ID: 162

Inhibitory Activities and Characterization of Greenly Synthesized Silver Nanoparticles using Culture Free Supernatant of *Lactobacillus plantarum*

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Abstract

This study is aimed at the biosynthesis, characterization and inhibitory activity of silver nanoparticles using *L. Plantarum* culture free supernatant (CFS). Silver nanoparticles (SNPs) were biosynthesized by *L. plantarum* and characterized using Visual detection, UV-Visible spectroscopy, Fourier Transformed Infra-red spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). The effect of the cultivation conditions on the biosynthesis of SNPs was carried out. Antibacterial activity of the biosynthesized silver nanoparticles by *L. plantarum* was done using agar well method. Biosynthesis of SNPs by *L. plantarum* was characterized by a strong plasmon resonance peak at 450 nm and a broad band at 350 - 550 nm. There were colour changes from yellow to brown. FTIR confirmed the presence of hydroxyl, aldehyde, carboxylic acid, amino acid, and esters which are responsible for the stability of silver nanoparticles (SNPs). The shape of the biosynthesized nanoparticles was spherical and partially aggregated with particle size ranging from 0.7- 10.0 nm. The antibacterial activity of the synthesized SNPs by *L. plantarum* (LPSNPs) ranged from 14 – 22 mm. *Staphylococcus aureus* and *Escherichia coli* were more susceptible to the synthesized SNPs. 24 hr incubation time, 25^{o}C, pH 9 and 5 mM gave the best SNPs production. The culture free supernatant of *L. plantarum* can be used for the synthesis of SNPs with highly effective antibacterial activities.

Key words: *Biosynthesized Silver nanoparticles, Antibacterial activity, Culture free supernatant, Scanning Electron Microscopy, L. Plantarum*

Introduction

Nanoscale technology is a new and essential domain in science. Nano-scale involves the use of matter on an atomic, molecular, and supra-molecular scale for industrial purposes (Anusha, 2020). Nanoscale technology has major applications in medical health, environmental science, biotechnology and physics (Scribenjarat et al., 2020). Nanoparticles synthesized by a biological approach are more desirable than that synthesized by chemical methods and has several advantages, including easy scaling up of the procedure, cost- effectiveness and its green nature (Hidayat et al., 2020).

Silver is the most used material in all of the nanotechnology; the desirable antimicrobial property activity of silver has drastically increased at the Nanoscale. Silver nanoparticles (AgNPs) are one of the most studied among metallic nanoparticles due to their effective antibacterial agents and possess a strong antimicrobial activity against bacteria, viruses, and fungi (Sreedevi et al., 2015). The reason for these antimicrobial properties is because silver ions and silver based compounds are highly toxic to a wide range of microorganisms including major species of drug resistant bacteria. These nanoparticles of silver have been studied as a medium for antibiotic delivery and synthesis composites for use as disinfecting filters and coating materials. They have extremely large surface area which permits the coordination of a vast number of ligands. Nano-silver has chemical and biological properties that are appealing to the consumer products, food technology, textile/fabrics, and medical industries. Nano silver also has unique optical and physical properties which are considered to have great potential for medical applications (Scribenjarat et al., 2020). Nano silver is an effective bactericidal agent against a broad spectrum of Gram-negative and Gram-positive bacteria which is due to interaction with the DNA of the bacteria cell, thus making them to lose the ability to replicate and finally leading to the death of the cell (Qasim et al., 2018).

Lactic acid bacteria are beneficial heterogeneous group of bacteria that has important roles in medicine, food industries and agricultural sector. Lactic acid bacteria are probiotics that are highly beneficial to the human body aiding in enhancing immune responses, preventing infection by enteropathogenic bacteria (*Escherichia coli*), treating and preventing diarrhoea (Bikila, 2015). However, more than one hundred species of the *Lactobacillus* genus are microorganisms' identified as commercial probiotics. Examples are *L. acidophilus, L. rhamnosus, L. reuteri, L. casei, L. Plantarum*. These microorganisms are well known for their ability to produce lactic acid as the main end-product of their anaerobic metabolism and for synthesizing a wide range of metabolites that beneficially

affect the nutritional, sensorial, and technological properties of fermented food products. Due to their essential beneficial properties, lactic acid bacteria have been extensively used as starter cultures, as probiotics and in the production of interesting compounds like nutraceutical products because of their resourceful metabolism (Ruiz Rodríguez et al., 2017a).

Lactobacillus Plantarum are Gram positive organisms, non-motile, acid-tolerant and non-sporulating facultative anaerobes that has significant properties of reducing blood cholesterol, increase resistance of low density lipoprotein oxidation, leading to decrease of blood pressure and inhibiting the growth of microorganism with the help of the metabolites produced (Dhewa et al., 2014). The resistance of microorganisms to some bactericidal agents and the use of a safe and eco-friendly antimicrobial agent has been a major issue. Green synthesis of nanoparticles is non-toxic, cost effective and eco-friendly in nature which has made it to attract the interest of numerous researchers. Examples are: the green synthesis of silver nanoparticles using supernatant of biosynthesized SNPs by *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 cultured in modified exopolysaccharides selection medium (Popoola and Adebayo-Tayo, 2017). The biosynthesis of silver nanoparticles with potent antimicrobial activity using lactic acid bacteria (Kabo et al., 2019). Also, there are research reports on bacterial synthesis of silver nanoparticles by culture free supernatant of lactic acid bacteria isolated from fermented food samples (Adebayo-Tayo et al., 2017). This research is aimed at the biosynthesis, characterization and antimicrobial activity of silver nanoparticles using the culture free supernatant (CFS) of *L. Plantarum.*

Materials and Methods

Culture Collection

Lactobacillus plantarum (LP) previously isolated from fermented food (locust beans) was collected from the culture collection of the Microbiology Laboratory, Department of Biological Sciences, KolaDaisi University. The culture was kept in a maintenance medium (De Mann Rogosa and Sharpe broth (LabM, UK) with 12% v/v glycerol). The stock culture were stored at 4ºC and sub-cultured regularly for its viability.

Test Organisms

Five test organisms: *Escherichia coli, Klebsiella sp, Staphylococcus aureus, C. albicans* and *Salmonella typhi* were obtained from the Department of Medical Microbiology, University College Hospital (UCH), Ibadan. The isolates were resuscitated on nutrient agar before use.

Biosynthesis of Silver Nanoparticles using the culture free supernatant of L. Plantarum

The culture free supernatant was produced by culturing *L. Plantarum* in sterile MRS broth. The inoculated broth was incubated microaerobically at 35ºC for 24 hrs. After incubation, the inoculated medium was centrifuged at 5000rpm for 20 mins and the supernatant obtained were used for the biosynthesis of SNPs.

1 ml of the culture free supernatant was challenged with 10 ml of freshly prepared 10 mM silver nitrate ($AgNO_3$) in deionized water under stirring conditions. The mixture was incubated at room temperature in a dark place for 24 - 48 hrs. Formation of brown colour indicates the formation SNPs (Popoola and Adebayo-Tayo, 2017).

Characterization of the Silver Nanoparticles

Visual detection and UV-Visible spectrophotometry characterization of the synthesized SNPs

The culture free supernatant treated with silver nitrate solution was observed for colour change in comparison to control as a visual method of detection of silver nanoparticle biosynthesis. Changes in colour from the initial colour of the various samples to brown indicate formation of silver nanoparticles.

The bio reduction of silver ions (Ag+) to silver nanoparticles (Ag°) in aqueous solution with various samples was monitored using UV-Visible spectrophotometer (Lambda 25UV/V, USA) with a resolution of 0.5 nm. The absorbance of the sample was read with the wavelengths ranging from 250 nm - 750 nm.

Fourier Transformed Infra-red spectroscopy (FTIR) analysis of synthesized SNPs

FTIR analysis was carried out to characterise the functional groups of SNPs. The synthesized SNPs were air dried at room temperature and used for FTIR analysis. The analysis of the dried SNPs was carried out using Potassium Bromide (KBr) pellet (FTIR grade) method in a ratio of 1:100. The spectrum was recorded using JASCO FT/IR-6300 Ibadan in the range of 450-4000 cm^{-1}. Fourier Transformed Infra-red spectrometer equipped with JASCO IRT-7000 Intron infrared Microscope using Transmittance mode operating at a resolution of 4 cm^{-1}.

Scanning Electron Microscopic (SEM) analysis of synthesized SNPs

SEM of the biosynthesized SNPs was used to determine the morphology of the SNPs. The aqueous solution of the biosynthesized SNPs were dried and subjected to scanning electron microscopy machine (Qantas 200 Environmental SEM, USA).

Effect of some physicochemical parameters on the biosynthesized SNPs

The effect of some physicochemical parameters like silver nitrate concentrations, incubation time, temperature and pH on the biosynthesized SNPs was evaluated.

The effect of incubation time (24 and 48 hrs), different concentrations of $AgNO_3$ (1mM - 5mM), temperature (25ºC, 35ºC and 45ºC) and pH (3, 5, 7, 9, 11 and 14) on the biosynthesized SNPs was evaluated.

Antimicrobial activity of SNPs

The antimicrobial analysis was done using agar well diffusion method on Gram positive and Gram negative bacteria: *Escherichia coli, Klebsiella sp, Staphylococcus aureus, C. albicans* and *Salmonella typhi*. A 24 hr old culture of each test organism was inoculated into 5 ml normal saline in a test tube and standardized to 0.5 Macfarland. A sterile swab stick was used to apply the suspension to the surface of freshly prepared Muller Hinton Agar (MHA) plates. The plates were allowed to dry and a sterile cork borer of 5 mm was used to cut uniform wells in the agar. Each well was filled with 40 µL biosynthesised SNPs. The plates were incubation at 37ºC for 24 hr. The zone of inhibition excluding well, for each case was measured and recorded (Abd El-Raheem et al., 2011).

Results and Discussion

Biosynthesis and characterization of SNPs using culture free supernatant of L. plantarum

The functions of biological synthesis in nano-scale technology and other fields of nanoscience are very significant in the biosynthesis of nanoparticles. In this study, culture free supernatant of *L. plantarum* was evaluated for the biosynthesis of silver nanoparticles (SNPs). Plate 1 shows the visual characterization of biosynthesized SNPs by *L. plantarum*. There was a colour change. The biosynthesis of SNPs was observed visually based on the colour changes observed after incubation. The colour change from golden yellow to dark brown indicates the production of silver nanoparticles which was as a result of the excitation of surface plasmon vibrations. The colour exhibited by the metallic nanoparticles could also be as a result of the coherent excitation of entire free electrons within the conduction band leading to surface plasmon resonance (SPR). This result is in correlation with the work of Popoola and Adebayo-Tayo, (2017) who worked on the green synthesis of silver nanoparticles using supernatant from *Lactobacillus casei* LPW2 cultured in modified exopolysaccharides selection medium. Abhishek and Rashmi, (2017) reported that the solution mixture turned into brown from yellow solution at room temperature suggesting the formation of SNPs.

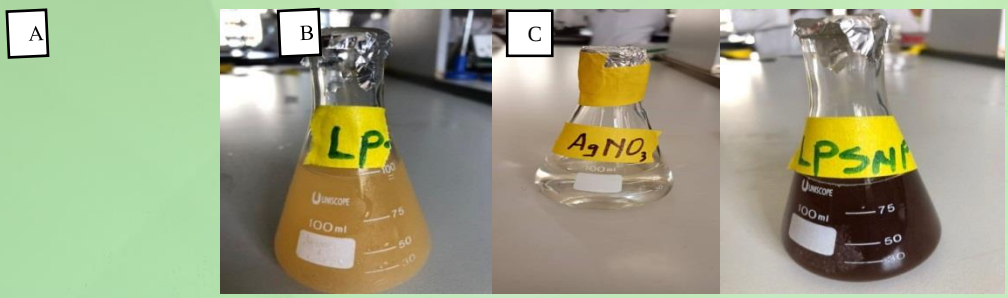

Plate 1. Visual Characterization of biosynthesized SNPs by *L. plantarum*
Key: A- CFS of *L. plantarum* (LP); B- $AgNO_3$; C- SNPs synthesized by CFS of *L. plantarum* (LPSNPs)

The biosynthesized SNPs produced were characterized with UV-visible Spectrophotometer. The spectra obtained from the biosynthesized SNPs by culture free supernatant of *L. plantarum* (LPSNPs) is shown in Figure 1. Surface Plasmon Resonance (SPR) peak was at 450 nm for LPSNPs indicating the formation of SNPs. LPSNP had the highest absorbance for the biosynthesis of SNPs and the least absorbance was recorded for LP. A broad band spectrum between 350 nm and 550 nm was observed for LPSNP and LP. The biosynthesized SNPs by culture free supernatant of *L. plantarum* (LPSNP) shows maximum production. The maximum production of the culture free supernatant indicates that the reduction of the ions occurs extra-cellularly through reducing agents released into the solution by the lactic acid bacteria. SNPs exhibits size and shape dependent on SPR bands. Similar studies have been reported by Sreedevi et al. (2015) who stated that the Surface Plasmon Resonance band in the SNPs solution of *Lactobacillus plantarum* was centred at 420 nm, indicating the particles are dispersed in the aqueous solution with no evidence of aggregation. This study is in correlation with the work of Soltani et al. (2015) who reported that the synthesis of SNPs by *Streptomyces somaliensis* showed a strong SPR peak at 450 nm. This result is similar to the study conducted by Kamani and Lim, (2013) who reported that SNPs showed a strong SPR peak at 400 – 550 nm with a broad band and size, indicating the formation of SNPs that varied in shape and size.

The biosynthesized SNPs were further characterized using Scanning Electron Microscopy. This is an important tool used to determine the morphology and size of the SNPs produced. The micrograph of SNPs synthesized by CFS of *L. plantarum* is shown in Plate 2. The SEM analysis confirmed that SNPs synthesized by CFS of *L. plantarum* was spherical and partially aggregated with particle size ranging from 0.7- 10.0 nm. The aggregation observation may be due to the drying process. The size differences are caused by concentration of reducing agent and the presence of stabilizer which is crucial to the size and shape control of nanoparticles in a biosynthesis

Turkish Journal of Agriculture - Food Science and Technology
Türk Tarım - Gıda Bilim ve Teknoloji Dergisi
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system. Similarly, Adebayo-Tayo et al. (2017) reported that the SEM for the biosynthesis of SNPs by *L. casei and L. fermentum* was partially aggregated with particle size ranged from 0.7-10 nm and 1.4 -10 nm.

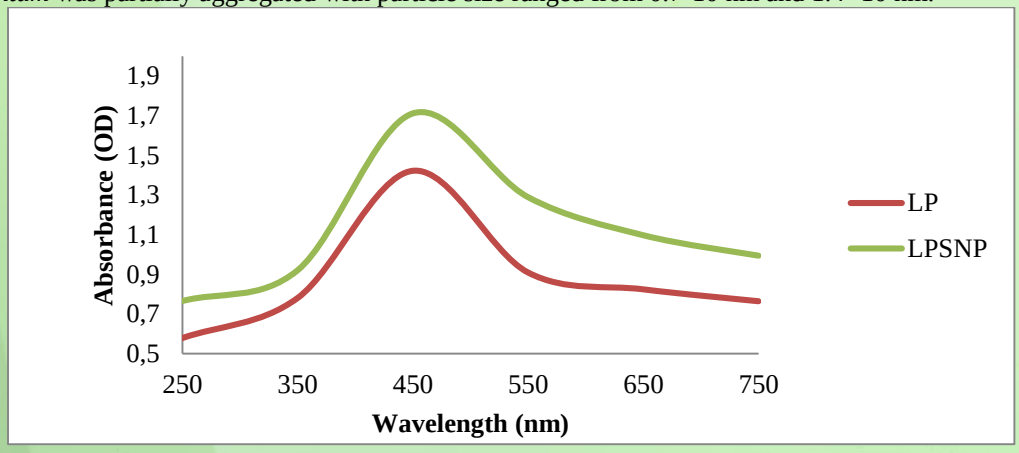

Figure 1. UV-Visible absorption spectra of SNPs synthesised by culture free supernatant of *L. plantarum*
KEY: LP- CFS of *L. plantarum*, LPSNPs – SNPs synthesized by CFS of *L. plantarum*

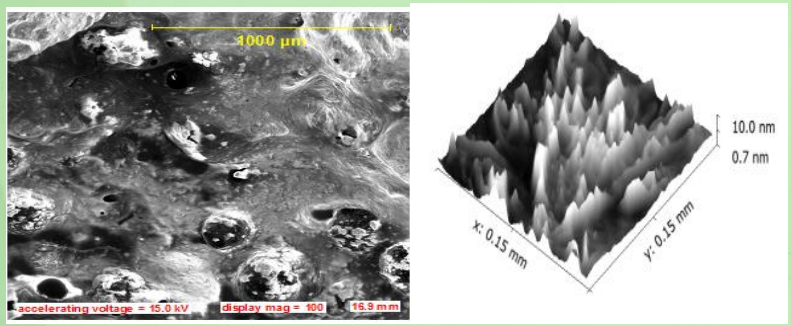

Plate 2. Scanning electron micrograph of LPSNPs (Silver nanoparticle synthesized by *L. Plantarum*

FTIR determines the functional groups present in samples. The FTIR spectrum of SNPs biosynthesised by cell free supernatant of *L. plantarum* is shown in Figure 2. 15 bands were present at 3401.08, 2098.88, 1642.74, 1458.7, 1415.73, 1121.51, 1080.53, 1037.6, 986.13, 735.56, 515.75, 505.77, 478.66, 471.77, and 456.21 cm^{-1} as shown in fig 3. The peak at 3401.08 cm^{-1} was observed to have the strongest and broadest absorption peak indicating a stretching vibration of hydroxyl group (OH). The peak at 2098.88 cm^{-1} indicates the presence of C=C stretching alkenes while the peak at 1642.74 cm^{-1}corresponding to the C=O carbonyl stretching frequency and carboxyl groups. The absorption peak at 1458.7 cm^{-1} indicates the presence of N-H bend of secondary amide and 1415.73 cm^{-1} peak shows the presence of C-N stretch of primary amide. The peak at 1121.51 cm^{-1} indicates C-H in plane bend of alkanes. The absorption peak at 1080.53 and 1037.6 cm^{-1} was identified to be the C-N stretch of aliphatic amines. The peak at 986.13 cm^{-1} showed the presence of C-O stretch of carboxylate while 735.56 cm^{-1} corresponded to the C-H stretching of mono-substituted benzene. The peaks at 515.75 cm^{-1} and 505.77 cm^{-1} showed the presence of S-S disulphide stretch. The absorption peaks at 478.66 cm^{-1}, 471.77 $cm^{-1}$and 456.21 cm^{-1} indicates the presence of acetylenic C-H bend of alkynes. All functional groups observed indicated that hydroxyl, aldehyde, carboxylic acid, amino acid, and esters may be responsible for the stability and bio-reduction of silver nitrate ($AgNO_3$) to silver nanoparticles (SNPs). These functional groups are present as a result of the presence of biomolecules from the cell membrane of *L. plantarum*, which was involved in the biosynthesis process. Carbonyl groups from the amino acid residues and peptides of protein has strong ability to bind silver, this protein form a coat covering the metal nanoparticles and aids its stabilisation in the medium. This is in accordance with the work of Balashanmugam et al. (2013) who reported that from FITR spectrum analysis, their SNPs were surrounded by proteins and amino acids which may be responsible for the stability. Also, Hidayat et al. (2020) reported the presence of proteins and amino acids which are a stabilising agent for SNPs. Akinsete et al. (2017) observed that the functional groups found in the biosynthesized SNPs by *B. ferruginea* includes carboxyl gourp, hyroxyl group, carbonyl group, secondary amides and aromatic conjugates and are responsible for the stability of SNPs.

The Effect of physicochemical parameters on the biosynthesized SNPs by culture free supernatant of L. plantarum

The UV-Visible spectra for the biosynthesis of SNPs by cell free supernatant of *L. plantarum* at different incubation time is shown in Figure 3a. SPR peak was at 450 nm for the SNPs at 24 hrs and 48 hrs and a broad

band at 350 - 550 nm was observed. The absorbance recorded for SNPs at 24 hrs was the highest while SNPs at 24 hrs had the least absorbance. The effect of incubation time on the biosynthesis of SNPs was evaluated and maximum absorbance was observed at 24 hrs with the SPR peak at 450 nm indicating the production of SNPs with different sizes and shapes. This is in accordance with the work Adebayo-Tayo et al. (2017) who reported that the UV-visible spectra of biosynthesized SNPs by *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 showed a broad band at 400 – 600 nm at 24 h and 48 hrs. Sreedevi et al. (2015) reported that at 48 hrs synthesis of SNPs were able to produce different shapes.

The effect of different concentrations (1 - 5 nm) of $AgNO_3$ on biosynthesis of SNPs by culture free supernatant of *L. plantarum* was evaluated. The UV-Visible spectra on the effect of different concentration of $AgNO_3$ on the biosynthesis of SNPs by culture free supernatant from *L. plantarum* is shown in Figure 3b. The SNPs showed strong SPR peak at 450 nm and highest broad band at 350 nm - 550 nm for all the concentrations. 5 mM had the highest absorbance followed by 4 mM and the least absorbance was recorded at 1mM concentration of $AgNO_3$. The ability of 5 mM $AgNO_3$ concentration to induce the highest SNPs biosynthesis may be due to the faster rate of bio-reduction with increased concentration of precursor salt. Also, SPR increases with increase in salt concentration. This result is in agreement with the report of Babu et al. (2013) who reported that the SPR peak intensity increased with the increase in silver nitrate concentration (1 to 5 mM). In contrary, the report of Annadurai et al. (2013) evaluated the effect of silver nitrate concentration using 1 mM to 5 mM and concluded that the optimum $AgNO_3$ concentration was 1 mM.

Temperature is one of the most important physical parameter on the synthesis of SNPs. Figure 3c shows the UV-visible absorption spectra for the effect of temperature on the biosynthesis of SNPs by culture free supernatant of *L. plantarum*. SPR peak was observed at 450 nm for all the temperatures and a broad band at 350 - 650 nm. The SNPs at 25ºC had the highest absorbance followed by SNPs at 35ºC and the least absorbance was recorded at 45ºC. The SNPs biosynthesis may be as a result of complete reduction of silver ions to SNPs. Denaturation of proteins capping occurs at higher temperature leading to higher rate of agglomeration. This result is in agreement with the work of Adebayo-Tayo and Popoola (2017) who reported that the highest production of biosynthesized SNPs by lactic acid bacteria was obtained at 28ºC. Muhammed et al. (2012) observed that with increase in temperature from 25ºC - 45ºC, the SPR peaks were sharp, yellowish-brown solution was obtained after 30 min of stirring the formation of SNPs and 45ºC was the optimum temperature required for the completion of the reaction. Figure3d shows the UV- visible spectra for the effect of different pH on the biosynthesis of SNPs by culture free supernatant of *L. plantarum*. The stabilized LPSNPs showed SPR peak at 450 nm for pH 7 and 9. Two peaks were observed for SNPs at pH 5 and pH 3 at 350 nm and 550 nm. The highest absorbance was recorded for SNPs at pH 9 followed by SNPs at pH 7 and the least absorbance was recorded for pH 5. The absorbance increased from 350 - 550 nm and then declined to 650 nm. At alkaline pH SNPs are stable while at low pH the protein structure gets affected, denatured and loses its activity. The ability of pH 7 to support SNPs biosynthesis may be attributed to the capping proteins secreted by the bacteria in the solution which was stable at high pH. Hydrogen ion are nucleophiles which play crucial role in maintaining the stability of SNPs by absorbing on it and in synthesis of smaller size SNPs by providing electrons for reduction of silver ions. Ability of pH 9, pH 7 and pH 11 to support the maximum biosynthesis of SNPs may be due to the fact that more nucleation regions are formed due to the availability of OH^- ions. Increases in pH increases more competition between protons and metal ions for negatively charged binding sites. This work is similar to the study of Sonal et al. (2015) who observed that pH 9 and pH 11 showed the maximum absorbance and peak at 425 nm and 436 nm respectively and there was no need for agitation of mixture in alkaline pH before all the silver ions supplied will be converted to SNPs. This is in accordance with the work of Afreen and Vandana (2011) who reported that absorption maxima show a sharp peak at pH 7.

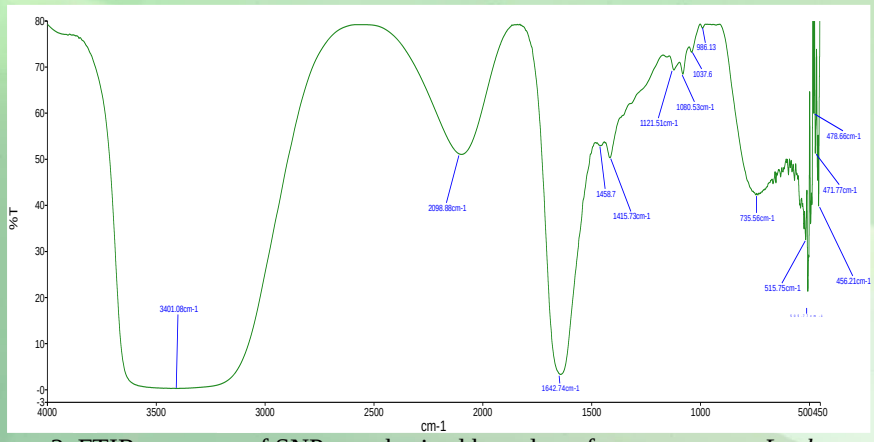

Figure 2. FTIR spectrum of SNPs synthesized by culture free supernatant *L. plantarum*

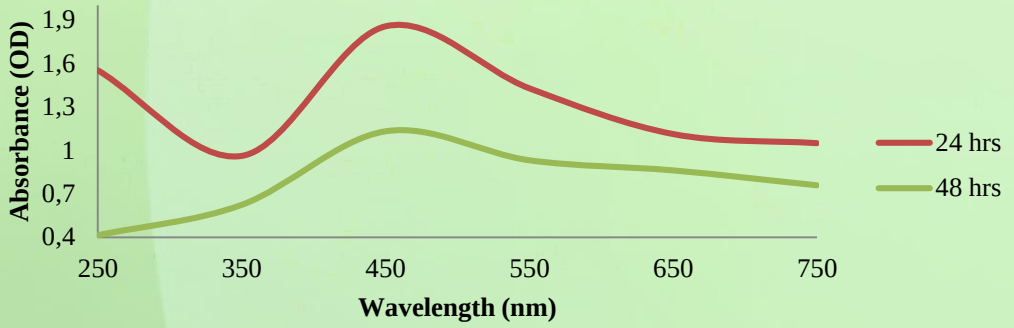

Figure 3a. UV-Visible absorption spectra on the effect of incubation time on the biosynthesis of SNPs by *L. Plantarum*

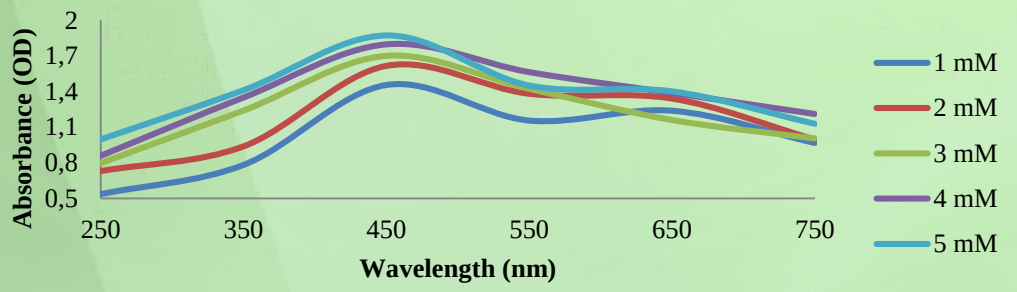

Figure 3b. UV-Visible absorption spectra of the effect of $AgNO_3$ concentration on the biosynthesis of SNPs by *L. Plantarum*

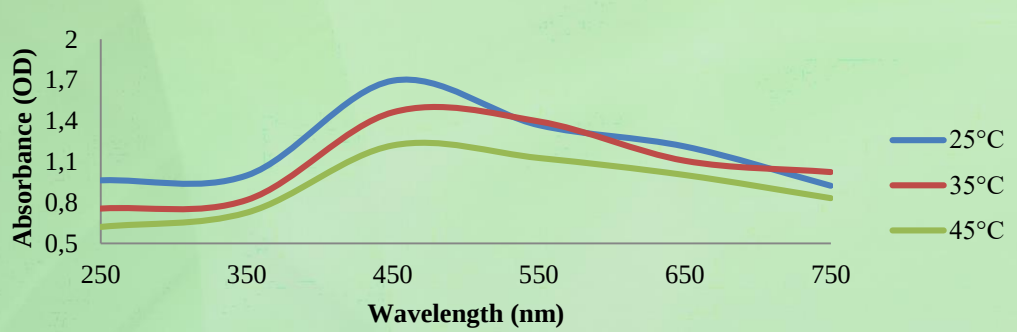

Figure 3c. UV-Visible absorption spectra of the effect of temperature on the biosynthesis of SNPs by *L. Plantarum*

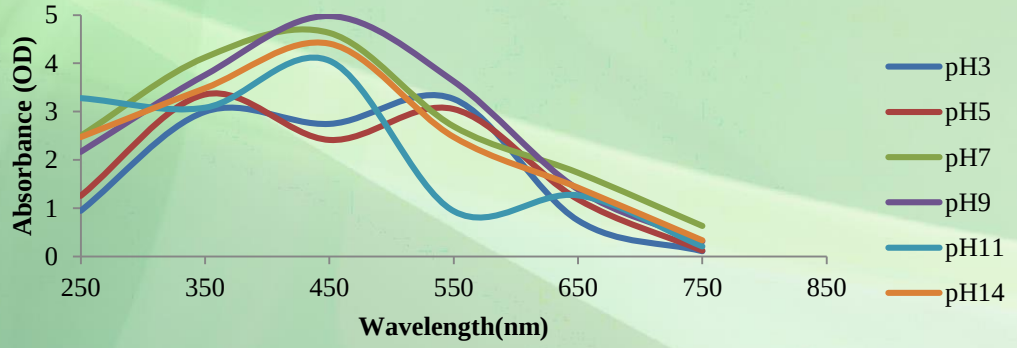

Figure 3d. UV-Visible absorption spectra of the effect of pH on the biosynthesis of SNPs by *L. Plantarum*

The Antimicrobial activity of the biosynthesized SNPs on the test organisms

The antimicrobial activity of LPSNPs, $AgNO_3$, LP and antibiotics (Ciprofloxacin) against some selected test organism were investigated.

Table 1 shows the antimicrobial activity of the biosynthesized SNPs. The zone of inhibition of LPSNP ranged from 15 - 22 mm. *S. aureus* had the highest susceptibility followed by *E. coli* and the lowest susceptibility was recorded against *Klebsiella sp.* The antimicrobial activity of Ciprofloxacin ranged from 14 - 20 mm. *S. typhi* had the highest susceptibility while the lowest susceptibility was recorded against *C. albicans.* The antimicrobial activity of LP and $AgNO_3$ ranged from 8 - 12 mm. The biosynthesized SNPs by culture free supernatant of *L. plantarum* (LPSNP) had the highest zone of inhibition.

The SNPs synthesized by *L. plantarum* exhibited varied antimicrobial activities against the test organisms. The antibacterial effects of SNPs are well established and several mechanisms of their bacterial effect have been proposed. The Gram-positive bacteria were more susceptible to the SNPs than the Gram-negative bacteria. Nanoparticles interact with DNA, thus losing its ability to replicate leading to cell death. The result is similar to work of Adebayo-Tayo and Popoola (2017) who reported that *Staphylococcus aureus*, a Gram positive organism was the most susceptible to their SNPs. Kabo et al. (2019) who reported the biosynthesis of silver nanoparticles with potent antimicrobial activity using lactic acid bacteria. This is also in accordance with the work of Adebayo-Tayo et al. (2017) who used the bacterial synthesis of silver nanoparticles by culture free supernatant of lactic acid bacteria isolated from fermented food samples. This is in contrast with the work of Hidayat et al. (2020), who reported that the inhibitory effects of SNPs were more pronounced on Gram-negative bacteria compared to Gram-positive bacteria due to the thickness of the cell wall and cell composition. Some of the antimicrobial properties exhibited by the fermented food strain (*L. plantarum*) may be as result of the low pH of the food as well as metabolites (SNPs) produced by LAB involved in the fermentation.

Table 1: Antimicrobial Activity of Silver Nanoparticles against Some Selected Test Pathogens

Test organisms	Zones of Inhibition (mm)				
	$AgNO_3$	LP	LPSNPs	Ciprofloxacin	
S. typhi	11	9	18	20	
S. aureus	10	10	22	18	
Klebsiella sp	10	9	15	14	
E. coli	9	12	20	19	
C. albicans	12	8	14	15	

Keywords; $AgNO_3$: Silver nitrate; LPSNPs: SNPs synthesized from *L. plantarum*; LP: Cell free supernatant of *L. Plantarum*

Acknowledgements

The authors acknowledge the management of KolaDaisi University for providing laboratory facilities and all the necessary assistances needed towards the completion of the research.

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