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Bacterial Synthesis of Silver Nanoparticles by Culture Free Supernatant of Lactic Acid Bacteria Isolated from Fermented Food Samples

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Authors' contributions

This work was carried out in collaboration between all authors. Author BCAT designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors AOP and OMA managed the analyses of the study, managed the literature searches and performed the statistical analysis. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Biosynthesis of silver nanoparticles (SNPs) using culture free supernatant (CFS) of Lactic acid bacteria (LAB).
Study Design: To biosynthesize, characterize and to determine the antibacterial potential of silver nanoparticle using culture free supernatant of lactic acid bacteria and to determine the effect of some parameters on SNPs biosynthesis.
Methodology: Biosynthesis and characterization of SNPs using CFS of LAB and the antibacterial activity.
Place and Duration of Study: Department of Microbiology, University of Ibadan, Ibadan, Oyo state, Nigeria between Jan to October 2016.
Results: The antibacterial potential of the CFS from the LABs was evaluated on some pathogenic bacteria and antibacterial activity ranged from 0 – 20 mm. The two LAB strains LPW2 and LPF6 had 93% and 86% relatedness to *Lactobacillus casei* strain WK2G-3A and *Lactobacillus fermentum*

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strain E10-15. The CFS of the LAB strains and $AgNO_3$ was able to produce SNPs. The biosynthesized SNPs had a strong Surface Plasmon Resonance (SPR) peak at 500 nm, varying shape (partially aggregated particles) and the sizes ranged from 0.7 - 10.0 nm and 1.4 – 10.0 nm for CFS SNPs from *Lactobacillus casei* and *Lactobacillus fermentum*. Carboxylic acid, protein, aldehydes, ester and hydroxyl groups are the functional groups responsible for SNPs formation. The antibacterial activity of the SNPs ranged from 11 – 29 mm. *Bacillus* sp. and *Streptococcus pyogenes* were found to be more susceptible to the biosynthesized SNPs. 28°C, pH 4 and 10 Mm $AgNO_3$ supported the highest SNPs production.

Conclusion: In conclusion the CFS from the LABs biosynthesized SNPs which exhibited antibacterial activity against some pathogenic microorganisms.

Keywords: Lactic acid bacteria; culture free supernatant; silver nanoparticles; SEM; FTIR; SPR.

1. INTRODUCTION

Nanoparticles exhibit unique physical, chemical and biological properties in term of their size, reactivity, surface area to volume ratio, magnetic and optical properties. Nanoscale (nanoparticles) science is the discipline that examines the unique behaviors and properties of materials that emerge at the size range of 1 to 100 nanometers. The reduction of materials' dimension has pronounced effects on the physical properties that may be significantly different from the corresponding bulk material [1]. Nanoparticles often show unique and considerably changed physical, chemical and biological properties compared to their macroscaled and bulk counterparts. Physical and chemical methods for nanoparticles synthesis are expensive and involve the production of toxic by-products which are environmentally not safe methods. An alternative method for synthesizing nanoparticles depends on biological system and the use of microbes as a tool for synthesis of new functional nanomaterial's has gained much interest in recent times [2]. Biosynthesis of nanoparticles is a kind of bottom up approach where the main reaction occurring is reduction/oxidation [3]. Recently, a simple and viable alternative to more complex chemical synthetic procedures to obtain nanomaterial's known as biosynthetic method employing both biological microorganism such as bacteria [4] and fungus [5] or plants extract [6,7,8] have emerged. Culture supernatant of microorganisms may act both as reducing and capping agents in SNPs production. The culture supernatants have been found to contain environmentally benign, yet chemically complex biomolecules such as enzymes or proteins, amino acids, polysaccharides, and vitamins which may be responsible for the reduction of Ag^+ ions [9]. The first report on bacterial mediated SNPs was given by Joerger et al. [4] in which *Pseudomonas stutzeri* AG259 was reported to synthesize Ag particles which were accumulated within the periplasmic space of bacterial cell of 200 nm. Depending on the position of the reduction enzymes, Fungi were found to be capable of reducing the metals ions into their corresponding nanometals either intracellularly or extracellularly [10]. Silver and its compounds are known to have antimicrobial properties. Early in the 19th century, 0.5% AgNO3 was used for the treatment and prevention of microbial infections such as *Ophthalmia neonatorum* [11]. Various hypotheses have been proposed to explain the mechanism of antimicrobial activity of silver nanoparticles. It is widely believed that silver nanoparticles are incorporated in the cell membrane, which causes leakage of intracellular substances and eventually causes cell death. Some of the silver nanoparticles also penetrate into the cells. It is reported that the bactericidal effect of silver nanoparticles decreases as the size increases and is also affected by the shape of the particles [12].

The bacteria that produce lactic acid as their major or sole fermentation product are sometime collectively called lactic acid bacteria (LAB). They belong to the low G+C Gram positive bacteria, Class Bacilli and Order Lactobacillales. LABS are non-sporing, devoid of cytochrome, catalase negative and usually non-motile. They are fastidious nutritionally. Energy is obtained by these bacteria through substrate level phosphorylation. They are facultative anaerobes and are sometimes classified as aerotolerant anaerobes [13]. LABS are widespread in nature and predominant microflora of milk and its products. They are one of the important groups of microorganisms in food fermentation and they produce variety of antimicrobial compounds such as ethanol, formic acid, acetone, hydrogen peroxide, diacetyl and bacteriocins which confer

preservative ability on them as a natural competitive means to overcome other microorganisms sharing the same niche [14]. The traditional and modern use of LAB in food production is due to their many functional characteristics. This study is aimed at the production, characterization and antibacterial potential of silver nanoparticles produced from culture free supernatant of lactic acid bacteria and the effect of some parameters on SNPs biosynthesis was evaluated.

2. MATERIALS AND METHODS

2.1 Culture Collection

Lactobacillus casei LPW2 and *Lactobacillus fermentum* LPF6 previously isolated from fermented food was obtained from the culture collection of the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Nigeria. The culture was streaked on de Man, Rogosa and Sharpe (MRS) agar and the plates were incubated for 24 hrs at 35°C [15]. The pure culture were kept in maintenance medium (MRS broth with 12% v/v glycerol) and the stock culture were stored at 4°C and sub-cultured from time to time to regulate its viability.

The pathogenic bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Bacillus* sp.) used in this study were obtained from The Department of Medical Microbiology, University College Hospital (UCH), Ibadan. The isolates were sub-cultured on plates of nutrient agar and incubated for 24 hours at 35-37°C. The colonies were then picked and stored in slants until when needed.

2.2 Identification of the LAB Strains

The LAB strains were identified based on microscopic, macroscopic and biochemical characterization. The colonial morphology was observed macroscopically. Gram staining, Catalase test, Indole test, Oxidase test, Citrate utilization test and Endospore staining were carried out [15,16].

Molecular characterization using 16S rRNA of the two EPS- producing LAB with the highest antagonistic potential was done. The DNA was extracted, amplified with universal primers: forward, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse, 5'-AAGGAGGTGWTCCARCCGCA-3' using Polymerase Chain Reaction (PCR). The purified PCR products were sequenced using automatic ABI 310 DNA Sequencer (Big Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin Elmer). Identification of the closest relatives were done by comparing to those available in the V2-V3 region of the 16S rRNA sequences using the GenBank DNA databases and the standard nucleotide-nucleotide BLAST algorithm. A phylogenetic tree was constructed by using the neighbor-joining method, which produced a unique final tree under the principle of minimum evolution using the MEGA5 programs [17].

2.3 Antimicrobial Activity of the CFS of LAB Strain against Some Selected Pathogens

The antimicrobial activity of the CFS of the LABs was done using a modified method of Omafuvbe and Enyioha [18]. The LAB strains were grown in MRS broth for 24 hrs, centrifuged at 5000 rpm for 20 minutes and the supernatant was used. 16 hrs old culture of the pathogens (*Escherichia coli*, *Staphylococcus* species, and *Bacillus* species) grown on nutrient agar at 37°C was suspended in saline. A lawn of the indicator strain was made by spreading the cell suspension over the surface of Mueller - Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 5 mm was used to cut uniform wells in the agar. Each well was filled with 60 µl culture free supernatant obtained from the LAB strains. The plates were incubated at 37°C for 24 hrs and observed for a zone of inhibition (ZOI) around the well.

2.4 Biosynthesis of SNPS Using Culture Supernatant from the LAB Strains

The CFS was produced by culturing the LAB in sterile MRS broth. The inoculated broth was incubated microaerobically at 35°C for 24 hrs. After incubation, the fermentation medium was centrifuged at 5000rpm for 20 minutes and the supernatant obtained were labeled as CFS and used for SNPs biosynthesis [19].

For the production of SNPs, 1 ml of the CFS was added to 10 ml of 10 mM aqueous solution of silver nitrate (AgNO3) prepared freshly in deionized water under stirring conditions and the mixture was incubated at room temperature in a

dark place for 24 – 48 hrs. Formation of yellowish brown colour indicates the SNPs formation.

2.5 Characterization of the Synthesized SNPs

2.5.1 Visual detection, UV-visible spectrophotometric and Scanning electron microscopic (SEM) analysis of the SNPs

The SNPs biosynthesized using the CFS was observed for the change in colour in comparison to control as a visual method of detection of silver nanoparticle synthesis. Changes in colour from colourless to yellowish brown indicate the formation of SNPs.

Spectrophotometric analysis of the bio-reduced silver ion (Ag^+) to silver nanoparticles (Ag^0) was determined using UV-Visible spectrophotometer with a resolution of 0.5 nm. The absorbance of the sample was read at the wavelengths of 200-800 nm.

SEM of the biosynthesized SNPs from the CFS was used to define the morphology of the SNPs. The aqueous solution of SNPs synthesized was dried and subjected to scanning electron microscopy (Qantas 200 Environmental SEM, FEI Company USA).

2.5.2 Fourier transform infra-red (FT-IR) analysis of the EPS and SNPs

The biosynthesized silver nanoparticles using the CFS of the LABs were further characterized using FTIR and the functional groups obtained were used for the SNPs characterization. The FTIR spectra of the SNPs were analyzed using FTIR spectroscopy (Shimadzu) operated at resolution of 4 cm^{-1}. For the measurement the dried samples were powdered with KBr pellets and pressed into a mold. The spectra were recorded at a wave range of 500-4000 cm^{-1}.

2.6 Antibacterial Potential of the Biosynthesized SNPs

The antibacterial activity of the biosynthesized SNPs was done using agar well diffusion method. Some selected pathogenic microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Bacillus* species) were used as indicator organisms. 24 hrs old culture of the pathogens grown on nutrient agar at 37°C was suspended in saline. A lawn of the indicator strain was made by spreading the cell suspension over the surface of Mueller - Hinton agar plates with a sterile cotton swab. The plates were allowed to dry and a sterile cork borer of diameter 7 mm was used to cut uniform wells in the agar. Each well was filled with 100 µl of the biosynthesized SNPs. The plates were incubated appropriately. After incubation at 37°C for 24 hrs, the plates were observed for zone of inhibition (ZOI) around the wells. Results were considered positive if the diameter (mm) of the ZOI was greater than 1 mm [20].

2.7 Effect of Some Parameters on SNPs Synthesis

The effect of temperature, pH and silver nitrate concentration on SNPs biosynthesis was evaluated. The effect of temperature (28°C, 35°C and 40°C), pH (4, 7 and 9) and different concentration of $AgNO_3$ (2 mM – 10 mM) on SNPs biosynthesis was investigated. The reaction mixture was incubated for 24 – 48 hrs and the biosynthesized SNPs was characterized.

3. RESULTS AND DISCUSSION

The morphological and biochemical characteristic of the LABs is shown in Table 1. The isolates are Gram positive rods and cocci, catalase and oxidase negative and non-spore forming. This observation corresponds to the description of LAB in Bergey's Manual of Systematic bacteriology [21]. This is similar to the work of Prabhu et al. [20] who isolated LAB from Yoghurt. The result is also in agreement with the work of Adebayo-Tayo and Onilude who isolated LAB form fermented foods [22]. According to Olasupo et al. [23] the presence of LAB in fermented food has been attributed to their ability to withstand the acidic condition as well as production of lactic acid in the food.

The antimicrobial potential of the LAB strains against some pathogens is shown in Table 2. The antimicrobial activity of the metabolites from the LAB against some pathogens ranged from 14 – 20 mm. *Staph. aureus* had the highest susceptibility to the metabolites of LPW2. *E. coli* CL and *Bacillus* sp. was `resistance to the metabolite of LPF6. All the indicator organisms were susceptible to the metabolites of LPW2.

Table 1. Morphological and biochemical characteristics of the LAB strains

Morphological and biochemical characteristics	Lab strains	
	LPW2	LPF6
Gram staining	+	+
Catalase test	-	-
Indole test	-	-
Oxidase test	-	-
Endospore staining	-	-
Citrate utilization test	-	-
Shape	Rod	Rod
Arrangement of cells	Singly	Chain

KEY: + = Positive, - = Negative

Table 2. Antibacterial activity of the LAB strains against some pathogens

S/N	Pathogens	Zone of inhibition (mm)	
		LPF6	LPW2
1	*Escherichia coli* CL	14[a]	19[b]
2	*Escherichia coli* Fd	0	17[d]
3	*Staphylococcus aureus*	16[a]	20[a]
4	*Bacillus* species	0	18[c]
5	*Klebsiella pneumoniae*	9[c]	10[f]
6	*Streptococcus pyogenes*	10[b]	12[e]
7	*Pseudomonas aeruginosa*	7[d]	8[g]

Mean with the same superscript along the same column are not significantly different (P≤ 0.05)

The antimicrobial effect by LAB could be due to the production of lactic acid, reduction of pH and low molecular mass compounds (LMM) such as ethanol, hydrogen peroxide, carbon-dioxide, diacetyl, acetone, formic acid, bacteriocin and others [24]. The observation is similar to the work of Nowroozi et al. [25] and Adebayo et al. [26] who reported the inhibitory activities of LAB on some indicator microorganisms.

Based on molecular identification, the 16S rRNA sequences obtained were compared with those of typed sequences in GenBank. LPW2 was identified as *Lactobacillus casei* with 93% similarities with *Lactobacillus casei* strain WK2G-3A. LPF6 was identified as *Lactobacillus fermentum* with 86% similarities with *Lactobacillus fermentum* strain E10-15. The use of molecular identification (16S rRNA) of isolate is a useful tool for actual characterization and comparison of genomes of closely related isolated. Savadogo et al. [27] similarly identified their LAB isolated from milk using molecular techniques.

3.1 Biosynthesis and Characterization of SNPs Using Culture Supernatant of LAB

The CFS of *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 were used for the biosynthesis of SNPs. Fig. 1a shows the SNPs produced by CFS of LPW2 after 24 hour of incubation. The reaction mixture turned yellowish-brown then deep brown in colour indicating the formation of silver nanoparticles.

Fig. 1b shows the SNPs produced by CFS of LPF06 after 24 hour of incubation. The reaction mixture turned deep brown in colour indicating the formation of silver nanoparticles. Production of SNPs using biological agent is a cost effective and ecofriendly method. Synthesis of silver nanoparticles using a probiotic microbe (*Brevibacterium linens*) was reported by Nithya and Ragunathan [28]. Similarly, synthesis of SNPs by bacteriocin producing *Lactobacillus* species isolated from yoghurt was reported by Prabhu et al. [20]. The traditional and modern use of LAB in industrial production is due to their many functional characteristics [29].

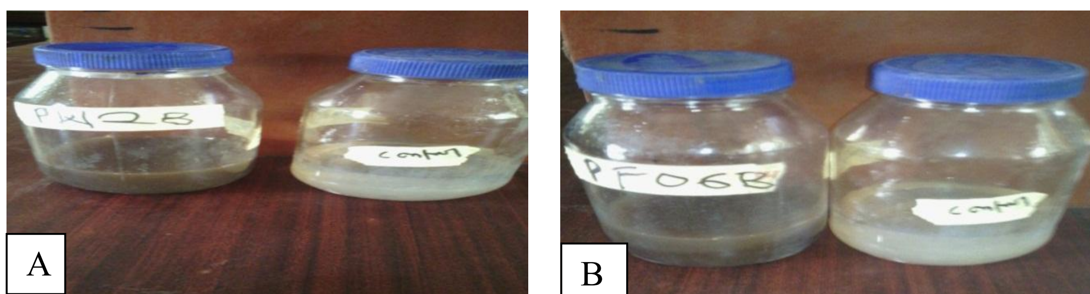

Fig. 1. Visual characterization of (A) *Lactobacillus casei* LPW2 SNPs (B) *Lactobacillus fermentum* LPF6 SNPs at 24 hrs

3.2 UV-Visible Spectrophotometric Analysis of the SNPs

The biosynthesized SNPs were characterized using UV-visible spectrophotometer. Fig. 2a and b shows the UV- visible spectra of the biosynthesized SNPs by *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 at 24 and 48 hrs incubation time. For *Lactobacillus casei* LPW2, a broad band at 400 and 600 nm was observed at 24 hrs and 48 hrs. The highest surface plasmon resonance (SPR) peak was at 500 nm. CFS of *Lactobacillus fermentum* LPF6 SNPs had a broad band peak at 400-600 nm with a strong SPR peak at 500 nm. This result is in agreement with the work of Kanmani and Lim. [30]. Typically, SNPs have maximum wavelength in the visible range of 400-500 nm [31]. The result is also similar to that of Prabhu et al. [20] who reported a SPR peak of 446 nm for their SNPs. Contrary to this, Shivashankar et al. [32] reported that the UV-Visible spectra of their SNPs showed characteristic surface plasmon absorption band at 386 nm.

3.3 Scanning Electron Microscopic Analysis of the SNPs

The SNPs were further characterized by scanning electron microscope. SEM is a useful tool for studying the size, shape and morphology of SNPs. Fig. 3a and 3b shows the SEM micrograph of the biosynthesized SNPs by CFS of *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6. The *Lactobacillus casei* LPW2 SNPs was spherical with the size ranged from 0.7 -10.0 nm and it was aggregated. *Lactobacillus fermentum* LPF6 SNPs was varying in shape and the size ranged from 1.4 – 10.0 nm and it was partially aggregated. The aggregation observed may be due to the drying process. Sadowski et al. [33] observed a similar situation in their work on synthesis of SNPs using *Penicillium* strains isolated from the soil. The procedure for the preparation of SNPs for SEM analysis including drying process can affect the shape and size of the SNPs.

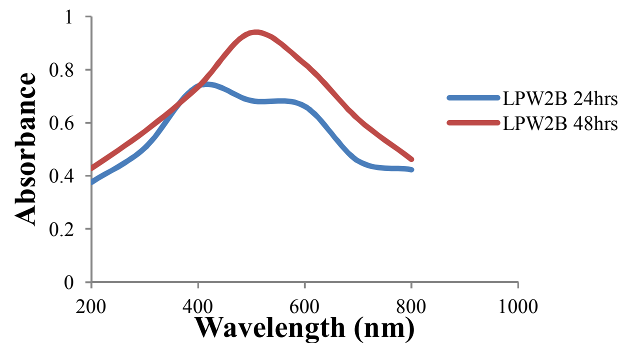

Fig. 2a. UV-visible spectra of *Lactobacillus casei* LPW2 SNPs

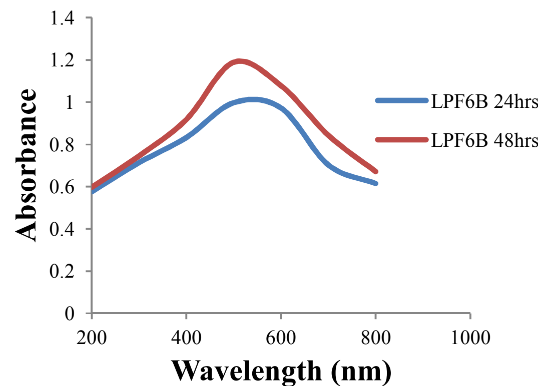

Fig. 2b. UV-visible spectra of *Lactobacillus fermentum* LPF6 SNPs

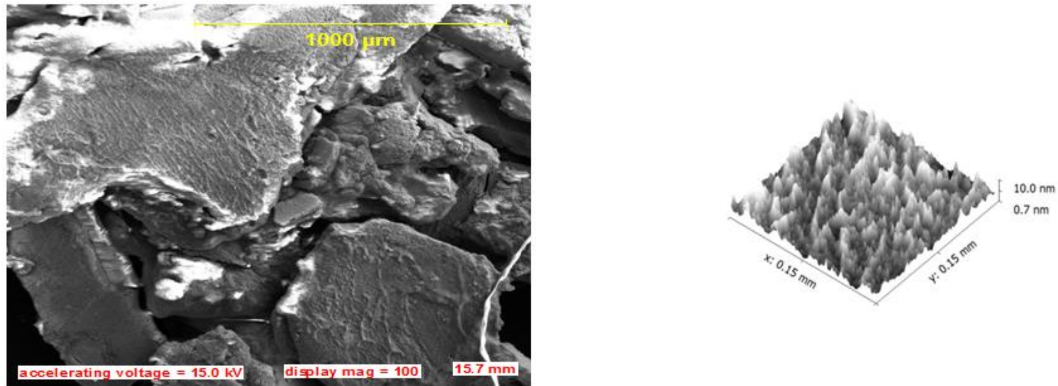

Fig. 3a. SEM micrograph of LPW2B SNPs

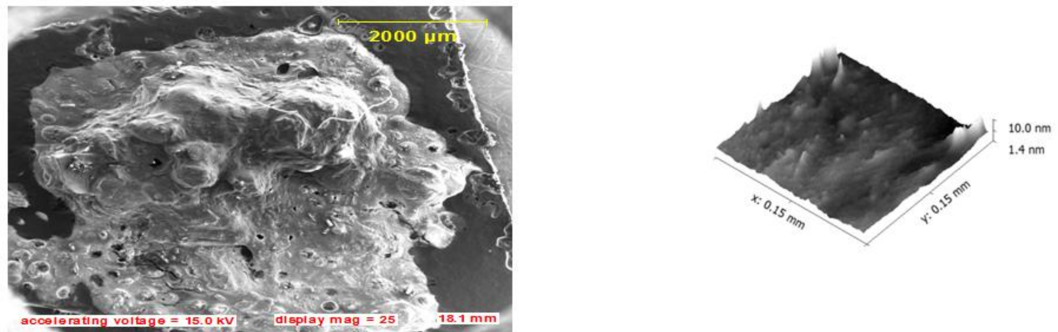

Fig. 3b. SEM micrograph of LPF6B SNPs

3.4 FTIR Spectrum of the Biosynthesized SNPs

The FTIR spectrum of *Lactobacillus casei* LPW2 SNPs is shown in Fig. 4a. 17 absorption peaks were observed and the absorption peaks ranged between 3296.46 cm^{-1} and 542.02 cm^{-1}. The prominent absorption peak at 3296.46 cm^{-1} corresponded to vibrational stretch of hydroxyl group (OH). The absorption peak at 2933.83 cm^{-1} indicated the C-H stretching vibration of an aldehyde. Moreover the peak at 2360.95 cm^{-1} indicated the presence of –COOH overtone acid group. The absorption peak at 1643.41 cm^{-1} corresponded to the carbonyl (C-O) and C=N stretch of amine. The absorption peaks at 1593.25 cm^{-1} and 1539.25 cm^{-1} indicated the presence of N-H stretch of primary amine and HO-C and C-OH deformation band. The absorption peaks at 1384.94 cm^{-1} and 1367.58 cm^{-1} could be attributed to C-H stretch. The absorption peaks at 1238.34 cm^{-1} and 1118.75 cm^{-1} corresponded to the C-O stretch of esters and carboxylic acids respectively. The peak at 1076.32 cm^{-1} and 1043.52 cm^{-1} indicated C-N stretch of aliphatic amines and C-O stretch of alcohol. The absorption peak around 827.49 cm^{-1} and 970.23 cm^{-1} indicated that linkages had occurred between monosaccharides. The absorption peaks between 781.20 cm^{-1}, 621.10 cm^{-1} and 542.02 cm^{-1} indicated the presence of C-OH out of plane bending, C-Cl of Chloroalkanes and S-S disulfide bonds. Therefore from the FTIR spectrum observed, it was clear that the SNPs were surrounded by carboxylic acid, aldehyde, esters protein and amino acids and these may be responsible for the biosynthesis and stability of the SNPs.

The *Lactobacillus fermentum* LPF6 SNPs was characterized by FTIR and the spectrum obtained is shown in Fig. 4b. The spectrum has 18 peaks ranging between 3840.40 cm^{-1} and 536.23 cm^{-1}. The peak at 3840.40 cm^{-1} could be attributed to N-H stretching vibration of primary amide. The strongest absorption peak at 3338.69 cm^{-1} indicated the stretching vibration of hydroxyl group (OH). The absorption peak at 2935.76 cm^{-1} could be attributed to C-H vibrational stretching of aldehyde. The peak at 2428.48 cm^{-1} indicated the presence of C≡C or C≡N bond. Also the absorption peak at 1763 indicated the presence

of C=O stretch of saturated esters. The absorption peak at 1635.69 cm^{-1} and 1593.25 cm^{-1} corresponded to C=O stretch of carboxylates and NH stretch of secondary amides. The absorption peak at 1456.3 could be attributed to OCH and COH deformation band or C-O stretch plus OH carboxylic acid. The absorption peak at 1384.90 cm^{-1} and 1244.13 cm^{-1} indicated C-N stretching vibration of aromatic amine and C-O stretch of esters and carboxylic acid. The peak at 1120.68 cm^{-1} and 1080.17 cm^{-1} corresponded to C-C stretch of ketones and C-O stretch of alcohol. Moreover, the peak at 1045.45 cm^{-1} indicated C-C, C-OH and C-H ring and side group vibrations. C-N stretching vibration of amine was shown by the absorption peak at 852.56 cm^{-1}. The absorption peak at 825.56 cm^{-1} could be attributed to CH bend of aromatic amine. 781.20 cm^{-1} absorption peak corresponded to C-H monosubstituted benzene. The absorption peaks at 619.17 cm^{-1} and 536.23 cm^{-1} indicated the presence of acetylenic C-H bend of alkynes and S-S stretch of disulfides. The functional groups observed indicated that carboxylic acid, esters aldehyde, amino acids and protein could be responsible for the synthesis and stability of the SNPs (Fig. 2b). Aldehyde, amino acids, ethers, esters carboxylic acids, hydroxyl groups among others may be the functional groups responsible for the production of SNPs. This is in agreement with the work of Khatami et al. [34] who reported carboxyl, hydroxyl, amine and others as the possible functional groups responsible for SNP formation.

3.5 Antibacterial Potential of the SNPs

The antibacterial activity of the biosynthesized SNPs is shown in Table 3. The zone of inhibition of CFS of *Lactobacillus casei* LPW2 SNPs ranged from 13 – 29 mm. *Bacillus* species had the highest susceptibility followed by *Strepococcus pyogenes*. The antibacterial

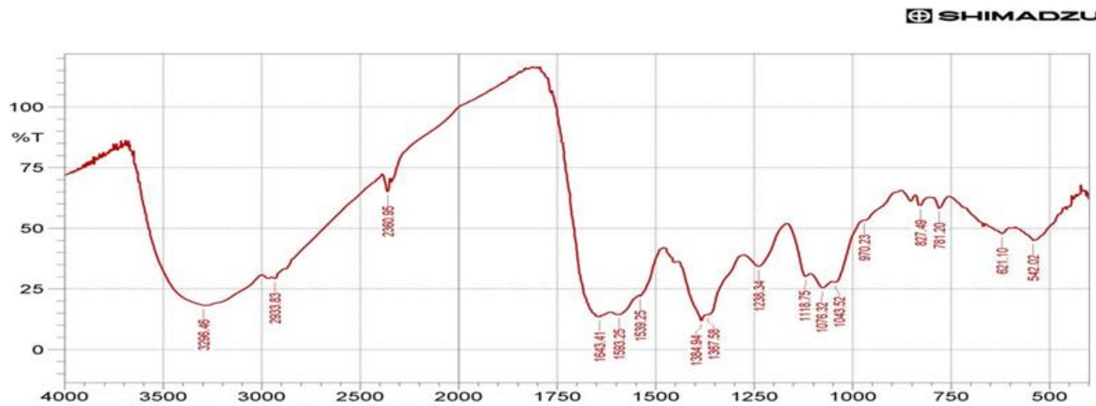

Fig. 4a. FTIR spectrum of *Lactobacillus casei* LPW2 SNPs

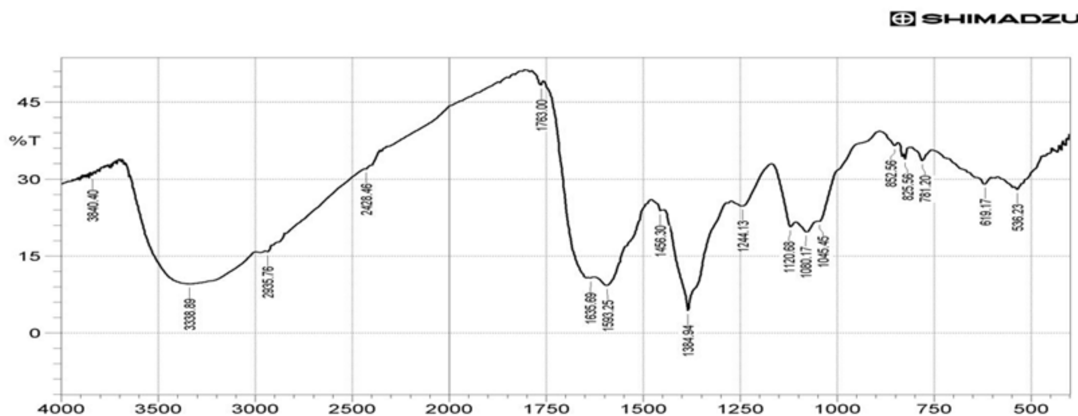

Fig. 4b. FTIR spectrum *Lactobacillus fermentum* LPF6 SNPs

Table 3. Antibacterial activity of the biosynthesized SNPs

Pathogens	Diameter of zones of inhibition			
	LPW2B SNPs	LPF6B SNPs	Ciprofloxacin	$AgNO_3$
Bacillus sp.	29 [a]	25 [a]	20 [b]	15 [a]
Streptococcus pyogenes	20 [b]	18 [b]	24 [a]	10 [c]
Staphylococcus aureus	14 [c]	12 [d]	17 [c]	11 [b]
Klebsiella sp.	14 [c]	13 [c]	15 [d]	9 [d]
Pseudomonas aeruginosa	13 [d]	11 [e]	10 [e]	8 [e]

Mean with the same superscript along the same column are not significantly different (P≤ 0.05)

activity of CFS of Lactobacillus fermentum LPF6 SNPs was evaluated and the zones of inhibition ranged from 11 – 25 mm. Bacillus species had the highest susceptibility followed by *Strepococcus pyogenes* and the least susceptibility was shown by *Pseudomonas aeruginosa*. The zones of inhibition of the Positive controls (Ciprofloxacin and $AgNO_3$) ranged from 8 – 20 mm. Generally the Gram positive pathogens were more susceptible to the SNPs compared to their Gram negative counterpart and *Lactobacillus casei* LPW2 SNPs showed more antibacterial activity. The antimicrobial properties of silver nanoparticles are well-established and several mechanisms for their bactericidal effects have been proposed. Toxicity of the SNPs is presumed to be size and shape dependent because small size nanoparticles may pass through cell membranes. Inside a bacterium, nanoparticles can interact with DNA, thus losing its ability to replicate which may lead to the cell death [12]. Prabhu et al. [20] also reported that *Staphylococcus aureus*, a Gram positive organism, was the most susceptible to their SNPs.

3.6 Effect of Temperature on SNPs Biosynthesis

Effect of temperature on the biosynthesis of SNPs by *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 is shown in Fig. 5a and b. For CFS of *Lactobacillus casei* LPW2 SNPs and the spectra revealed that at all the temperature ranges, two SPR peaks at 300 and 500 nm was observed. The highest peak was observed at 28°C followed by 35°C and 40°C (Fig. 3a). For CFS of *Lactobacillus fermentum* LPF6 SNPs (Fig. 3b), two SPR peaks were observed at 300 nm and 500 nm. The highest peak was observed at 28°C. Temperature is one of the important physical parameter on the synthesis of SNPs. Effect of temperature on SNPs synthesis was evaluated and the optimum temperature for the production of SNPs in this work was 28°C. This result is in agreement with the work of Prabhu et al. [20] that produce their SNPs at room temperature. However, the result is in contrary to the work of Annadurai et al. [35] reported that the synthesis of silver nanoparticles increased when the reaction temperature was increased by using the leaf extract of *Coleus aromaticus* and it was concluded that higher temperature (70°C) was optimum for nanoparticles synthesis.

3.7 Effect of pH on SNPs Biosynthesis

The effect of pH on the biosynthesis of SNPs by CFS of *Lactobacillus* casei LPW2 and *Lactobacillus fermentum* LPF6 was shown in Fig. 6a and b. For *Lactobacillus casei* LPW2 SNPs pH 4 was the best with the formation of a strong SPR peak between 500 nm and 600 nm. The absorbance increased from 200 nm – 600 nm and then declined further. At pH 7 a strong peak was observed at 400 nm. Also, a strong SPR peak at 400 nm was observed for pH 9. Fig. 4b shows the spectra of *Lactobacillus fermentum* LPF6 SNPs biosynthesized at different pH. The highest absorbance was observed at pH 4 with SPR peak at 600 nm. pH 7 has a strong peak 400nm. A broad band was observed at pH 9 between 400 nm and 600 nm. The ability of pH 4 to support the highest SNPs production by the LAB supernatant is not in agreement with the work of Muhammad Amin et al. [36] who reported the reduction rate of silver ions increases with an increase in pH.

3.8 Effect of Different Concentration of $AgNO_3$ on SNPs Biosynthesis

The UV-Visible spectra for the effect of different concentrations (2 – 10 mM) of silver nitrate ($AgNO_3$) solutions on biosynthesis of *Lactobacillus casei* LPW2 and *Lactobacillus*

fermentum LPF6 SNPs is shown in Fig. 7a and b. The SPR peak of the biosynthesized SNPs from *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 produced with different concentration of $AgNO_3$ (2 – 10 mM) ranged from 400 – 600 nm and 10 mM $AgNO_3$ supported the highest SNPs formation with SPR peak at 500 nm. The result is in agreement with the report of Babu Sumi Maria et al. [37] that the SPR peak intensity increased with the increase in silver nitrate concentration (1 to 10 mM) indicating faster rate of bioreduction with increased concentration of precursor salt. In contrast, the report of Annadurai et al. [35] evaluated the effect of silver nitrate concentration using 1mM to 5mM and concluded that the optimum silver nitrate concentration was 1 mM.

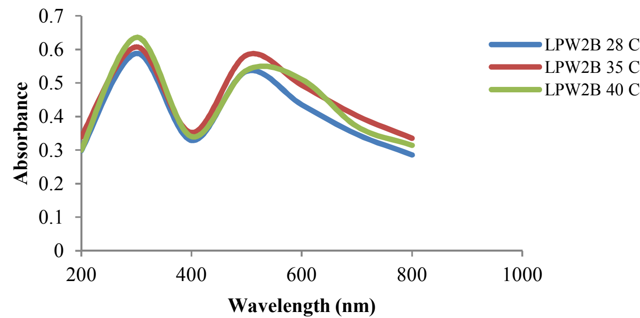

Fig. 5a. UV-visible spectra of LPW2B SNPs at different temperature

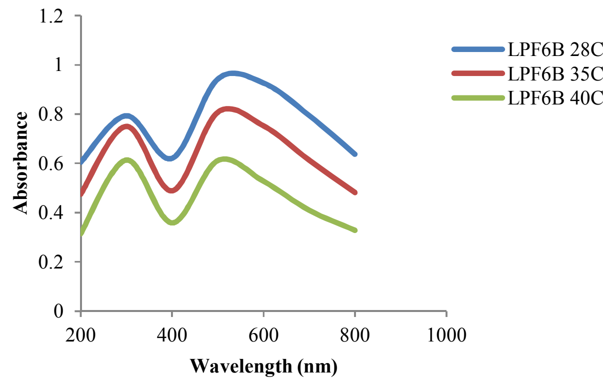

Fig. 5b. UV-visible spectra of LPF6B SNPs at different temperature

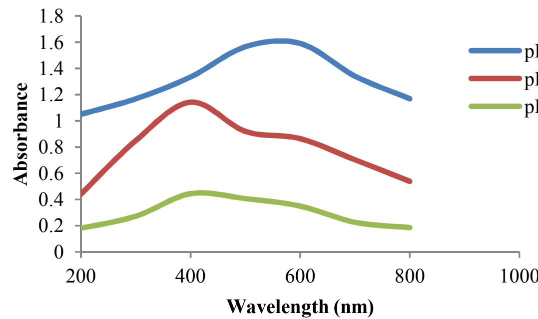

Fig. 6a. UV-visible spectra of biosynthesized *Lactobacillus casei* LPW2 SNPs at different pH

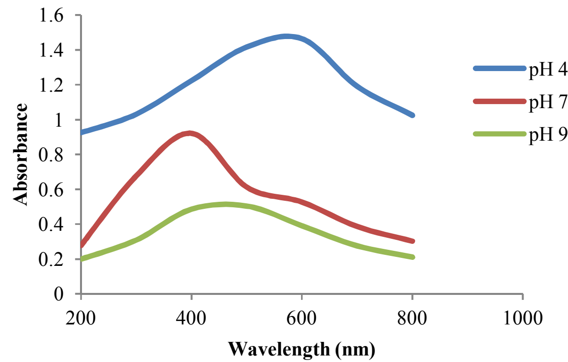

Fig. 6b. UV-visible spectra of LPF6B SNPs at different pH of production

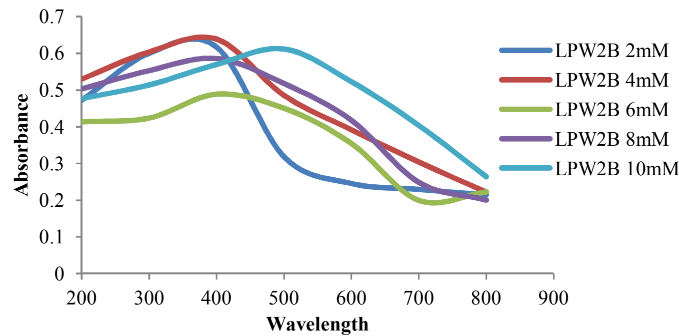

Fig. 7a. UV-visible spectra of LPW2B SNPs at different concentrations of $AgNO_3$

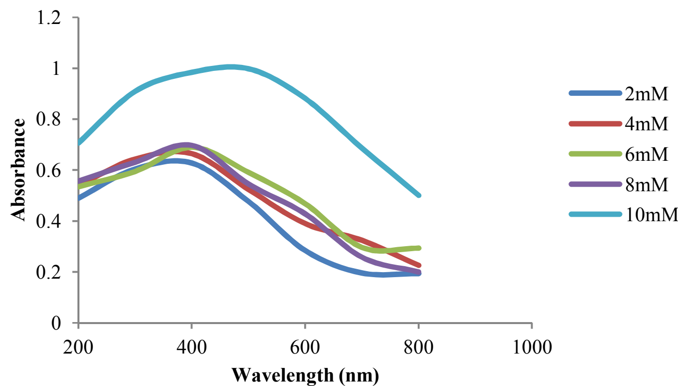

Fig. 7b. UV-visible spectra of LPW2B SNPs at different concentrations of $AgNO_3$

4. CONCLUSION

In conclusion, supernatant from *Lactobacillus casei* LPW2 and *Lactobacillus fermentum* LPF6 was able to bioreduce $AgNO_3$ for the biosynthesis of SNPs. 28°C, pH 4 and 10 mM was the best condition for SNPs production by the supernatant of the LAB strains. SEM analysis of the SNPs revealed partially aggregated SNPs with varying shape and size. FTIR analysis of the biosynthesized SNPs revealed the presence of Carboxyl, hydroxyl, amino acids and proteins. The biosynthesized SNPs had antibacterial activity against the indicator organisms. Finally,

for production of stable and functional SNPs, sample preparations and drying process of the SNPs should be taken into consideration.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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