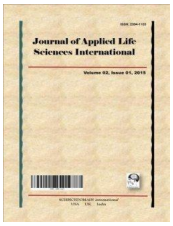

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Green Synthesis of Silver Nanoparticles Using Supernatant from *Lactobacillus casei* LPW2 Cultured in Modified Exopolysaccharides Selection Medium

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Authors' contributions

This work was carried out in collaboration between both authors. Author ATBC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author PAO carried out the bench work under the supervision of author ATBC. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: Biosynthesis of silver nanoparticle using supernatant from *L. casei* cultured in modified exopolysaccharide selection medium.
Study Design: To bio-reduced $AgNO_3$ using supernatant from *L. casei* cultured in modified exoploysaccharides selection medium and to evaluate the antibacterial potential of the biosynthesized SNPs.
Place and Duration of Study: Department of Microbiology, University of Ibadan, Ibadan, Oyo State, Nigeria between Jan to December 2016.
Methodology: Production and characterization of SNPs using supernatant from *L. casei* cultured in modified Exopolysaccharides selection medium and to evaluate the antibacterial activity of the SNPs.
Results: Nanotechnology has to do with the manufacture of materials at the nanometer level.

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Clusters of atoms in the size range of 1 – 100 nm are known as nanoparticles. Biosynthesis of silver nanoparticles (SNPs) using supernatant of lactic acid bacteria (LAB) culture in modified Exopolysaccharides selection medium (mESM) was investigated. The supernatant obtained from *L. casei* LPW2 cultured in modified exopolysaccharides selection medium was used in the bio-reduction of $AgNO_3$. The reaction mixture turned deep brown after 24 hrs of incubation indicating the formation of SNPs. The SNPs was characterized with UV-Visible spectrophotometer and it had a broad band between 400 – 600 nm with strong surface plasmon resonance at 500 nm. The FTIR analysis revealed the presence of carboxylic acids, hydroxyl group, amino acids and protein as the possible functional groups responsible for the bioreduction of silver to its nanoparticles. The antibacterial potential of the SNPs was evaluated and the zone of inhibition ranged from 13 – 24 mm with *Bacillus* sp. being the most susceptible. The minimum inhibitory concentration (MIC) of the biosynthesized SNPs was investigated and an MIC of 3.125% was observed on the tested pathogens.

Conclusion: In conclusion, supernatant from LAB cultured using mESM can be used for the production of SNPs with potent antibacterial activity on Gram positive bacteria.

Keywords: L. casei; supernatant; mESM; SNPs; MIC; $AgNO_3$.

1. INTRODUCTION

Lactic acid bacteria (LAB) are classified as Gram positive, non-motile, fastidious, catalase negative, devoid of cytochrome, non-sporulating, acid tolerant organism that produce lactic acid as a major or sole product of fermentative metabolism [1]. They are categorized into different genera based on morphology, mode of glucose fermentation, configuration of the lactic acid produced, ability to grow at high salt concentrations and other growth physiology [2]. They have found application industrially and this is because of their fermentative ability as well as their health and nutritional advantages [3]. Exopolysaccharides are high molecular weight polysaccharides found external to the cell of microorganism. The LAB genera known for production of EPS are *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and *Pediococcus* [4]. Types of EPS produce by LAB based on location and sugar constituent are intracellular polysaccharides and extracellular polysaccharides and homo and hetero exopolysaccharides respectively. EPS produced by LAB are widely used to improve the body and texture of fermented food products [5]. Polymeric Exopolysaccharides produced by microorganisms were complex natural biological polymer mixture of proteins and polysaccharides. It played an important role in the transport of metals such as Ag^+ [6].

The word nano is from the Greek word meaning dwarf. According to the National Cancer Institute, Nanotechnology can be defined as development of technology at the atomic, molecular, or macromolecular level of approximately 1–100 nm to create and use structures, devices, and systems that have novel properties [7]. The use of biological system for nanoparticles production can be classified under wet nanotechnology and is referred to as green or biological method of nanoparticles synthesis. Green synthesis of nanoparticles is an alternative to chemical and physical methods because of its many advantages such as cost effectiveness and environment friendliness [8]. This study aimed at the biosynthesis of Silver nanoparticles by *Lactobacillus casei* EPS-Producing lactic acid bacteria.

2. MATERIALS AND METHODS

2.1 Culture Collection

EPS- producing *Lactobacillus casei* 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 were stored at 4°C and sub-cultured from time to time to regulate its viability.

2.2 Inoculum Preparation

Sterile MRS broth (10 mL) was inoculated with 0.5 ml of the fresh culture. The inoculated medium was incubated for 24 hrs at 30°C. The resulting culture was then transferred to Modify Exopolysaccharide Selection Medium (mESM) [9] containing 5% (w/v) skim milk (Oxoid), 0.35%

yeast extracts (Oxoid), 0.35% peptone (Difco), and 5% glucose (BDH) and incubated at 30°C for 16 hrs. For the large scale production, 10 ml inocula of the 16-hour old culture were used.

2.3 Production of Silver Nanoparticle Using Biosynthesis of SNPs by the LAB

The clarified supernatant obtained from LAB culture grown in mESM, treated with 17% (w/v) of 80% trichloroacetic acid solution and centrifuged at 12,000 rpm at 4°C for 15 min was used for the production of SNPs. 1 ml of the LAB-mESM supernatant was challenged with 10 ml of freshly prepared 10 mM silver nitrate (AgNO3) 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.

2.4 Characterization of the Synthesizes SNPs

2.4.1 Visual detection of the SNPs

The LAB-mESM 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 yellowish brown indicate formation of silver nanoparticles.

2.4.2 UV-visible spectrophotometry characterization of the SNPs

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

2.4.3 Scanning electron microscopic (SEM) and Fourier transform infra-red (FT-IR) analysis of the SNPs

SEM of the biosynthesized SNPs was used to define the morphology of the SNPs. The aqueous solution of SNPs synthesized were dried and subjected to scanning electron microscopy (SEM).

Characterizations of the biosynthesized SNPs were done 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}. 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}.

2.4.4 Antibacterial potential of the SNPs

The antibacterial potential of the biosynthesized SNPs was done using agar well diffusion method [10]. 18 hrs old young culture of *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Bacillus* sp. 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 at 37°C for 24 hrs. After incubation the plates were observed for zone of inhibition (ZOI). Results were considered positive if the diameter (mm) of the ZOI was greater than 1mm [11].

2.4.5 Determination of minimum inhibitory concentration (MIC) of the biosynthesized SNPs

MIC was carried out to find out the lowest concentration of the biosynthesized SNPs that will inhibit the growth of selected pathogenic microorganism was done using the modified method of Balashanmugam et al. [12]. Different concentration (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) of the LAB-mESM SNPs was prepared by using a two-fold dilution with sterile distilled water. 5 ml of sterile water was dispensed into five different sterile test tubes and 5 ml of the first dilution taken and dispensed into the second dilution etc. A lawn of the indicator strains 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 6 mm was used to cut uniform wells in the agar. Each well was filled with 100 µL of the synthesized SNPs. The lowest dilution of the SNPs at which zones of inhibition was observed against the indicator organisms is regarded as

the minimum inhibitory concentration for each SNPs. Ciprofloxacin was used as positive control.

3. RESULTS AND DISCUSSION

3.1 Biosynthesis and Characterization of SNPs

The visual study of the biosynthesized LAB-mESM SNPs is shown in Plate 1. After 24 hrs of incubation, the reaction mixture turned yellowish-brown then deep brown in colour indicating the formation of SNPs. This result correlates with that of Phanjom and Ahmed [13] who reported the formation of dark brown colour for their SNPs and attributed the colour formation to the surface plasmon resonance of silver. Similarly, Manzoor-ul-haq et al. [14] confirmed the formation of their SNPs based on colour change from yellow to dark brown within 24 hrs.

3.2 UV-Visible Spectrophotometric Analysis of the SNPs

The LAB-mESM biosynthesized SNPs were characterized with UV-Visible spectrophotometer and the spectra obtained were given in Fig. 1. The biosynthesized SNPs had a broad band between 400 and 600 nm after 24 hrs of incubation. While 48 hrs incubation period, the biosynthesized SNPs had a strong SPR peak at 500 nm. The intensity of the SNPs is time dependent as the spectrum at 48 hrs was higher and more stable. According to Basavaraj et al. [15] there is a correlation between the position and shape of the plasmon absorption of nanoparticles and particle size, dielectric constant and surface adsorbed species. This result is in agreement with that of Kanmani and Lim [16] who reported the formation of SNPs with strong SPR between 400 and 550 nm. It is also in agreement with the work of Dhanalakshmi and Rajendran [17] that observed that the reaction media of their *Tridax procumbens* mediated SNPs has absorbance peak at 460 nm.

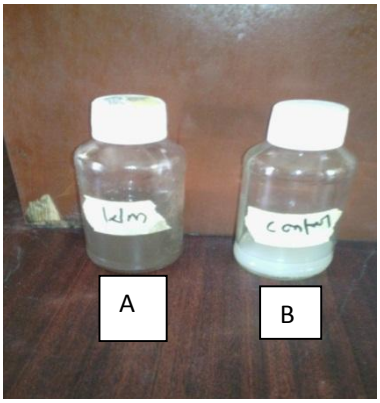

Plate 1. Visual characterization of *Lactobacillus casei* LPW2-mESM SNPs at 24 hours

The scanning electron micrograph of the SNPs biosynthesized using the supernatant of *L. casei* culture using mESM is shown in Fig. 2. The particle size ranged from 0.2 – 10 nm with aggregated shape. Different sizes and shapes of nanoparticles have been reported by several authors [16,18].

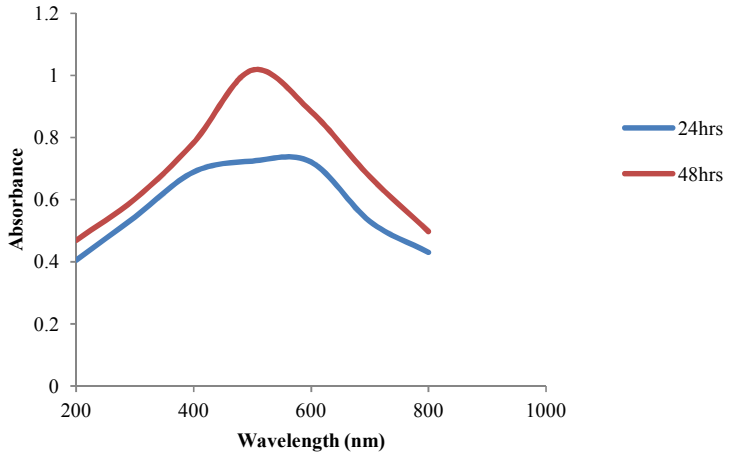

Fig. 1. UV-visible spectra of *Lactobacillus casei* LPW2- mESM SNPs

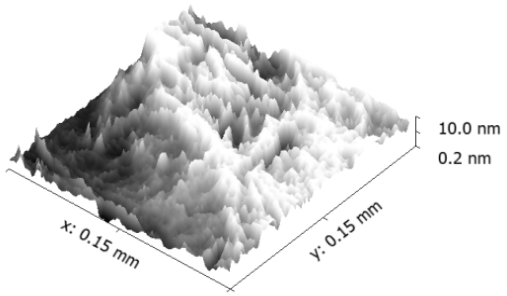

Fig. 2. SEM micrograph of SNPs from supernatant of *L. casei* culture using mESM

3.3 FTIR Spectrum of the Biosynthesized SNPs

Fig. 3 shows the FTIR spectrum of the biosynthesized supernatant of *Lactobacillus casei* LPW2- mESM SNPs and absorption peaks ranging from 3383.26 cm^{-1} to 551.66 cm^{-1} were observed. The broadest absorption peak observed at 3383.26 cm^{-1} indicated the presence of bonded OH stretching vibration and aliphatic N-H stretch of primary amine. The absorption peak at 2933.83 cm^{-1} corresponded to C-H stretching vibration of aldehydes. The peaks at 2360.95 cm^{-1} could be attributed to -COOH overtone acid group. The absorption peaks at 2077.40 cm^{-1}, 1627.97 cm^{-1} and 1597.11 cm^{-1} corresponded to C=C stretching of alkenes, C=O stretch of carboxylates, C=C terminal olefin RCH=CHR and N-H stretch of secondary amide. The peak at 1384.94 cm^{-1} indicated symmetrical C-H bend. Moreover, the peak at 1261.49 cm^{-1} corresponded to C-N stretch of aromatic primary amine and C-O stretch of carboxylic esters. The absorption peaks at 1118.75 cm^{-1} to 1057.03 cm^{-1} corresponded to C-H in plane bend, C-N stretch of amine and C-O stretch of alcohol. The absorption peaks at 997.23 cm^{-1} could be attributed to C-H, C-C and C-OH ring and side group vibration of carbohydrates.

The absorption peaks at 875.71 cm^{-1} to 925.86 cm^{-1} indicated that linkages had occurred between the monosaccharides. The absorption peak at 777.34 cm^{-1} corresponded to C-H stretching vibration of mono-substituted benzene. The peak at 599.86 cm^{-1} and 551.66 cm^{-1} corresponded to acetylenic C-H bends of alkynes and S-S disulfide stretch respectively. From all indications with the observed functional groups, aldehydes, esters, carboxylic acids, amino acids and proteins may be responsible for the bioreduction of silver nitrate to SNPs. The strong ability of the amino acid residues and carbonyl groups to bind to silver has been reported by Balaji et al. [19]. Similarly, Manzoor-ul-haq et al. [14] reported the presence of protein and amino acid residues as the stabilizing agent of their SNPs.

3.4 Antibacterial Potential of the Biosynthesized SNPs

The antibacterial potential of the biosynthesized SNPs is shown in Table 1. The zone of inhibition (ZOI) of the biosynthesized SNPs ranged from 13 -24 mm. *Bacillus* species had the highest susceptibility while

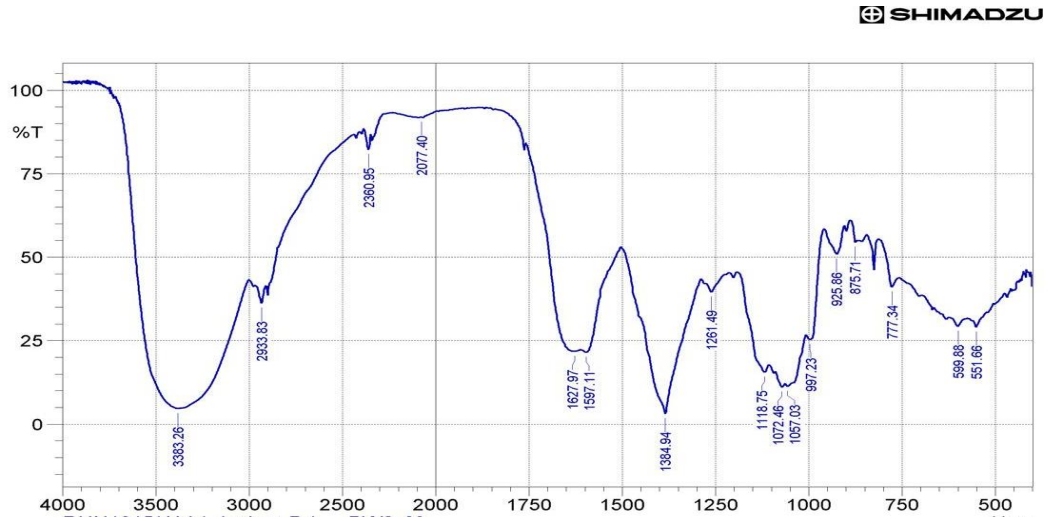

Fig. 3. FTIR spectrum of *Lactobacillus casei* LPW2- mESM SNPs

Table 1. Antibacterial activity of the biosynthesized *Lactobacillus casei* LPW2- mESM SNPs

S/N	Pathogens	Diameter of zones of inhibition		
		SNPs	Ciprofloxacin	$AgNO_3$
1	*Bacillus* sp.	24	20	15
2	*Streptococcus pyogenes*	22	24	10
3	*Staphylococcus aureus*	15	17	11
4	*Klebsiella* sp.	16	15	9
5	*Pseudomonas aeruginosa*	13	10	8

Table 2. Determination of MIC of *Lactobacillus casei* LPW2- mESM SNPs on some pathogens

S/N	Pathogens	Zones of inhibition (mm)					
		50%	25%	12.5%	6.25%	3.125%	Control
1	*Salmonella typhi*	14	12	12	11	11	12
2	*Pseudomonas aeruginosa*	20	18	18	16	15	15
3	*Escherichia coli*	15	13	11	10	10	14
4	*Bacillus* sp.	17	15	15	14	13	15

Pseudomonas aeruginosa was the most resistant. For the positive control, the ZOI ranged from 10-20 mm and 8-15 mm for the Ciprofloxacin and $AgNO_3$ respectively. The antibacterial activity of the biosynthesized SNPs was slightly more effective than the commercially available antibiotic and tested $AgNO_3$ tested. The well-developed surface of SNPs which provides the maximum contact with the environment could be responsible for their high antibacterial effect and their toxicity is presumed to be size and shape dependent [20].

3.5 Minimum Inhibitory Concentration (MIC) of the Biosynthesized SNPs

Table 2 shows the MIC of *Lactobacillus casei* LPW2- mESM SNPs and the ZOI ranged from 11-14 mm, 15-20 mm, 10-15 mm and 13-17 mm for *Salmonella typhi*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus* species respectively. *Pseudomonas aeruginosa* was the most susceptible while *Salmonella typhi* was the least susceptible to the biosynthesized SNPs. The biosynthesized *Lactobacillus casei* LPW2-mESM SNPs had MIC of 3.125% on all the tested pathogens. The MIC was defined as the lowest concentration of the nanoparticles that inhibited the visual growth of the test organisms.

This result is in agreement with the work of Zarei et al. [21] who reported that their SNPs had an MIC of 3.12 on their food borne pathogens. Balashanmugam et al. [13] also checked the MIC of their SNPs on some pathogens and *Escherichia coli* and *Bacillus subtilis* have the highest values.

4. CONCLUSION

The supernatant of LAB-mESM was able to bioreduce the $AgNO_3$ for SNPs production. The SNPs had antibacterial activity against the tested pathogens. The SNPs had MIC of 3.12% on majority of the pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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