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ORIGINAL ARTICLE

Biogenic synthesis and antimicrobial activity of Silver nanoparticle using exopolysaccharides from Lactic Acid bacteria

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Abstract

Nanotechnology provides the ability to engineer the properties of materials by controlling their size, and this has driven research toward a multitude of potential uses for nanomaterials. This study aimed at biosynthesis and characterization of silver nanoparticles (SNPs) using exopolysaccharides (EPS) of lactic acid bacteria (LAB) and the antimicrobial potential of the biosynthesized SNPs against some pathogenic bacteria. EPS production by the EPS- producing *Lactobacillus casei* (LPW2E) and *Lactobacillus fermentum* (LPF6) using submerged fermentation ranged from 256-640.9 mg/L. The EPS produced by the two LABs were used for the biosynthesis of SNPs. The SNPs were characterized by colour changes from colourless to yellowish brown and deep brown after 24 hrs of incubation. The UV-visible spectrophotometer was further used to characterize the SNPs. The SNPs had strong surface plasmon resonance band at 500 nm. Scanning electron microscopic (SEM) analysis showed that the SNPs varied in shape and were partially aggregated. The particle size ranged from 0.2 nm–10 nm and 0.0–10 nm. The FTIR analysis indicated the presence of functional groups such as hydroxyl, carboxyl, ester, aldehydes among others which may be responsible for the reduction, capping, and stabilization of the SNPs. The SNPs had antibacterial activity against the test pathogens and the zones of inhibition ranged between 12-26 mm. In conclusion, this study demonstrated that EPS could be used for the production of stable SNPs with antibacterial activity.

Keywords: *Exopolysaccharides; FTIR; Lactic acid bacteria; SEM; Silver nanoparticles.*

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INTRODUCTION

Nanotechnology is the science involving the synthesis and stabilization of various nanoparticles. Nanobiotechnology represents an economic alternative to chemical and physical methods of nanoparticles formation [1]. A nanoparticle is defined as a small object that behaves as a whole unit in terms of its transport and properties. The physical, chemical and biological properties of the nanoparticles differ from the bulk materials. Formerly, nanoparticles are synthesized through physical and chemical methods. These Physical and chemical methods for nanoparticles synthesis are expensive and involve the production of toxic by-products which are environmentally not safe methods hence the need for an alternative method. Biological method of nanoparticles synthesis emerges as a means to produce nanoparticles that are safe and eco-friendly. The synthesis of metal nanoparticles using biological systems is an expanding research area due to the potential applications in nanomedicines [2]. Bacteria, Fungi, Yeast and Algae are the microorganisms explored for their potentials in nanoparticles synthesis [3, 4]. Of all the metals used in nanoparticles synthesis, Silver has been the most studied. Silver nanoparticles of a size smaller than 100 nm contain about 10,000-15,000 silver atoms and they received the greatest attention due to their wide spectrum of antimicrobial activity [4].

Polysaccharides are a diverse group of biological macromolecules that are found to occur in several organisms. They are natural, non-toxic, and biodegradable polymers that cover the surface of most cells and play important roles in various biological mechanisms such as

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immune response, adhesion, infection, and signal transduction [5]. The EPS produced by LAB can be applied as natural additives as well as being produced in situ. All these polysaccharides can be homopolymers or heteropolymers of neutral sugars (pentoses and hexoses) or anionic sugars (hexoses), substituted or non-substituted with non-sugar compounds attaining linear or ramified final conformations [6, 7]. Functions like gliding motility, protection against osmotic shock, predation, desiccation and detoxification of toxic compounds, nutrient sequestering and chelation of metals etc. have also been attributed to microbial EPS. The polysaccharides are now emerging as stabilizing and reducing agents for nanoparticles synthesis as natural polysaccharides such as chitosan, heparin, gum acacia polymer and gum kondagogu, were used in the preparation of silver nanoparticles [8]. This is because the macromolecular chain of EPS contains many hydroxyl groups that strongly associate with metal ions which enable the control of shape size and particle dispersion [9]. Nanoparticles have found applications in all fields of human life, including medicine, surgery, cosmetics, biophysics, chemistry, space, electronics, agriculture, sports, and more [10]. This study is aimed at production and characterization of SNPs using EPS of LAB.

EXPERIMENTAL

Culture collection

EPS- producing *Lactobacillus casei* and *Lactobacillus fermentum* previously isolated from fermented food was collected from the culture collection of the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Nigeria. The culture was kept in maintenance medium (MRS broth with 12% v/v glycerol) and the stock culture was stored at 4 ^{o}C and sub-cultured from time to time to regulate its viability.

Inoculum preparation

The working culture was prepared by transferring 0.5 ml of the stock frozen culture to 10ml of MRS broth and incubated for 24 hrs at 30^{o}C. The resulting culture was then transferred to modify Exopolysaccharide Selection Medium (mESM) containing 5% (w/v) skim milk (Oxoid), 0.35% yeast extracts (Oxoid), 0.35% peptone (Difco), and 5% glucose (BDH) and incubated at 30 ^{o}C for 16 hrs [11]. For the large scale production, 10 ml inocula of the 16-hour old culture were used.

Production, Isolation and Quantification of EPS by the isolates using submerged fermentation

The isolates were grown in MRS broth for 24 - 48hrs and were then transferred to the modified exopolysaccharide selection medium (mESM) [11]. This was done in the ratio 1:10 (1ml of the isolate in broth to 10ml of mESM) and incubated for 24hrs. The fermented broth was boiled in a water bath at 100^{o}C for 15minutes to inactivate the enzymatic activities of the LAB and then cooled to room temperature. The EPS was isolated by treating the fermented broth with 17% (v/v) of 80% trichloracetic acid (TCA) solution and centrifuged at 16,000 rpm for 30 min. The EPS were precipitated by adding 3 volumes of cold absolute ethanol and stored overnight at 4^{o}C. The sample was centrifuged (12,000 rpm for 15 min) and the recovered precipitates were redissolved in distilled water and recovered by adding 20 ml of water. The EPS production was expressed as mg/L. The total amount of carbohydrates in the polysaccharides was determined by the phenol-sulfuric acid method described by Dubois *et al.* [12]. Glucose was used as standard and the result was expressed in milligrams of glucose per liter.

Biosynthesis of SNPs using EPS produced by LAB

The biosynthesis of silver nanoparticles using the EPS was done according to the modified method of Kanmani and Lim [9] with some slight modifications. 20ml of EPS solution was mixed with 20ml of 10mM aqueous solution of silver nitrate ($AgNO_3$) prepared freshly in deionized water under stirring conditions. The mixture was incubated at room temperature in a dark place for 24-48 hrs. Formation of yellowish brown colour indicates the SNPs formation.

Characterization of the biosynthesized SNPs

Visual detection and UV-visible spectrophotometer of the SNPs

EPS from LAB-treated with $AgNO_3$ solution 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 silver nanoparticles.

The reduction of silver ions (Ag^{+}) to silver nanoparticles (Ag^{0}) was spectrophotometrically identified by the UV-Visible spectrophotometer with a resolution of 0.5 nm. The absorbance of the sample was read using UV-visible spectrophotometer at the wavelengths of 200-800 nm.

Scanning electron microscopic (SEM) analysis of the SNPs

SEM of the biosynthesized SNPs 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).

Fourier Transform Infra-Red (FT-IR) analysis of the EPS and SNPs

The biosynthesized SNPs 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 a resolution of 4cm^{-1}. The dried samples were powdered with KBr pellets and pressed into a mold and spectra were recorded at a wave range of 500-4000 cm^{-1}.

Antibacterial activity of the SNPs

The antibacterial activity of the biosynthesized SNPs was done using agar well diffusion method described by Shivashankar *et al*. [13]. 18hrs old culture of the pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyrogens*, *Klebsiella pneumoniae* 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 7 mm was used to cut uniform wells in the agar. Each well was filled with 100 µl of the various biosynthesized SNPs. The plates were incubated appropriately. After incubation at 37 °C for 24 hrs, the plates were observed for the zone of inhibition (ZOI) around the wells.

Table 1: Production of EPS by the LAB strains using submerge fermentation.

S/N	Isolate Code	EPS Production (mg/L)
1	LABLPOG4	275
2	LABLPOG7	432
3	LABLPF6	518.7
4	LABLPF19	464.7
5	LABLPF22	256
6	LABLPW2	640.9
7	LABLPW6	458.9
8	LABLPW6b	346.3
9	LABLPW13	480.6
10	LABLPW14	356.5

RESULTS AND DISCUSSION

Production of EPS by the EPS-producing LAB

The EPS- production by the LAB strains using submerged fermentation is shown in Table 1. Some of the LAB strains were able to produce EPS in reasonable quantity. EPS production ranged from 256mg/L-640.9 mg/L. The highest yield was obtained from LPW2 followed by LPF6 while the least yield was obtained from LPF22. All the LAB strains produced a reasonable quantity of EPS.

Some of the LAB strains screened on modified MRS agar were positive for EPS production. This result agreed with the work of De Vuyst *et al*. [14] who reported that LABs are capable of producing EPS. This result is in agreement with the work of Van den Berg *et al*. [11] who reported that 30 out of their 607 LAB were capable of EPS production. The yield of the EPS in this study was high and ranged between 256-640.9 mg/L. This is in agreement with the work of Savadogo *et al*. [15] who reported EPS yield of between 160-740mg/L. Similarly, Mostefaoui *et al*. [16] reported a high yield of EPS among the *Lactobacilli* strains. A small amount of nitrogen present in the EPS production medium may account for the higher yield in EPS observed in this study. This agrees with the findings of Garcia-Garibay and Marshall [17] and Adebayo-Tayo and Onilude [18] who reported the production of EPS from LAB grown in modified MRS broth.

Characterization of the biosynthesized SNPS

Visual detection and UV-visible spectrophotometer analysis of the SNPs

The addition of silver nitrate to EPS followed by incubation at room temperature resulted in the production of SNPs as the colourless solution turned yellowish brown (Fig. 1a and 1b).

The spectrum of the characterized LPW2E SNPs and LPF6E SNPs using UV-Visible spectrophotometer is shown in Fig. 2a and 2b. For sample LPW2E SNPs, at 24hrs a broadband between 400 nm and 500nm was observed which signify the presence of spherical shape SNPs. The peak shows that the SNPs have a sharp Plasmon absorption band of 500 nm at 48hrs.

For sample LPF6E SNPs, the broadband was observed between 300nm and 600nm for the SNPs at 24hrs. At 48hrs, the absorbance increased from 200 nm, peaked at 500 nm and then declined down to 800 nm. The SPR peak was observed at 500nm for the 48hrs incubation time and it was stable. Generally, the majority of the SNPs peaked at 500nm and the spectra at 48hrs were higher and more stable.

The biosynthesis of SNPs was done using the EPS produced by the selected EPS- producing LAB strains and the colour change was observed. The formation of SNPs can usually be confirmed by colour change using visual detection. Kanmani and Lim [9] also observed a colour change in their EPS SNPs from colourless to yellowish brown. This result is in agreement with the work of Shivashankar *et al*. [13] who observed a change in colour of the SNPs from fungal filtrate from colourless to yellowish brown.

Characterization of the SNPs can also be done by measuring the surface plasmon resonance (SPR) with a UV-Visible spectrophotometer. SPR is important in the determination of metal nanoparticle size which increases with wavelength. According to Hebeish *et al*. [19] SNPs exhibit unique and tunable optical properties due to their strong SPR transition. A UV-visible spectrophotometer can provide other information about morphology, size, and stabilization. The

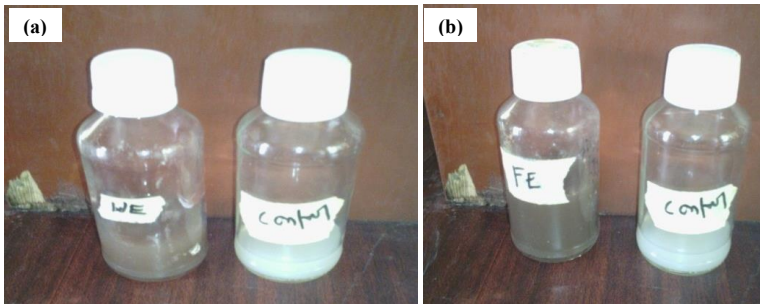

Fig. 1: a and b) Visual characterization of LPW_2 EPS SNPs (a) and LPF6 EPS SNPs (b) at 24 hours.

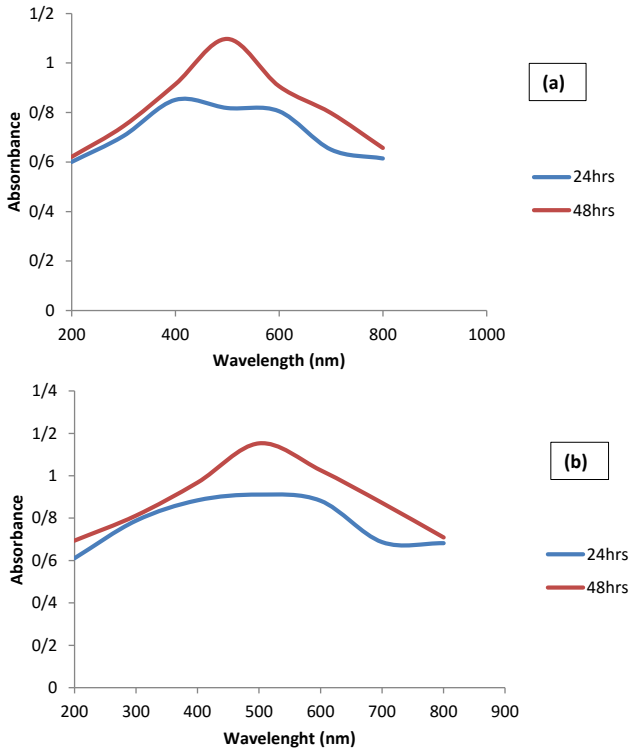

Fig. 2: a) UV-Visible spectra of LPW2E SNPs, b) UV-Visible spectra of LPF6E SNPs.

biosynthesized EPS SNPs in this study showed a strong SPR peak at 500nm. Typically, SNPs have a maximum wavelength in the visible range of 400-550nm [20]. The observation is in agreement with the work of Kanmani and Lim [9] whose SNPs had a strong SPR peak between 400-550 nm with a broadband. The results are also in agreement with the work of Valencia *et al*. [21] who reported the formation of SNPs with SPR peak between 411 and 414nm. However, the result is not in agreement with the work of Shivashankar *et al*. [13] whose SNPs had an SPR band at 386nm.

Scanning electron microscopic analysis of the SNPs

The SNPs were further characterized by SEM. Fig. 3a and 3b shows the SEM micrographs of sample LPW2E SNPs and LPF6E SNPs. For sample LPW2E SNPs the shape and size of the SNPs vary and the SNPs were partially aggregated with particle size ranged from 0.2 -10nm. For sample LPF6E SNPs the SNPs were partially aggregated and the particle size ranged from 0.0–10nm.

SEM is an important tool for SNP's characterization. The SNPs in this study have shapes ranging from rectangular to spherical. Similarly, production of SNPs with varying shapes has been reported by Kanmani and Lim [9]. The SEM analysis of the SNPs revealed that they were partially aggregated. This may occur due to the drying process. Sadowski *et al*. [22] also observed a similar thing and concluded that sample preparation including drying can affect the shape and size of the SNPs.

FTIR Analysis of the SNPs

The FTIR spectrum of LPW2E SNPs is shown in Fig. 4a. 17 absorption peaks ranging from 3381.33 - 551.66 cm^{-1} were observed. The peak located at 3381.33 cm^{-1} and 3284.88 cm^{-1} could be attributed to the N-H stretching vibration of amide and OH stretching vibration of the hydroxyl group. The absorption peak at 2928.04 cm^{-1} and 2875.96 cm^{-1} indicated C-H stretching vibration of aldehyde. The absorption peak at 2360.95 corresponded to the – COOH overtone acid group. The peak at 1743.71 cm^{-1} could be attributed to C=O stretch of saturated esters. The absorption peak at 1654.98 indicated the presence of C=O stretching of the amide or carboxyl group. The absorption peak at 1543.10 cm^{-1}, 1384.94 cm^{-1} and 1259.56 cm^{-1}

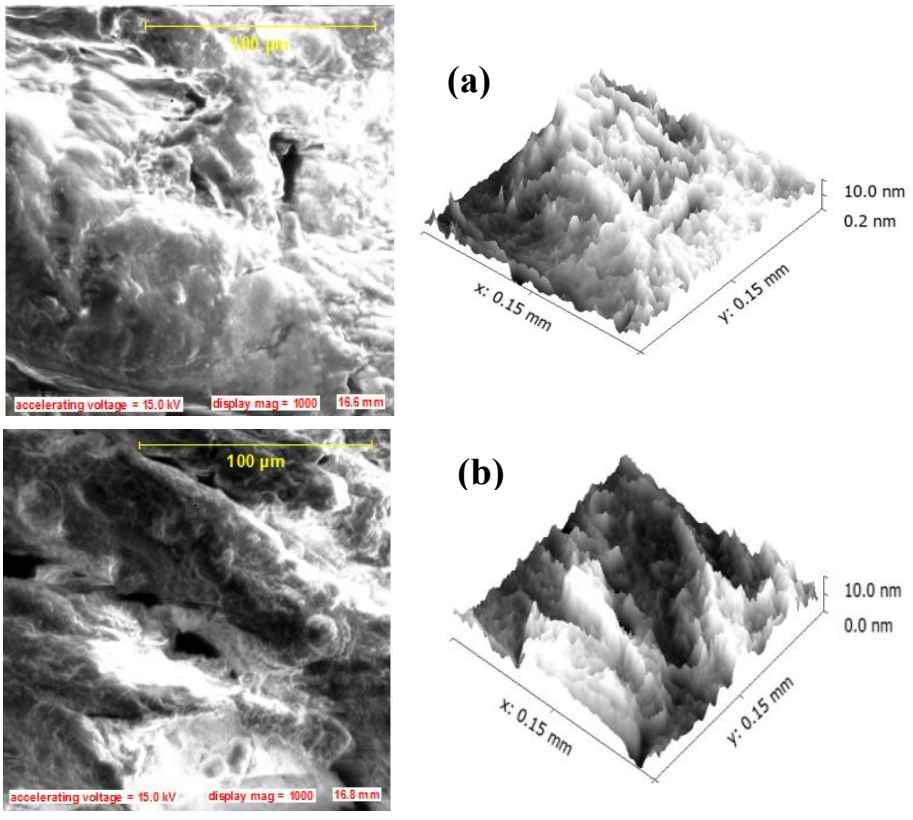

Fig. 3: a) Scanning electron micrograph of LPW2E SNPs, b) Scanning electron micrograph of LPF6E SNPs.

corresponded to CN bending vibration of amide II, C-H stretching vibration of aromatic and aliphatic amine and C-O stretch of carboxylic esters. The absorption peak at 1116.82 cm^{-1} and 1072.46 cm^{-1} corresponded to C=O stretch, C-H in the plane bend of mono- substituted alkenes and OH stretch of alcohol. The absorption peak at 991.44 cm^{-1} indicated the presence of C-H, C-C, and C-OH ring and side group vibration of carbohydrates. The absorption peaks at 827.49 cm^{-1} and 925.86 cm^{-1} indicated that linkages had occurred between the monosaccharide. The absorption peak at 777.34 cm^{-1} corresponded to C-H stretching of mono-substituted benzene while the peak at 551.66 cm^{-1} could be attributed to the S-S stretch of disulfides. All the functional groups observed indicated that monosaccharides, aldehydes, hydroxyl, carboxyl, esters and amino acids could be responsible for the formation of SNPs (Fig. 4a).

Exopolysaccharide produced by LPF6E was characterized using FTIR and 21 bands were present at 3389.04, 2974.33, 2901.04, 2360.95, 1618.33, 1568.18, 1539.25, 1435.09, 1390.72, 1305.85, 1273.06, 1078.24, 1051.24, 922.00, 889.21, 840.99, 736.83, 669.32, 570.95, 532.37 and 447.50 cm^{-1} as shown in Fig. 4b. The peak at 3389.04 has been identified as O-H stretch of alcohol. The peaks at 2974.33 and 2901.04 indicated the presence of C-H symmetrical stretch. The peak at 2360.95 was also attributed to the presence of COOH overtone. The peak at 1618.33 could be attributed to the presence of C=O stretch of amide. The peaks at 1568.18 and 1539.25 could be attributed to NH bend. The presence of CH_2 plus COH and CCH was shown by the peaks at 1435.09, 1390.72 and 1305.85. The peaks at 1273.06, 1078.24 and 1051.24 indicated the presence of C-O stretch of alcohol. The presence of C-O, CCH and vibration ring of pyranose was shown by the peak at 922. The peaks 889.21, 840.99 and 736.83 showed the presence of CH, CCO, and CCH functional groups. The peaks at 669.32 up to 447.50 indicated the presence of acetylenic CH of alkynes.

From the observations in the spectrum, the presence of -COOH and OH groups was an indication that the sample was an exopolysaccharide. The FTIR spectrum revealed that the EPS is complex polysaccharide containing different functional group in addition to functional group reported earlier in polysaccharide structure (Fig. 4b).

FTIR Spectrum of LPW2E SNPs

Fig. 4c shows the FTIR spectrum of LPF6E SNPs. The peak at 3344.68 cm^{-1} indicated the presence of N-H stretch of a primary amide (bonded) and OH stretching vibration. The peak at 2928.04 cm^{-1} could be attributed to C-H stretch of aldehyde. The peak at 2366.74 cm^{-1} indicated the presence of –COOH overtone acid group. C=O stretch of saturated esters was given by the absorption peak at 1745.64 cm^{-1}.

The absorption peak at 1651.12 cm^{-1} referred to the C=C terminal olefin. Absorption peaks at 1568.18 cm^{-1} and 1541.18 cm^{-1} corresponded to the N-H secondary amide. The peaks at 1437.02 cm^{-1}, 1384.94cm^{-1}, and 1263.42 cm^{-1} could be attributed to the C-O stretch plus OH carboxylic acids, symmetrical C-H bends and C-O stretch of carboxylic esters respectively. Moreover, the peak at 991.44 cm^{-1} corresponded to the C-H out of plane bend indicating the presence of a carbohydrate. The peak at 887.28 cm^{-1} could be C-O stretch or C-N vibration of amine. The absorption peak at 840.99 cm^{-1} could be attributed to C-H skeletal. 603.74 cm^{-1} and 549.73 cm^{-1} peaks indicated the presence of acetylenic C-H bend of alkynes and S-S stretch of disulfide respectively. The absorption peaks between 840 cm^{-1} and 887.28 cm^{-1} indicated that linkages had occurred between the monosaccharides. The peaks observed for the EPS stabilized SNPs differ from the pure EPS produced by the same organism. The hydroxyl and the carboxylic groups in the polysaccharide, esters, aldehyde and some amino acids may be responsible for the synthesis and stabilization of the LPF6E SNPs (Fig. 4c).

The possible functional groups of the EPS responsible for reduction and stabilization of the SNPs was analyzed by FTIR. The FTIR of the SNPs differs from the pure EPS. This observation suggests that there is a strong interaction of silver with the EPS functional groups. Pandey *et al*. [23] pointed out that the hydroxyl groups in polysaccharides have an efficient coordinating ability with silver. Aldehyde, amino acids, esters, ether, carboxyl and hydroxyl groups among others may be the functional groups responsible for the SNPs formation. This result is in agreement with the observation of Udayasoorian *et al*. [24] and Nanda and Raghavan [25] who equally characterized their SNPs and EPS SNPs by FTIR.

Antibacterial activity of the SNPs

The synthesized SNPs were evaluated for their antibacterial activity against some pathogenic

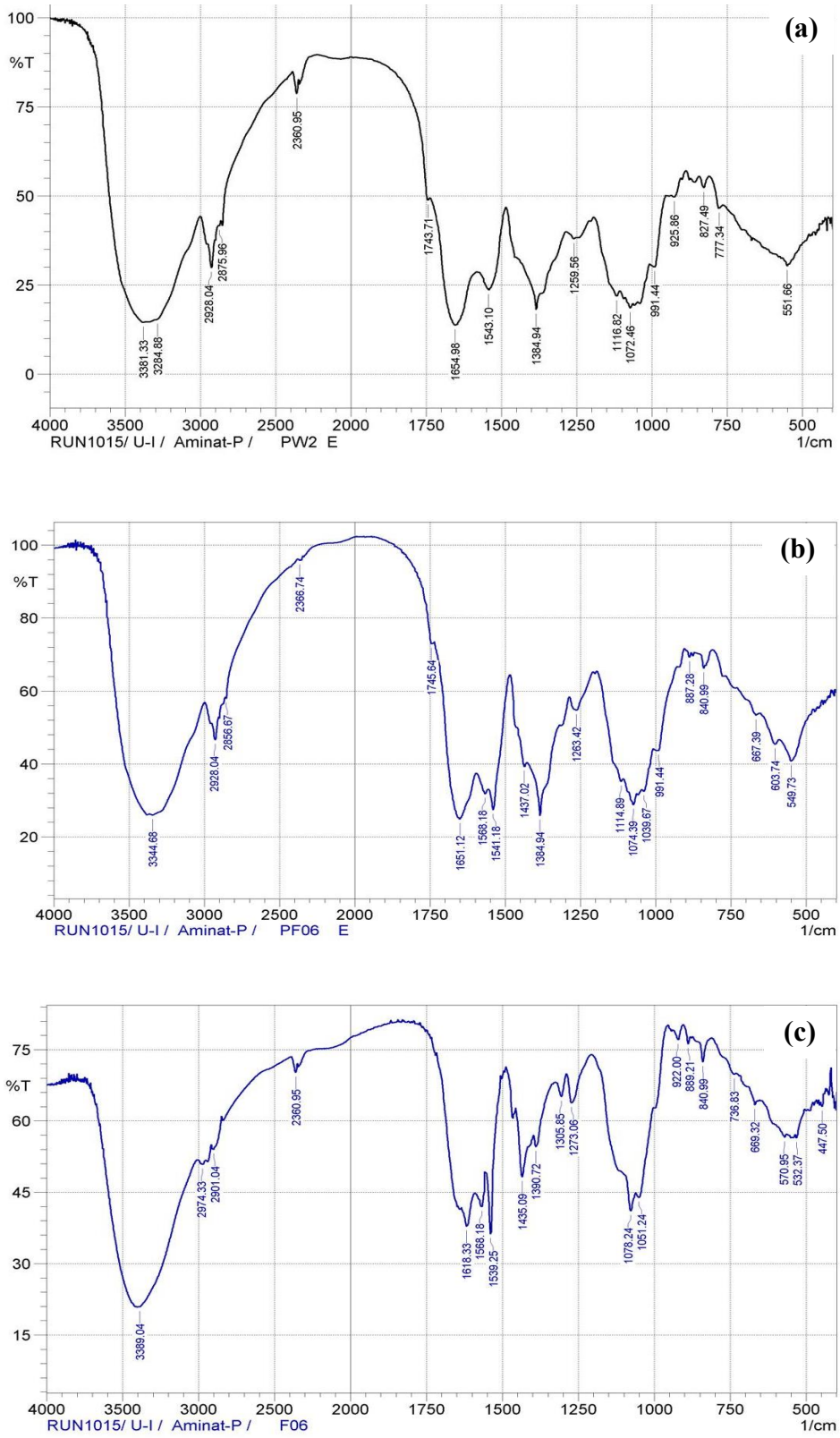

Fig. 4: a) FTIR Spectrum of LPW2E SNPs, b) FTIR Spectrum of LPF6E SNPs, c) FTIR Spectrum of Exo-polysaccharide produced by LABLPF6E SNPs.

Table 2: Antibacterial activity of the synthesized SNPs.

Pathogens	Diameter of zones of inhibition			
	Lactobacillus casei EPS SNPs	*Lactobacillus fermentum* EPS SNPs	Ciprofloxacin	$AgNO_3$
Bacillus sp.	25	26	20	15
Streptococcus pyogenes	23	22	24	10
Staphylococcus aureus	15	12	17	11
Klebsiella sp.	15	15	15	9
Pseudomonas aeruginosa	13	12	10	8

bacteria as presented in Table 2. LABLPW2 EPS SNPs and LABLPF6 had the highest antibacterial activity against *Bacillus* species with a zone of inhibition 25mm and 26mm. *Bacillus* species was the most susceptible to the two SNPs followed by *Streptococcus pyrogens* with a zone of 23mm and 22mm respectively. *Pseudomonas aeruginosa* had the least susceptibility to the two SNPs. The positive control (Ciprofloxacin and $AgNO_3$) had antibacterial activity against the pathogen but not as much as the biosynthesized SNPs.

The antibacterial activity of the SNPs is well established and several mechanisms for their bactericidal effects have been proposed. It is reported that the bactericidal activity decreases as the size increase and it is also affected by the shape of the particles. The biosynthesized EPS SNPs had antibacterial activity against the test pathogens. This result is similar to the work of Valencia *et al*. [21] who reported that their SNPs produced from soluble starch has antibacterial activity against *Staphylococcus aureus*. Similarly, Rajawat and Qureshi [2] reported that their SNPs were effective against pathogenic *Salmonella typhi*.

In this study, Gram-positive bacteria were more susceptible to the SNPs than their Gram-negative counterpart. Wei *et al*. [26] observed a similar situation in their work where *Bacillus subtilis* was more susceptible to the SNPs than *E. coli*. This result is also in agreement with the work of Yakout and Mostafa [27] who observed that the Gram-positive bacteria were more susceptible to their SNPs formed using soluble starch. In contrary, this work is not in agreement with the work of Priyadarshini *et al*. [28] that reported that *Escherichia coli* showed a greater inhibition zone compared to *Bacillus cereus*. The results contrast the report of Gurunathan *et al*. [29], where *Pseudomonas aeruginosa* and *Shigella flexneri* were more susceptible to the SNPs than *Staphylococcus aureus* and *Streptococcus pneumoniae*.

CONCLUSION

EPS from the EPS- producing *Lactobacillus casei* (LPW2E) and *Lactobacillus fermentum* (LABLPF6) was able to reduced $AgNO_3$ for the production of SNPs. Changes in colour which indicate SNPs was observed. The biosynthesized SNPs had a strong SPR at 500 nm and it was partially aggregated with varying shape and sizes. The SNPs had a varied functional group. The EPS stabilized SNPs had antibacterial activity against both Gram-positive and Gram-negative bacteria with the former showing more susceptibility hence can be used as broad-spectrum antimicrobial. The biosynthesized LPW2 EPS SNPs and LPF6 SNPs had the highest antibacterial activity against some of the test pathogens compare to Ciprofloxacin and $AgNO_3$.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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