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Research Article
Microbial Synthesis and Characterization of Silver Nanoparticles by *Lactococcus lactis* TNM-B1 and its Antimicrobial Properties

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Abstract

Background and Objectives: The use of bio-nanomaterial as a result of environmental remediation procedure, reliability and eco-friendliness has recorded an outstanding development in nanotechnology science. This work studied the biosynthesis of silver nanoparticles (AgNPs) by *Lactococcus lactis* TNM-B1from fermented tigernut milk and its antibacterial properties against clinical isolates. **Materials and Methods:** The AgNPs were characterized by visual observation such as; UV-visible spectroscopy (absorption peak between 400-450 nm for the cell free extract and silver nitrate ratios (1:1, 1:4, 1:9, 3:7) at different concentrations of metal ions (1, 2 and 3 mM) and control, without the metal ions), Fourier Transform Infra-Red Spectroscopy (FTIR) and X-ray Diffraction (XRD). **Results:** The AgNPs characterized by visual observation revealed the ability of the microbial system to form extracellularly silver nanoparticles at 35°C, 160 rpm and 72 h. The UV-visible spectroscopy showed peak at 400 nm which confirmed the presence of nanoparticles of cell-free extract and silver nitrate. The FTIR spectroscopy ascertained the presence of protein functional group as a stabilizing agent and XDR spectra revealed several peaks over the spectrum of 2θ values of 32.1, 46.2, 57.6 and 78.0° corresponding to the 111, 200, 220 and 311 planes as nanocrystals, respectively and AgNPs structural elucidation from high-resolution scanning electron microscopy appeared as convex light core, mono-dispersed with average 15.26 mm width diameter. **Conclusion:** This study provided an insight into the potential use of nanoparticles as an alternative choice drug against antibiotic-resistant strains. It showed significant antimicrobial activity against Gram-negative (*Klebsiella pneumonia*, *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeroginosa*) except *Proteus mirabilis* compared with ciprofloxacin having observable zones of inhibition.

Key words: Synthesis, silver nanoparticle, *Lactococcus lactis* TNM-B1, characterization, antimicrobial properties

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

For over three decades now, nanotechnology especially the nanoscience of silver metal using the microbial system has attested scientific transmutation simply because of the joint action of both physical and chemical processes such as; size, distribution and morphology. Their synergetic activity, optical, electronic, magnetic and antimicrobial characteristics have also been widely investigated[1-5]. Microbial synthetic came into being as a substitute for the conventional chemical and physical mechanism which has constituted a major clampdown in term of cost efficiency despite the advantages of attaining appreciable nanoparticle sizes. There has been great compatibility of the biological approach of nanoparticle synthesis with green chemistry concept. The former offers greater stability and appropriate dimensions as they synthesized by using a one-step procedure than the latter[6-8]. These have prompted its potential application as exceptional absorbers for the production of nano-sized materials. However, the application of microbial systems by bioreduction of the metal ions to nanomaterials tends to quell some of these challenges posed by the conventional mechanism. More interestingly, the advent of Surface Plasmon Resonance (SPR) with increased peak intensity has authenticated nano-biomolecule synthesis as a result of the electronic transition within metallic nanoparticle structure. Zhang *et al.*[9] exemplified the bio-nanoparticle technology by either intracellular or extracellular procedures. Intracellular involves grabbing of metallic target ions from the environment and transforming into metal element through enzymes generated within the cell, whereas interaction involving extracellular trapping of metal ions on the cell surface and ions reduction in the presence of enzymes account for the extracellular means. Moreover, the extracellular biosynthesis entails simpler downstream process and little technicality which favors the quest for potential applications for large-scale production of silver nanoparticles.

The applications of micro-organisms and its microbial enzymes, polysaccharides, biodegradable polymers and biological systems have been reported to be reliable, nontoxic, clean eco-friendly and green experimental protocols for synthesis of nanoparticles by Akinsiku *et al.*[4], Korbekandi *et al.*[10-12], Iravani and Zolfaghari[13], Korbekandi and Iravani[14], Iravani *et al.*[15], Abo-State and Partila[16] and Aljabali *et al.*[17]. This bioscience and its applications have been appropriate in drug delivery, cancer treatment, gene therapy and DNA analysis. Others include antibacterial agents, biosensors, enhancing reaction rates, separation science, luminescence tagging, labeling and Magnetic Resonance Imaging (MRI) and its potential in polymer industry[18-20]. Due to the compatible *in vivo* screening, the conjugated bimetallic nanoparticle has been reportedly used by Vaseem *et al.*[21] to synthesize glucose-capped nickel nanoparticles (GNiNPs) via aqueous solution method in which glucose bi-functioned as capping and reducing agent. The synthesis of nanostructures has also been extended to nanowires and assembly of nanoparticles by using biological templates such as; DNA, proteins, viruses and S-layers[22].

Among microbes, bacteria have gained wider view as the best candidate for nanoparticle production because of their ability to resort to specific defense mechanisms to quell stress like toxicity of heavy metal ions or metals and could grow and survive even at high metal ion concentrations as in *Pseudomonas aeruginosa* and *P. stutzeri*[22,23]. The aim of this research work was to screen potential micro-organisms for the biological synthesis and characterizations of silver nanoparticles and evaluate the antimicrobial properties of the nanoparticles on clinical isolates.

MATERIALS AND METHODS

The experimental study was carried out in the Microbiology Laboratory of the Department of Biological Sciences, College of Natural and Applied Sciences, McPherson University, Seriki Sotayo, Obafemi Owode Local Government Area of Ogun state, Nigeria, between October, 2017-June, 2018.

Chemicals/media/reagents: Silver nitrate was purchased from Sigma Aldrich (St. Louis, USA), Mueller-Hinton agar (Lab M. Heywood, the UK), Nutrient agar (Biolab Budapest, Hungary), Nutrient broth (Biomark laboratories, India) were also purchased. All chemicals and reagents used were of analytical grade (ANALAR).

Test micro-organisms: Bacterial isolates from fermented tigernut milk were screened for their potential to synthesize nanoparticles. Freshly cultured clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *S. saprophyticus*, *Salmonella typhi* and *Proteus mirabilis* used to investigate the antimicrobial properties of the synthesized silver nanoparticles (AgNPs) were obtained from the Department of Medical Microbiology and Parasitology, Sacred Heart Hospital, Lantoro, Abeokuta, Ogun state, Nigeria.

Bacterial cultures for screening process: Bacteria cells from fermented tigernut milk were subjected to the screening programme. Potential pure cultures were maintained on nutrient agar in a MacConkey bottle slant at 27°C as well as sub-cultured bimonthly to regulate its viability during the period of study.

Molecular identification: The extraction of total 16S rRNA and DNA, Polymerase Chain Reaction (PCR) of the extract and DNA sequencing were carried out at the Bioscience Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. The DNA was amplified by using standard PCR in order to determine the phylogenetic grouping of the genomic DNA sample. The 16S rRNA and DNA sequence were submitted to the National Center for Biotechnology Information (NCBI) database and the sequence was compared with available 16S rRNA sequence by using an automatic alignment tool (Blast). The construction of the phylogenetic tree was generated by Phy ML and the visualization of the tree by Tree Dyn using the online program (www.phylogeny.fr). The MEGA program was used for drawing the tree.

Screening of cultures for silver accumulation and nanoparticle synthesis: Cultures were grown up aerobically in test tubes containing 10 mL nutrient broth and incubated[24] at 30°C at 160 rpm for 72 h. The biomass was separated using Whatman filter paper and washed thrice with distilled water to remove any nutrient that might interact with the silver ions. The biomass was re-suspended in 10 mL distilled water and pH adjusted with NaOH to 6. The cell-free extract and silver nitrate (1:1, 1:4, 1:9 and 3:7) was added separately to the reaction vessel with different concentrations of silver nitrate (1, 2 and 3 mM) and control (without the silver nitrate) and ran along with the experimental conditions. The reaction between these supernatants and Ag^+ ions were carried out in dark conditions.

Characterization of silver nanoparticles: The reduction of Ag^+ ions in the sample was checked and preliminary detection of silver nanoparticles carried out by the visual color change of the filtrate. The optical properties of the synthesized silver nanoparticles were analyzed using UV-visible spectroscopy. About 2 mL of the sample was withdrawn at different time intervals to record the pH and the absorbance. For this, the nanoparticle containing samples were subjected to absorption analysis at 300-600 nm range using UV-vis spectrophotometer.

Fourier Transform Infrared (FTIR) spectroscopy was employed to analyze the various functional groups present in the AgNPs powders using TENSOR 27 series FTIR spectrometer, Germany. The KBr pellet technique was used in which a ratio of 1:99% of the synthesized AgNPs powder and KBr were mixed in a mortar and pestle and then compressed to form a pellet of 2 mm diameter. All FTIR spectra data were obtained in the range of 400-4000 cm^{-1} with 64 times scanning and a resolution of 4 cm^{-1}. The phase and purity of the fabricated AgNPs powders were determined by X-ray Diffraction (XRD) using X-pert PRO, PANalytical, Netherlands diffractometer-90° (wavelength = CuKα1). The diffraction patterns were collected over a range of $2\theta = 10$ an incremental step size of 0.02. The various peaks evolved were established by standard Joint Committee of Powder Diffraction Society (JCPDS). The morphology and particle size of AgNPs were obtained by Scanning Electron Microscopy (SEM) (VERGAJ TESCAN).

Antimicrobial susceptibility assays using agar well diffusion method: The antimicrobial potency of the AgNPs was determined for its antibacterial properties against the clinical isolates such as; *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *S. saprophyticus*, *Klebsiella pneumonia*, *Proteus mirabilis* and *Salmonella typhi* using the agar well diffusion technique[25]. For the antibacterial test, the pathogenic organisms were grown up in test tubes containing 10 mL nutrient broth for 24 h. The bacteria cell suspensions (200 µL) were evenly spread on molten Mueller-Hinton agar plates (40°C). A well of 3 mm diameter using sterilized cork-borer was formed on the agar plate and 40 µL of the suspended AgNPs was added into wells cut out in the center of the plate and incubated at 37°C for 24 h. Ciprofloxacin (50 mg mL^{-1}) was used in the same volume as the positive control and silver nitrate as a negative control. After incubation, the zones of inhibition were measured. The assays were conducted in triplicate for each organism.

RESULTS

Screening and identification of bacterial isolates: Bacterial isolate screened from fermented tigernut milk was found to have the potential to form silver nanoparticles as observed by a change in the color of the reaction. The selected isolate was characterized by 16S rDNA sequencing-based method. The 16S rDNA sequence of the isolate was submitted to NCBI with accession number MH712043 and identified as *Lactococcus lactis* TNM-B1.

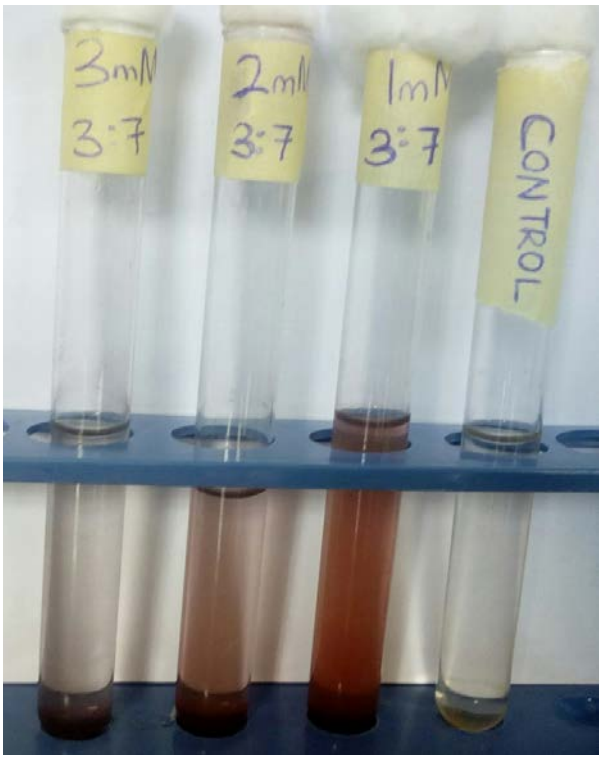

Fig. 1: Bioreduction process of silver dispersion at various concentrations with control (silver nitrate) remains colourless

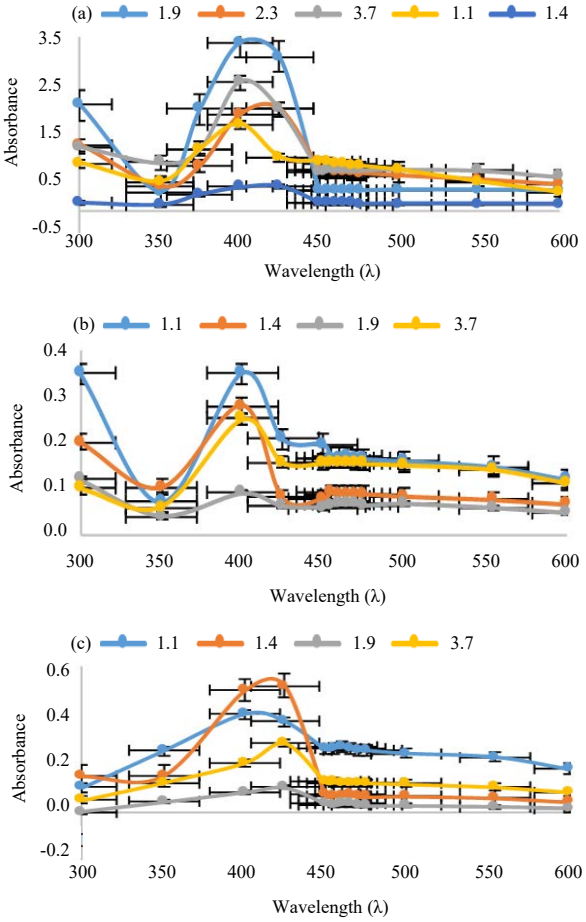

Fig. 2(a-c): UV-vis spectra analysis of silver nanoparticles at various concentrations of $AgNO_3$, (a) 1 mM, (b) 2 mM and (c) 3 mM at different ratio of cell supernatant and metal ions
Error bars with standard error

Synthesis and characterization of silver nanoparticles: The isolate was cultivated on nutrient broth medium for 24 h. Subsequently, the cleaned, cleared and cell-free supernatants were used as catalysts (reducing agent) for AgNPs synthesis.

Silver nitrate vulnerability to the cell-free supernatants of *Lactococcus lactis* TNM-B1 developed in a time-dependent color change of the reaction mixture from colorless to brown (Fig. 1), stipulating AgNPs biosynthesis. However, the production of AgNPs was looked into overtime by UV-vis absorption spectrum scanning in the range of 300-600 nm. As demonstrated in the UV-vis spectra, the absorbance intensity gradually increased with time without any wavelength shift in which the maximum absorbance was obtained, indicating a continuous reduction of silver nitrate and consequently, an increase in AgNPs concentration. Silver nanoparticles are known to have an intense absorption peak in UV absorption spectra due to its surface plasmon excitation. For the culture supernatant of *Lactococcus lactis* TNM-B1, an absorption peak was obtained between 400-425 nm for the cell-free extract and metal ions (at ratio 1:1, 1:4, 1:9 and 3:7) by using different concentrations of metal ions (1, 2 and 3 mM) which confirmed the presence of nanoparticles (Fig. 2a-c). Ratio 1:9 (1 mM concentration) of the resultant culture was noted to have the highest absorbance at 400 nm. Whole, ratio 1:1 and 1:4 were noted to have highest absorbance at 2 and 3 mM concentrations, respectively. The color change and UV absorption data analysis thus, confirms the reduction of silver nitrate to silver nanoparticles by the culture supernatant *Lactococcus lactis* TNM-B1. FT-IR spectrum was used to recognize the presence of the various functional groups present in the biomolecules that could contribute to Ag^+ ions reduction. Figure 3 showed the FT-IR spectrum concur with the of the AgNPs. The bands appearing between 3709.54-3453.03 cm characteristic of stretching modes for the O-H bonds, while those obtained at 1714.48-1844.14 cm^{-1} were allocated to C=O functional group. The band at 1644.73 cm^{-1} was ascribed to the C=C bond of the biomolecule, while those at 1362.85 and 656.13 cm^{-1}

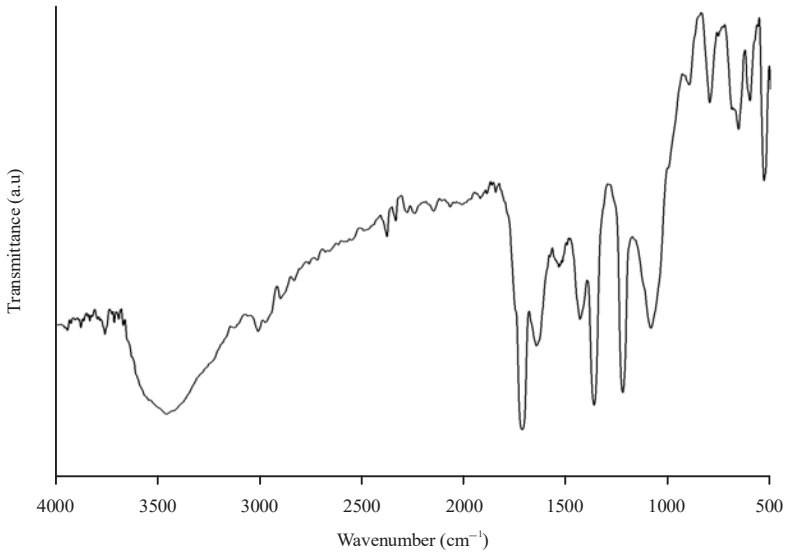

Fig. 3: FTIR spectrum of synthesized $AgNO_3$ from *Lactococcus lactis* TNB B1

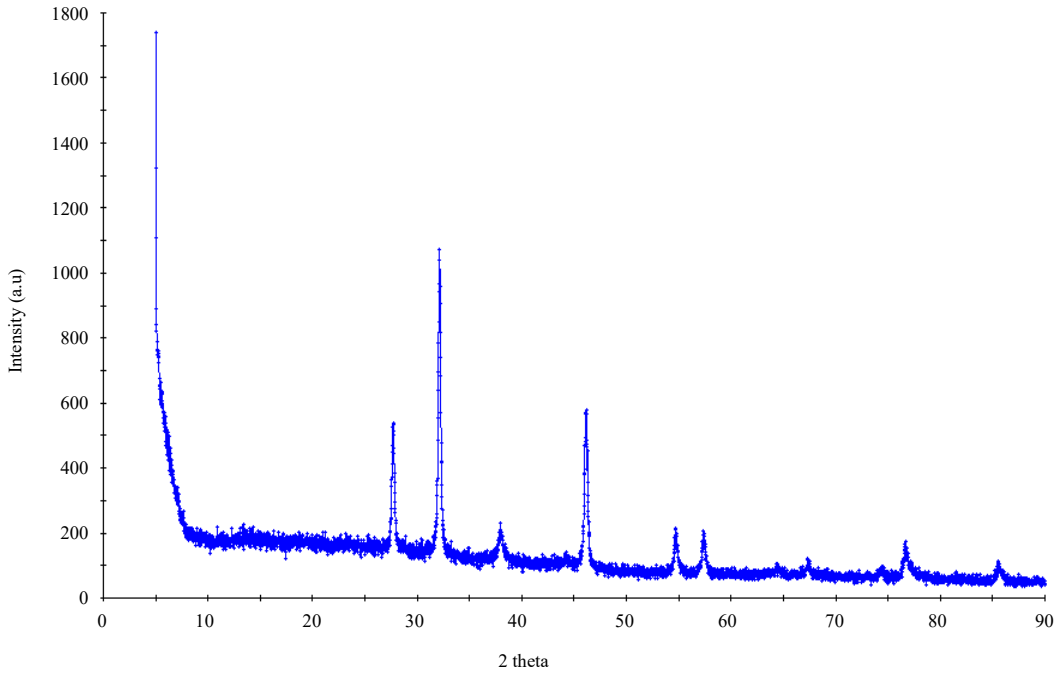

Fig. 4: XRD spectrum of synthesized $AgNO_3$ from *Lactococcus lactis* TNB B1

represented the C-H bonds of alkanes and alkynes functional groups, respectively. Meanwhile, the functional group of aliphatic Ag^+ to Ag^o when the band attained 1085.52 cm^{-1} with C-amines also involved in the reduction of AgN stretching vibrations of the aromatic and aliphatic amines. The XRD spectrum for the synthesized AgNPs was shown in Fig. 4. The spectra unveiled several SEM peaks over the entire spectrum of 2θ values, ranging from 10-90° images of the silver nanoparticles sample at different magnifications (Fig. 5a-b). The images showed that most of the silver nanoparticles were predominate as a convex light core with a compact arrangement and 15.23 and 15.28 mm width diameters, respectively using advanced software named 'VEGAJ TESCAN'.

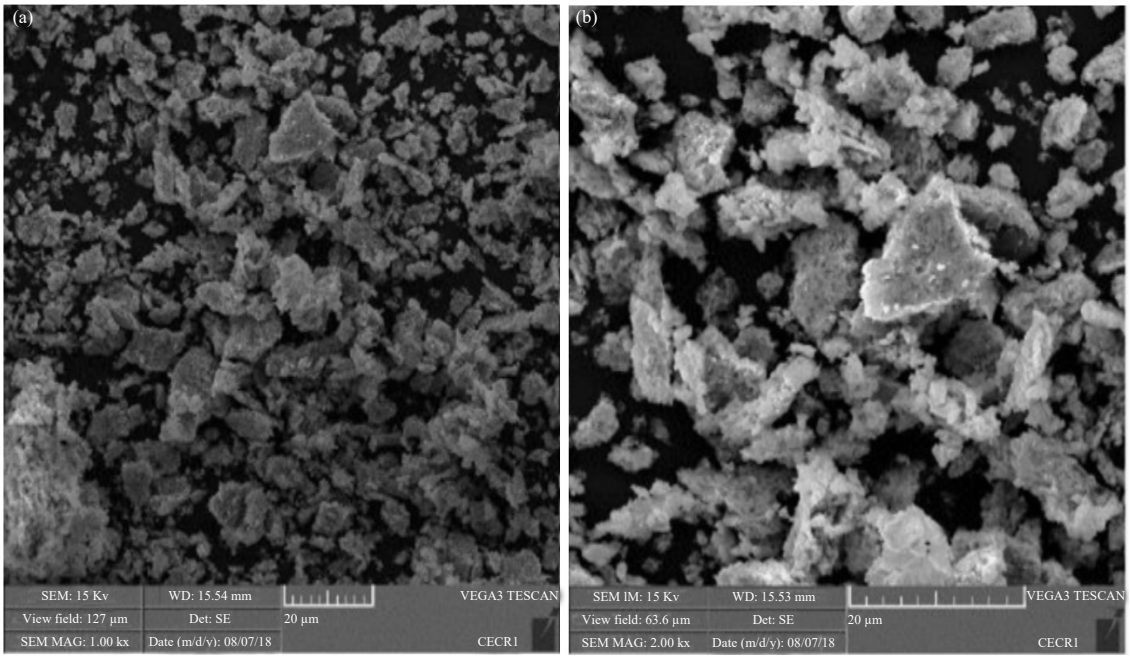

Fig. 5(a-b): Scanning Electron Microscopy (SEM) images of synthesized $AgNO_3$ by using *Lactococuus lactis* TNB B1

Table 1: Zone of Inhibition (ZI) of the ciprofloxacin and nanoparticles on the clinical isolates

	Zone of inhibition (mm)	
Bacterial isolates	Antibiotics	Nanoparticles
Klebsiella pneumonia	3.9	2.5
Salmonella typhi	2.8	2.4
Escherichia coli	4.0	3.7
Staphylococcus saprophyticus	2.6	2.5
Staphylococcus aureus	2.6	Nil
Pseudomonas aeriginosa	2.4	2.2
Proteus mirabilis	2.8	Nil

Antimicrobial susceptibility assays: Quite a large number of synthetic metallic compounds are known to exert antimicrobial properties and are used as a bactericidal agent, however, silver compounds have proven as a potent antibacterial agent. Metallic nanoparticle shows effective antimicrobial potential against Gram-positive and Gram-negative bacteria. In present study, the biosynthesized AgNPs showed significant antimicrobial potency against most of the Gram-negative clinical isolates (*Salmonella typhi*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*) except *Proteus mirabilis*. Among the two Gram-positive bacterial clinical isolates tested, *Staphylococcus saprophyticus* showed an observable clear zone of inhibition, while *Staphylococcus aureus* revealed no significant effect. The zones of inhibition of each isolate were presented in Table 1 and *E. coli* had the highest zone of inhibition 3.7 mm. Negative control wells containing silver nitrate derived from the bacterial cultures showed no zone of inhibition, while positive control wells containing antibiotics ciprofloxacin showed observable zones of inhibition on all the plate. It has been discovered from nature that the development of biomimetic have brought about the advancement of nanomaterials.

DISCUSSION

In this study, AgNPs was biosynthesized using a cell-free extract of *Lactococcus lactis* TNM-B1. The incorporation of silver nitrate to the cell filtrate bring about color change from colorless to brown indication a fundamental formation of AgNPs. However, the microbial screening procedure often resulted in a color change for silver nanoparticle synthesis. This has been ascribed to the excitation of surface plasmon vibration in the silver nanoparticles as the basis for the formation of brown color[26]. Saravanan *et al.*[27] explored the cell supernatant of *Bacillus megaterium* (NCIM 2326) for the biosynthesis of silver nanoparticles when a pale yellow color changed to brown due to the reduction of silver ions to silver nanoparticles. This agreed to the fact that a change in color as obtained in the experiment can be considered as an indication of silver nanoparticles formation. UV-vis spectroscopy which evaluated the absorption spectra of silver nanoparticles formed further confirmed the collective excitation of the

conductivity of metal electrons. Thus, methods based on UV-vis spectroscopy have been shown to be an effective technique for the analysis of nanoparticles[28]. Selected isolates revealed an absorption band between 400-425 nm when UV-vis spectra of the synthesized silver nanoparticles was investigated. The existence of such peak, appropriated to a surface plasmon, was also well reported for silver nanoparticles as in the case of *Neurospora crassa*[29]. The technique behind the extracellular production of nanoparticles using micro-organisms is not entirely recognized within the scope of knowledge. It has been showed that nitrate reductase released by microbes assist in the bioreduction of aqueous metal ions to metal nanoparticles[30]. This was investigated in *Bacillus licheniformis* where nitrate reductase secreted by the bacteria was found to be responsible for the reduction of silver ions to nanoparticles[28]. The FTIR spectra apted to recognize the presence of the various functional groups in the biomolecules which could potentially contribute to the reduction of Ag^+ ions. The XRD spectra showed various peaks over the entire spectrum of 2θ values which ranged from 10-90°. A comparison of our XRD spectra with the standard ascertained that the AgNPs formed in this study were nanocrystals as witnessed by the peaks at 2θ values of 32.1, 46.2, 57.6 and 78.0° corresponding to the 111, 200, 220 and 311 planes for silver, respectively[31]. Biologically synthesized AgNPs are promising therapeutic agents demonstrated significant antimicrobial activity[32,33]. Quite a number of biosynthesized nanoparticles have been described and characterized for their potential to hinder microbial systems. The investigation for novel nanoparticles with well-defined biological and physicochemical properties remains at the leading edge of nano-technological research. The synthesized nanoparticles were evaluated for their antibacterial ability against clinical isolates. Accordingly Singh *et al.*[34] have reported that antimicrobial activity is dose and size-dependent with more pronouncement on Gram-negative than Gram-positive bacteria. Undoubtedly, the Gram-negative cell wall which composed of thin peptidoglycan layer is more receptive to AgNPs permeation as compared with the Gram-positive cell wall that is made up of thicker peptidoglycan layer with an effective barrier against the AgNPs penetration[35]. Marambio-Jones and Hoek[36] reported that silver ions of AgNPs attached to the cell surface (negatively charged) change the physical and chemical properties of cell membranes and impede functions such as; permeability, respiration, electron transport and osmoregulation. Rajeshkumar and Malarkodi[37] also substantiated that the silver nanoparticles may directly interact with the microbial cells.

CONCLUSION

This article provides insights into the production of AgNPs through reductive reactions that are influenced by bacterial origin. The newly isolated strain of *Lactococcus lactis* TNM-B1 produced extracellularly was able to catalyze the synthesis of AgNPs. It also garnered a technical approach to an alternative drug of choice against antibiotic-resistant strains.

SIGNIFICANCE STATEMENT

This study discovered the applications of nanoparticles in contract to conventional antibiotic drugs in biomedical industries that can be beneficial as chemotherapy for bacterial infections to secure human healthy living. This study will help the researcher to uncover the critical areas of pharmaceutical that many researchers were not able to explore. Thus, a new theory on the development of eco-friendly technologies in material synthesis is of considerable importance to expand their biological applications.

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