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Original Article

Green Synthesis and Antimicrobial Activities of Silver Nanoparticles using Cell Free-Extracts of *Enterococcus* species

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Abstract

Cell-free extracts of six strains of *Enterococcus* species obtained from fermented foods were used for the green synthesis of silver nanoparticles (AgNPs), which was characterized by UV-Vis spectroscopy, Fourier-transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM). The biosynthesized AgNPs were dark brown in colour having surface plasmon resonance in the range of 420-442 nm. The spherical shaped AgNPs had sizes of 4-55 nm, whose formations were facilitated by proteins as indicated by the presence of peaks 1,635-1,637 and 3,275-3,313 cm^{-1} in the FTIR spectra. The energy dispersive x-ray (EDX) showed prominent presence of silver in the AgNPs colloidal solution, while the selected area electron diffraction was typified by the face-centred crystalline nature of silver. The particles inhibited the growth of multi-drug resistant clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *Proteus vulgaris,* and also potentiated the activities of ampicillin, ciprofloxacin and cefuroxime in the AgNPs-antibiotic synergy studies. In addition, the prospective relevance of the particles as nanopreservative in paints was demonstrated with the inhibition of growth of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *A. flavus* in AgNPs-paint admixture. This report further demonstrates the green synthesis of AgNPs by strains of *Enterococcus* species.

Keywords: antibiotics, biological routes, colour change, drug-resistance, paint preservative

Introduction

Nanotechnology is a branch of knowledge that is concerned with the synthesis of particles at nano-scale level. The biological routes of synthesis of nanoparticles are important alternatives to the use of chemicals and other methods that often have toxic effects on the ecosystem (Sneha *et al*., 2011). The importance of nanotechnology is increasing rapidly because of the manipulation of materials at nano-scale level for different applications. Over the years, nanoparticles have been synthesized by physical, chemical and biological methods. Many nanoparticles have been synthesized using bacterial metabolites, and these include gold (Ojo *et al*., 2016), ZnS (Malarkodi and Annadurai, 2012), TiO_2 (Malarkodi *et al*., 2013), silver (Rajeshkumar *et al*., 2013; Lateef *et al*., 2015a, b, c) and silver-gold alloy nanoparticles (Ojo *et al*., 2016).

Out of the different types of nanoparticles, AgNPs are the most effective, with good antimicrobial efficacy (Gong *et al*., 2007). They have been widely applied in the production of solar cells and batteries, as catalysts in chemical reactions, bio-labelling and as antimicrobials

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(Joerger *et al*., 2000; Magudapathy *et al*., 2001). It is also used in the treatment of burns, as dental materials, in textile fabrics, treatment of water, and in sunscreen lotions (Duran *et al*., 2007). AgNPs have been successfully synthesised using plant extracts (Kannan *et al*., 2013; Lateef *et al*., 2015d; Adelere and Lateef, 2016; Azeez *et al*., 2017; Lateef *et al*., 2016a; Lateef *et al*., 2016b; Lateef *et al*., 2016c), bacteria (Priyadarshini *et al*., 2013; Lateef *et al*., 2015a, b, c; Lateef *et al*., 2016d), fungi (Selvi and Sivakumar, 2012; Lateef and Adeeyo, 2015), and even spider cobwebs and paper wasp nest (Lateef *et al*., 2016e; Lateef *et al*., 2016f; Lateef *et al*., 2016g). The antimicrobial efficacy of AgNPs is due to its ability to be ionized in aqueous medium to silver ions with pronounced antimicrobial action (Choi *et al*., 2008). Factors that influence the antimicrobial activities of nanoparticles include dimension, morphology, type of the particles and crystallinity. As a result, there is intense investigation by researchers to synthesize nanoparticles for diverse applications.

In the current work, strains of *Enterococcus* species previously isolated from traditional fermented vegetable condiments and local cheese '*wara*' (Oladipo *et al*., 2013; 2014a, b; 2015) that have probiotic potentials were evaluated for the green synthesis of AgNPs, using the cell-free extracts. The study also investigated the biomedical and industrial applications of biosynthesized AgNPs as antimicrobial agents.

The use of *Enterococcus* species in nanobiotechnology is at infancy as there are few reports of the use of *E. faecalis* (Chandrakanth *et al*., 2014) for the synthesis of AgNPs, ZnO nanoparticles (Ashajyothi *et al*., 2014), AuNPs (Ashajyothi and Chandrakanth, 2014) as well as the synthesis of CdS nanoparticles by marine isolate of *Enterococcus* sp. (RMAA) (Rajeshkumar *et al*., 2014). However, the present study further demonstrates the capabilities of different species of *Enterococcus* for the green synthesis of AgNPs. The study is the first reference to the use of *E. gallinarum* and *E. casseliflavus* for the synthesis of AgNPs.

Materials and Methods

Microorganisms

Six strains of *Enterococcus* species used in this study were isolated as described by Oladipo *et al*. (2013). They were characterized using both biochemical and molecular techniques. Molecular identification to species level was performed by 16S rRNA analysis. Genomic DNA of the species were isolated and the DNA fragments were amplified; amplification was carried out in a thermal cycler with each reaction mixture comprising the template DNA along with master mix and primers designated as FD1 (5′-AGAGTT TGATCCTGGCTCAG - 3′) forward and RD1 (5′-AAGGAGGTGATCCAGCC- 3′) for reverse. The amplified products with the expected sizes were subsequently sequenced and compared with sequences in the database of National Centre for Biotechnological Information (NCBI) (BLAST, 2016). The sequences were subsequently submitted to GenBank for accession numbers.

Preparation of cell-free extracts

Brain heart infusion broth was prepared, sterilized and inoculated with fresh culture of the isolates (1×10^6 cfu/ml). The cultured flasks were incubated at 37 °C for 72 h. After incubation, centrifugation was carried out at 4,000 rpm at 10 °C for 15 min (Lateef *et al*., 2015a), and the supernatants were used for the synthesis of AgNPs without further purification.

Synthesis of AgNPs

The bacterial supernatant (1 ml) was added separately to the reaction vessel containing 10 ml of 1 mM silver nitrate ($AgNO_3$) and placed under sunlight for the photoactivation of silver to aid the synthesis of AgNPs. The formation of AgNPs was monitored through visual observation of change in colour, and measurement of the absorbance in the range of 270-800 nm on a UV-Visible spectrophotometer (Genesys 10 UV Thermoelectron Corporation, UK).

The fingerprints of biomolecules responsible for the formation of AgNPs were elucidated using Fourier transform-infrared (FTIR) spectroscopic analysis (BUCK M530 Spectrophotometer, Buck, USA) on the powder sample of AgNPs as previously reported (Lateef *et al*., 2015a). The morphology and size of the particles were studied using transmission electron microscope JEM-1400 (JEOL, USA) operated at 200kV. Few drops of colloidal AgNPs were applied on the copper grid (3.05 mm) (Agar Scientific, Essex, UK). This was then layered with 0.3% formvar that was prepared in chloroform. After settling of the particles on the copper grid, excess fluid was removed and then air-dried for TEM viewing.

Antimicrobial activities of AgNPs

It was carried out by agar diffusion method as previously described (Lateef *et al*., 2015a; Lateef and Ojo, 2016), using clinical isolates of *Proteus vulgaris*, *Escherichia coli* and *Klebsiella pneumoniae* originally sourced from LAUTECH Teaching Hospital, Ogbomoso. Inoculum obtained as 18-h culture ($\sim 10^6$ cfu/ml) was used for the seeding of Mueller-Hinton agar (Lab M Ltd., UK) plates. AgNPs of different concentrations were dispensed as 100 µl into wells of 7 mm that were bored on the plates using cork borer. This was followed by the incubation of the plates at 37 °C for 24 h, after which zones of inhibitions were monitored.

Antibacterial susceptibility test

The clinical bacterial isolates used were evaluated for their vulnerability to some commonly used antibiotics as previously demonstrated (Lateef *et al*., 2015a; Lateef and Ojo, 2016). Standard antibiotics discs obtained from Abtek Biologicals Ltd. (UK) were used. The composition of the Gram negative discs were: ofloxacin (Ofl), 5; ceftazidime (Caz), 30; cefixime (Cxm), 5; augmentin (Aug), 30; cefuroxime (Crx), 30; nitrofurantoin (Nit), 300; gentamicin (Gen), 10; ciprofloxacin (Cpr), 5. In the other hand, Gram positive discs consisted of gentamicin (Gen), 10; cefuroxime (Crx), 30; erythromycin (Ery), 5; ceftriaxone (Ctr), 30; ofloxacin (Ofl), 5; Ceftazidime (Caz), 30; augmentin (Aug), 30 and cloxacillin (Cxc), 5. The experiment was carried on Mueller-Hinton plates, where

cultures were observed at 48 h of incubation at 37 °C. The inhibitory zones were examined and interpreted (Chortyk *et al*., 1993) taken cognizance of the appropriate breakpoints (Andrews, 2005).

Synergistic studies on antibiotic-AgNPs mixtures

Studies were conducted on the antimicrobial synergy between the AgNPs and some commonly dispensed antibiotics such as cefuroxime, ampicillin and ciprofloxacin. The agar-diffusion method as previously described was used (Lateef *et al*., 2015a; Lateef *et al*., 2016e). The antibiotics were dissolved in sterile distilled water to obtain concentrations of 500 µg/ml and 1 mg/ml. Then, bacterial isolates were exposed to the antibiotics using 100 µl of each antibiotic dispensed into the wells. The second part of the experiment on antimicrobial synergy was carried out by combining 50 µl of the antibiotic with 50 µl of AgNPs (100 µg/ml), to which the test isolates were exposed. The plates were then incubated and observed for zones of inhibition as previously stated.

Evaluation of antimicrobial properties of synthesized AgNPs as preservative in paint

The potential preservative action of the synthesized AgNPs against bacteria and fungi was carried out through the introduction of AgNPs into emulsion paint as previously described (Lateef *et al*., 2015a). Commercially available white emulsion paint was procured and prepared according to the instructions of the manufacturer. The paint was dispensed as 19 ml in McCartney bottles and autoclaved at 121 °C for 15 min. Thereafter, the paints were inoculated with 1 ml (~10^6 cfu/ml) of 18 h broth cultures of *S. aureus* and *P. aeruginosa*. For *Aspergillus flavus* and *A. niger*, 1 ml (~10^6 cfu/ml) of 48 h culture was used. In the control, samples of emulsion paint were inoculated with the test organisms alone, whereas for the test, samples of the paint containing 1 ml of 100 µg/ml of AgNPs were inoculated with the test organisms. The bottles were incubated at 37 and 30 ± 2 °C for 48 h for bacteria and fungi, respectively. After the period of incubation, contents of the bottles were withdrawn and 1 ml was plated on nutrient agar (bacteria) and potato dextrose agar (fungi) using the pour plate method. The incubation of plates was done at 37 °C for bacteria and 30 ± 2 °C for fungi for up to 48 h, and thereafter checked for growth.

Results and Discussion

Bacterial isolates

The strains of *E. gallinarum, E. faecium* and *E. casseliflavus* used in the study were isolated from traditionally fermented vegetable condiments (*iru*, *ogiri*, *okpehe*, *ugba*) and *wara*. These strains were Gram-positive cocci, catalase negative, oxidase negative, and non-spore formers, with the ability to grow in the presence of 6.5% NaCl at pH 9.6 and at 10 and 45 °C. The strains were able to hydrolyze esculin, pyrrolidonyl-β-naphthylamide and arginine, but were unable to hydrolyze starch. They also showed different sugar fermentation patterns. Further confirmation of identity was carried out by 16S rRNA sequencing, and accession numbers assigned to each strain as shown in Table 1.

Enterococcus species are members of the family of lactic acid bacteria (LAB) that is widely distributed in nature. They have a history of being used as starter cultures in fermented foods largely due to the unique flavours that they produce, and their health-promoting activities (Oladipo *et al*., 2014b; Oladipo *et al*., 2015). Some of them may have antimicrobial activities against food deteriorating microorganisms, making them useful in food preservation (Oladipo *et al*., 2015). Also, different probiotic supplements, for both man and animals, include *Lactobacillus* and *Enterococcus* species (Pollmann *et al*., 2005; Tompkins *et al*., 2008; Vankerckhoven *et al*., 2008). To a large extent, there is dearth of information on the use of *Enterococcus* to produce nanoparticles. Its relevance in nanotechnology include the use of the marine isolate of *Enterococcus* sp. (RMAA) for the green synthesis of CdS nanoparticles (Rajeshkumar *et al*., 2014), and *E. faecium* to synthesize AgNPs (Chandrakanth *et al*., 2014). However, there is no report on the evaluation of *E. gallinarum and E. casseliflavus* for the green synthesis of AgNPs.

Biosynthesis and characterization of AgNPs

The formation of AgNPs was characterized with the development of colour, which was produced as a result of the reduction of silver ion by biomolecules present in the cell-free extracts. The intensity of the colour increased as the bio-reduction of silver ions progressed and stabilized when the reaction was completed. The formation of AgNPs was facilitated by the cell-free extracts within a period 10 min, with the dark brown colour which stabilized in 20 min as shown in Fig. 1. Several colours, including light yellow, yellow brown and dark brown, have been reported for colloidal solutions of AgNPs (Das *et al*., 2014; Emeka *et al*., 2014; Lateef *et al*., 2015a, b, c, d; Netala *et al*., 2016). The variations in colours have been ascribed to the activities of different types of biomolecules that act as bioreductants in the transformation of silver metal to nanoparticles of different sizes and shapes that affect the surface plasmon resonance of the particles. The excitation of surface plasmon vibrations in metal nanoparticles leads to the development of colour (Mulvaney, 1996).

Table 1. *Enterococcus* species used in this study

Isolate code	Names of isolates	Source of isolation	Accession number
C103	*Enterococcus gallinarum* strain C103	*Ogiri*	JF774410
U8	*E. gallinarum* strain U82	*Ugba*	JF774412
T10	*E. faecium* strain T77	*Okpehe*	JN645284
T7	*E. gallinarum* strain T71	*Okpehe*	JF774411
IP11	*E. faecium* strain IP27	*Iru*	JN645285
W14	*E. casseliflavus* strain W194	*Wara*	JN645289

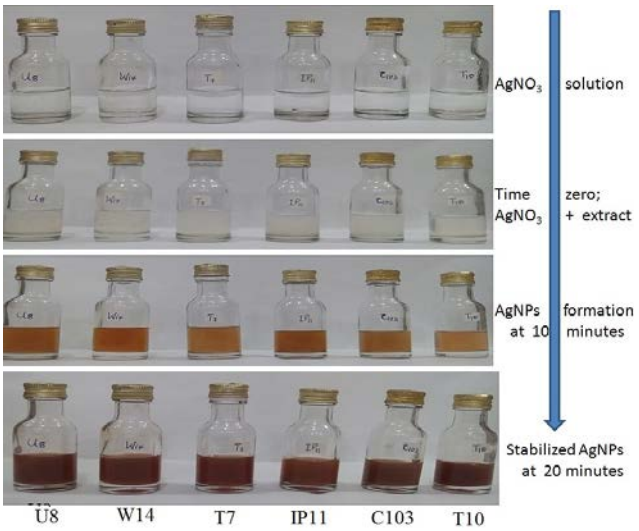

Fig. 1. Biogenic synthesis of AgNPs using cell-free extracts of strains of *Enterococcus* species

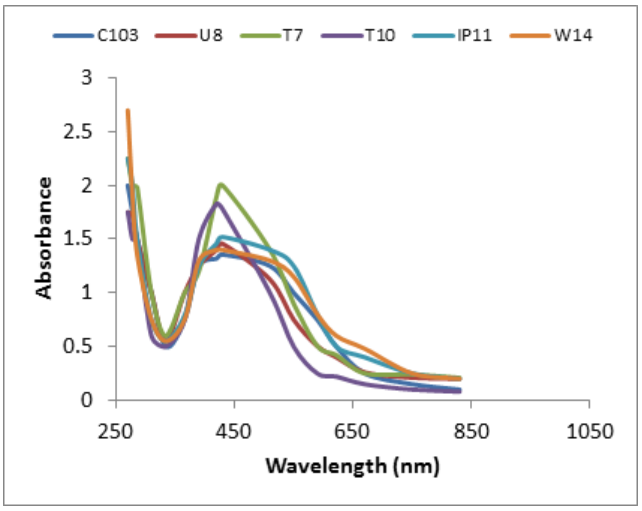

Fig. 2. UV-Vis absorption spectra of biosynthesized AgNPs

The UV-Vis absorption spectra of the biosynthesized AgNPs are as presented in Fig. 2. The biosynthesized AgNPs displayed surface plasmon resonance in the range of 420-442 nm, which is within the range of values previously reported for AgNPs (Shaligram *et al*., 2009; Kannan *et al*., 2013; Emeka *et al*., 2014; Kathiraven *et al*., 2015; Lateef *et al*.,2015b, d; Lateef and Adeeyo, 2015; Anandalakshmi *et al*., 2016). However, using cell-free extract of a strain of *E. faecium*, Chandrakanth *et al*. (2014) reported the formation of AgNPs, which absorbed maximally at 309 nm. The particles obtained in the present study were stable without significant changes in absorption spectra when stored for about three months at room temperature.

The FTIR absorption spectra showed strong peaks at 3,305.99 and 1,635.64 cm^{-1} for *E. gallinarum* C103 cell-free extract mediated AgNPs; 3,313.71 and 1,635.64 cm^{-1} for *E. gallinarum* U8; 3,275.13 and 1,637.56 cm^{-1} *for E. gallinarum* T7; 3,309.85 and 1,635.64 cm^{-1} for *E. faecium* T10; 3,313.71 and 1,635.64 cm^{-1} for *E. faecium* IP11; 3,304.06 and 1,635.64 cm^{-1} for *E. casseliflavus* W14 cell-free extract mediated AgNPs (Fig. 3). The bands 3,275-3,313, and 1,635-1,637 cm^{-1} correspond to the –NH2 of amines or -OH stretch of carboxylic acid, C=C stretch of alkenes, C=O stretch of amides or N-H bend of 1° amine

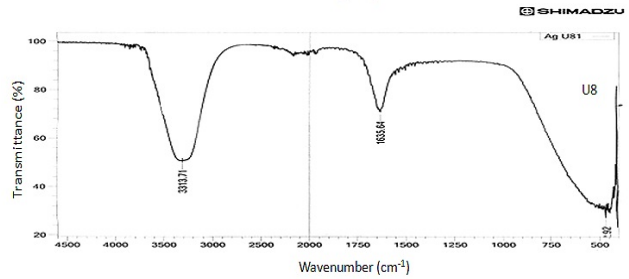

Fig. 3. FTIR spectrum of a typical biosynthesized AgNPs

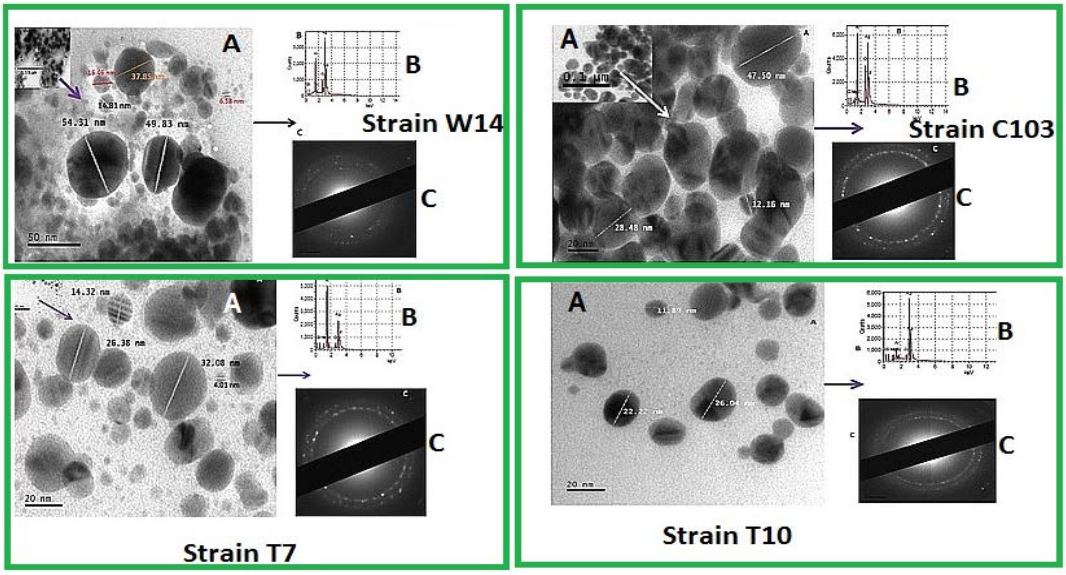

Fig. 4. Transmission electron micrographs (A), selected area electron diffraction patterns (B) and energy dispersive x-ray signals (C) of some of the biosynthesized AgNPs

respectively. This is an indication that proteinous molecules present in the cell-free extracts of strains of *Enterococcus* were involved in the biosynthesis of AgNPs.

Microscopic analysis using TEM (Fig. 4a) showed that the biosynthesized AgNPs were nearly spherical, having a size range of 4-33 nm for *E. gallinarum* T7, 5-38 nm for *E. faecium* IP11, 12-50 nm for *E. gallinarum* C103, 5-41 nm for *E. gallinarum* U8, 10-26 nm for *E. faecium* T10, and 5-55 nm for *E. casseliflavus* W14 cell-free extract mediated AgNPs. The polydispersed form of the AgNPs was corroborated by the broadness of the UV-Vis spectra as earlier presented. The EDX patterns (Fig. 4b) showed the predominant presence of silver in the AgNPs solutions with the characteristic ring-like SAED patterns (Fig. 4c) typical of the face-centered cubic crystalline structure of silver (Shameli *et al*., 2011; Salem *et al*., 2014; Shankar *et al*., 2014). From the foregoing, it can be concluded that the cell-free extracts of *Enterococcus* sp. can be applied for the biogenic and eco-friendly synthesis of AgNPs, which adds to the biotechnological relevance of the bacterial isolates.

Table 2. The antibacterial activities of biosynthesized AgNPs against some bacterial isolates

Isolates	Zones of inhibition (mm)		
	A	B	C
E. coli (T10)	12	14	15
K. pneumoniae (T10)	12	13	15
K. pneumoniae (C103)	12	12	14
P. vulgaris (C103)	11	11	13.5
E. coli (W14)	11	11	15
K. pneumoniae (W14)	10	10	12
K. pneumoniae (U8)	8	10	11
E. coli (U8)	11	11	12
K. pneumoniae (T7)	10	12	12
E. coli (T7)	10	11	11.5
K. pneumoniae (IP11)	11	12	13

T10, C103, W14, U8, T7 and IP11 are strains of *Enterococcus* species as defined in Table 1; A, B and C are concentrations of AgNPs of 60, 80 and 100 µg/ml respectively; each value is average of two readings.

Antimicrobial activities of biosynthesized AgNPs

The biosynthesized AgNPs showed inhibitory activities against some clinical isolates of bacteria (Table 2). The AgNPs at concentrations of 60-100 µg/ml inhibited the growth of multi-drug resistant strains of *E. coli*, *P. vulgaris* and K. *pneumoniae* with zones of inhibition of 8-15 mm. The limited inhibitory actions might be due to the multi-drug resistant nature of the isolates used in the present study. The antibiotic susceptibility of the isolates showed the drug resistance patterns as follow: *K. pneumoniae* (Caz, Crx, Gen, Cpr, Ofl, Aug, Amp), *E. coli* (Caz, Crx, Gen, Cpr, Ofl, Aug, Nit, Amp) and *P. vulgaris* (Caz, Crx, Cpr, Ofl, Aug, Nit, Amp). The results are in agreement with earlier published works on the antibacterial activities of AgNPs (Salem *et al*., 2014; Shankar *et al*., 2014; Lateef *et al*., 2015a, b, d; Lateef *et al*., 2016a, e). In a similar work, Chandrakanth *et al*. (2014) reported inhibitory zones of 14-19 mm by AgNPs biosynthesized using *Enterococcus faecium*

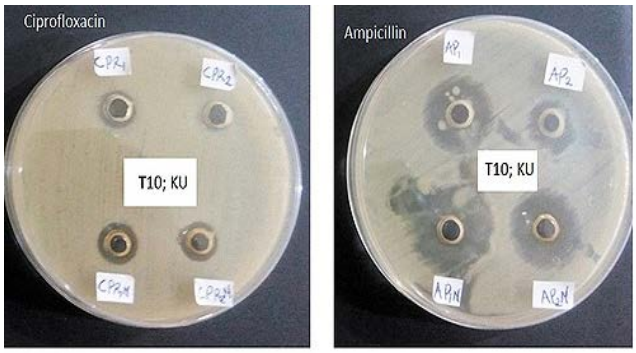

Fig. 5. The synergistic activities of biosynthesized AgNPs with antibiotics on some clinical bacterial isolates

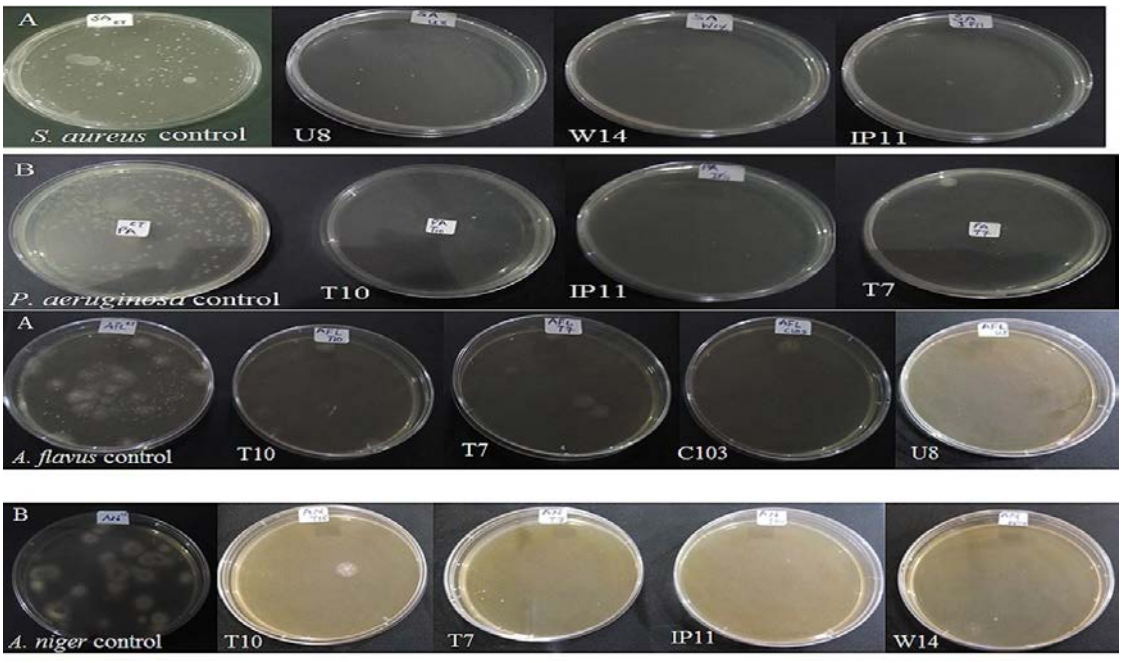

Fig. 6. The antibacterial and antifungal activities of biosynthesized AgNPs as additives in emulsion paint

against multi-drug resistant strains of *E. coli*, *K. pneumoniae* and *S. aureus*.

In AgNPs-antibiotics synergistic studies, it was discovered that the presence of AgNPs led to the improved performance of antibiotics such as ampicillin, ciprofloxacin and cefuroxime against drug-resistant strains of *K. pneumoniae*. Improved performance of 13.6-71.4, 8.3-57.1 and 71.4-85.7% were obtained for ampicillin, ciprofloxacin and cefuroxime respectively when used in combination with AgNPs (Fig. 5). It is noteworthy that in most cases, the two levels of antibiotic concentrations of 0.5 and 1 mg/ml used alone did not inhibit the growth of the test isolates. Therefore, the inclusion of AgNPs might have rendered the isolates susceptible, either by acting as drug carrier or by enhancing entry of antibiotics as a result of damage to the bacterial cell wall. These results are similar to those previously reported (Devi and Joshi, 2012; Lateef *et al*., 2015a; Lateef *et al*., 2016b; Lateef *et al*., 2016e) on the synergistic antibacterial effects of AgNPs on some antibiotics against Gram positive and negative bacteria. The susceptibility of drug-resistant bacteria as offered by AgNPs-antibiotics synergy could be a promising panacea to solving problems of curtailing the prevalent drug-resistance phenomenon among bacteria in clinical practice (Lateef *et al*., 2005).

Similarly, the incorporation of biosynthesized AgNPs into emulsion paint yielded pronounced antimicrobial efficacy, which has potential of protecting the paint against microbial attack and biodeterioration. Remarkable antibacterial and antifungal activities were noticed through the drastic reduction of growth of *S. aureus*, *P. aeruginosa*, *A. niger* and *A. flavus* leading to the appearance of a single or few colonies after 72 h of incubation as against dense growth that were obtained on the control plates (Fig. 6). The results are in agreement with those reported earlier (Lateef *et al*., 2015a; Lateef *et al*., 2016b; Lateef *et al*., 2016e). It is envisaged that the AgNPs can act as nanopreservative in paint to prevent microbial deterioration thereby extending the shelf life of paints and painted surfaces.

Conclusions

In the current study, green synthesis of AgNPs using the cell-free extracts of six strains of *Enterococcus* species was successfully carried out. The spherical biosynthesised AgNPs of 4-55 nm in size displayed good antibacterial activities against multi-drug resistant strains of bacteria in both single and synergistic studies. The antimicrobial properties of the particles were demonstrated in paint, resulting to elimination of *S. aureus*, *P. aeruginosa*, *A. niger* and *A. flavus* when the particles were used as additives. The study has shown the relevance of *Enterococcus* species as biotechnological tool in the green synthesis of AgNPs.

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