the cell wall of the microorganisms.[22] From the results, it can be therefore be concluded that there is a correlation between the concentration (effective dose) of the AgNPs and the class of the bacteria treated owing to differences in the cell wall structure, thickness and composition.

CONCLUSION

The present study demonstrates an eco-friendly method of extracellular synthesis of AgNP through different LAB isolates. Biosynthesized AgNPs from this study have a potential to be used as bio disinfectants for controlling different pathogenic microorganisms from different surfaces. Further studies are required to develop topical ointments incorporated with AgNPs to aid against multi-drug resistant pathogenic microorganisms. Silver nanoparticles from this study can also be used as a coating agent for surgical devices, instruments and wound healing bandages.

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