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Biosynthesis and antibacterial activity of silver nanoparticles

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ABSTRACT

In the present study extracellular synthesis of silver nanoparticles (AgNPs) was achieved using cultural supernatant of bacterial isolate *Aeromonas dhakensis* AS3. Biosynthesis of AgNPs was completed within 120 min by incubating cell free supernatant with silver nitrate solution under illumination. Brown color appearance of solution due to surface plasmon resonance (SPR) and absorption maxima centered at 405 nm was indicated formation of AgNPs. Fourier transform infrared spectroscopy (FTIR) spectrum analysis revealed the presence and association of possible biomolecules with AgNPs during synthesis. Atomic force microscopy (AFM), Field emission scanning electron microscopy (FE-SEM) and High resolution transmission electron microscopy (HR-TEM) showed spherical nanoparticles with an average size of 5 nm. X-ray diffraction (XRD) and Energy Dispersive X-ray (EDX) spectrum confirmed crystallinity and purity of AgNPs. The synthesized AgNPs was found to have significantly independent as well as combined activity against multidrug resistant extended spectrum β-lactamases (ESBLs) producing *Acinetobacter junii*, *E.coli* and *Klebsiella* spp. harboring TEM and/or CTX-M genes. This work demonstrates the possible use of biosynthesized AgNPs to combat ESBLs producing pathogens. Copyright © 2016 VBRI Press.

Keywords: ESBLs; aeromonas dhakensis AS3; biosynthesis; silver nanoparticles; antibacterial activity.

Introduction

Study on metal nanoparticles has gained significance in recent time due to their extensive application in the diverse branches of sciences and technology **[1-3]**. Metal nanoparticles (*viz*. silver, gold, palladium, titanium, copper and iron etc.) exhibit unique size and shape dependent physical, chemical and biological properties as compared with their bulk counterparts **[4, 5]**. Among metal nanoparticles, AgNPs have been widely studied due to their various bioreactive features like antimicrobials **[6, 7]**, anti-cancer **[8, 9]**, drug delivery **[10]**, biomolecular detection **[11]** and catalysis **[12, 13]**.

Nanoparticles are synthesized mainly by three methods including physical, chemical and biological **[14, 15]**. Although physical and chemical methods ensure production of well characterized nanoparticles but use of enormous energy and generation of toxic byproduct make them 'not a prefer' approach **[16, 17]**. Therefore, development of non toxic *i.e.* green chemistry process for the synthesis of nanoparticles is an emerging demand of nanotechnology to avoid conventional hazard of chemical toxicity **[18-21]**. Green chemistry process emphasizes on the usage of microorganisms that has offered a reliable, eco friendly biofactory for the production of nanoparticles **[22, 23]**. Both unicellular and multicellular organisms are able to synthesized AgNPs **[24, 25]**. Among the microorganisms bacteria are mostly chosen for synthesis of nanoparticles due to their growing success, easy handling and genetic manipulation **[26, 27]**. The first biosynthesis of AgNPs was carried out using silver mine bacterial isolate *Pseudomonas stutzeri* AG259 **[28]**. The reaction rate of bacterial mediated synthesis is slow as compared to chemical methods, which limit the large scale production at industrial level **[29, 30]**. Recently it has been reported that visible light catalyze the rate of AgNPs biosynthesis within short time periods; that in turn may be applied in industries for large scale production **[31, 32]**.

The increasing prevalence of extended spectrum *β*-lactamases (ESBLs) producing *Enterobacteriaceace* creates great health care problems throughout the world **[33, 34]**. ESBLs are enzymes imparting resistance to bacteria towards expanded spectrum β-lactam antibiotics up to 3rd and 4th generation cephalosporins **[35]**. The first plasmid mediated TEM type ESBL producing bacteria was isolated from a patient blood sample named Temoniera in 1965 and now more than 300 variants of ESBLs have been reported from almost all parts of the world (www.lahey.org) **[36]**. The ESBLs producing bacteria are not only restricted in clinical setting but also arise in the aquatic environment **[37, 38]**. In river aquatic ecosystem bacteria from various origins (urban waste water, animal wastage, hospital and industrial effluent etc.) are able to mix and transfer their resistant genes to clinically important bacteria for development of human pathogen with novel resistant mechanisms **[39-43]**. Prevalence and increase in ESBLs producing multidrug resistant bacteria obliges the scientific community to develop new alternative therapy to prevent them. Silver nanoparticles (AgNPs) have already been proven to be a significant antimicrobial agent and some previous study reported that it's also effective against ESBLs positive bacteria **[44-46]**.

In present study, we have demonstrated rapid and efficient synthesis of AgNPs using *Aeromonas dhakensis*

AS3 isolate. Furthermore, the antibacterial activity of synthesized AgNPs was evaluated against bla_{TEM} and/or bla_{CTX-M} types of ESBLs producing various environmental isolates. Based on literature survey, extracellular synthesis of AgNPs using this bacterial isolate has not been reported so far.

Experimental

Materials

All the culture media and antibiotics were purchased from Himedia, India. Silver nitrate ($AgNO_3$) was obtained from Merck Ltd., India. Sterile Milli-Q water and glassware were used throughout the experiments.

Isolation and identification of the bacteria

Effluent water sample was collected from Sahibabad Site-IV industrial area, Uttar Pradesh, India. The collected sample was serially diluted and spread on Luria agar (LA) plates and incubated at 37 °C, overnight. After incubation, morphologically distinct 11 isolates were streaked on LA plates to obtain pure colonies and named AS1 to AS11. All the isolates were screened for extracellular synthesis of AgNPs and based on their efficiency, isolate AS3 was selected and identified by 16S rDNA sequences.

Extracellular synthesis of AgNPs

For extracellular synthesis of AgNPs, the selected isolate was freshly inoculated in a 250 ml Erlenmeyer flask containing 100 ml of Luria Broth (LB) and incubated at 37 °C for 24h with a continuous agitation at120 rpm. After incubation the culture supernatant was obtained by centrifugation at 9000 rpm at 4 °C for 10 minutes and stored at 4 °C for further use. Cell free culture supernatant was separately mixed with freshly prepared 1mM $AgNO_3$ solution at 1% (v/v) ratio in a reaction vessel and incubated at 40 °C under 20W (≈1200 lumens) compact fluorescence light. Culture supernatant at the same ratio without addition of $AgNO_3$ and only $AgNO_3$ (1mM) solution was used as control. The time dependent synthesis of AgNPs was monitored by visual observation of color change and measuring absorbance spectra in the scanning range of 300-800 nm by a double beam UV-visible Spectrophotometer (Labtronics LT-2800).

Isolation and purification of AgNPs

The synthesized AgNPs was isolated from solution by simply centrifugation at 10000 rpm at 4 °C for 30 min as described earlier **[47]**. The clear supernatant was discarded and pellet was washed four times by sterile Milli-Q water to remove the impurities. The isolated AgNPs was dried in hot air oven at 40 °C and powdered nanoparticles were used for characterization.

Characterizations

The FTIR spectrum of powder AgNPs was recorded by Bruker Tensor 37 instrument using spectral range between 4000-600 cm^{-1} with a resolution of 4 cm^{-1}. For TEM analysis, a drop of aqueous AgNPs solution placed on carbon coated copper grids and images were obtained by HR-TEM (Tecnai G^2 200kV TEM (FEI) instrument). Further, the morphology of particles was observed by FE-SEM (Zeiss Sigma) instrument equipped with energy dispersive X-ray spectroscopy (EDX). Synthesized AgNPs was spin coated on the glass substrate and their morphology as well as size was studied by AFM (Bruker nanoscope V) using intermittent mode. The crystalline nature of particles were examined by X pertPro PANalytical X-ray diffractometer instrument using Cu-Kα radiation (k=1.54 Å) operating at 45 kV with 40 mA.

Isolation and identification of ESBLs producing bacteria

The ESBL producing bacterial isolates were obtained from Delhi stretch of river Yamuna, India. For this, collected water samples were serially diluted and plated on LA with cefotaxime (1mg/L) to isolate resistant bacteria. Colonies with different morphology were selected from each plate and streaked on Luria Agar (LA) to obtain pure culture. All isolates were screened for ESBL production by preliminary test and Phenotypic Disc Confirmatory Test (PDCT), as per guidelines of Clinical Laboratory Standards Institute (CLSI-2014). *K. pneumonia* ATCC 700603 and *E. coli* 25922 were used as ESBL positive and negative control respectively. All the isolates that showed ESBL positive phenotype were characterized by PCR using specific primers for bla_{TEM} and bla_{CTX-M} genes. Finally, those isolates harboring TEM and/or CTX-M resistant determinant were identified by 16s rRNA gene amplification by designed primers and sequences analysis.

Antibacterial activity of AgNPs

The antibacterial properties of synthesized AgNPs were investigated against ESBLs producing bacterial isolates by well diffusion method. In brief, bacterial suspension was spread on Mueller Hinton Agar (MHA) plates and made well of 6 mm diameter by cork borer. The different concentrations of 20 µl AgNPs solution was placed to each well and incubated at 37 ºC for 18 h. At the same time cell free culture supernatant and cefotaxime were used as control. After incubation the clear zone of inhibition (ZOI) in the petri plates were measured in millimeters. The minimum inhibitory concentration (MIC) was determined by broth micro dilution method using 96-well microtiter plates. Further, bacterial suspension was taken from the MIC well with no growth and streaked on LA plates. The lowest concentration showing no growth on LA after incubation was considered as minimum bactericidal concentration (MBC). The synergistic effects of AgNPs with eight different classes of antibiotics were evaluated by disc diffusion methods. For that, antibiotic disc was impregnated with 20 µl of AgNPs (20µg/disc) and incubated at 37 ºC for 18 h to check their synergism. The antibiotics used in this experiment were Trimethoprim (TR), Imipenem (IPM), Amikacin (AK), Colistin (CL), Chloramphenicol (C), Rifampicin (RIF), Cefoxitin (CX) and Ciprofloxacin (CIP). Further, in order to evaluate synergistic antibacterial activity of AgNPs with various antibiotics, the fractional inhibitory concentration index (FICI) was determined by two dimensional checkerboard assay in a 96-well microtiter plate. The FIC index was calculated as follows:

The fractional inhibitory concentration index (FICI) = [MIC of A in combination/MIC of A alone] + [MIC of B in combination/MIC of B alone]
Where A is AgNPs and B is antibiotic.

The FICI values were interpreted as follows: Synergy (FICI <0.5), partial synergy (0.5≤ FICI <1.0) and antagonism (FICI >4.0) **[48, 49]**.

Results and discussion

Isolation and identification of the bacteria

In this study, a total of eleven bacterial isolates collected from industrial effluents were screened for the extracellular synthesis of AgNPs. Isolate AS3, showed maximum synthesis was further characterized by 16S rDNA sequence. Analysis of the sequence data showed maximum homology with *Aeromonas dhakensis*. The sequence data has been submitted to NCBI with an accession number KP791800.

Extracellular synthesis of AgNPs

Synthesis of AgNPs occurred when bacterial isolate AS3 supernatant were mixed with silver nitrate (1 mM) solution and incubated at 40 ºC under illumination. AgNPs could not be detected in control conditions (Culture supernatant and $AgNO_3$ alone). Our observations are in line with earlier report using psychrophilic bacteria **[50]**. The AgNPs synthesis was relatively faster and completed within 120 min. Synthesis of nanoparticles significantly improved under illuminated conditions which might be due to excitation of biomolecules present in supernatant, releasing electrons for the reduction of Ag^+ to Ag(0) **[30, 31, 51]**. This may be speculated that AgNPs synthesis occurred due to the combined effect of visible light and biomolecules present in AS3 supernatant.

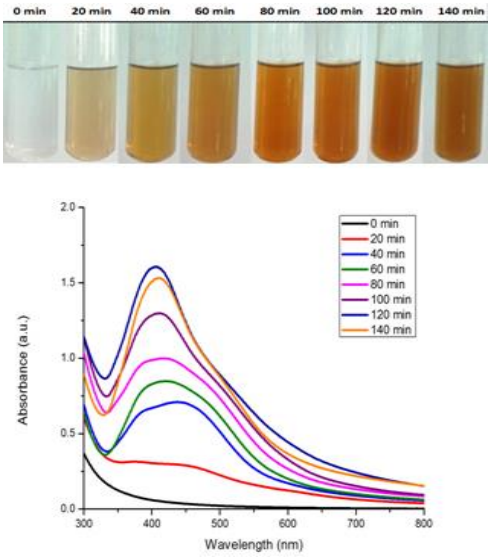

Fig. 1. UV-vis spectra of AgNPs synthesized at different time intervals.

Characterizations

The appearance of brown color in the reaction vessel due to SPR suggested the formation of AgNPs **[2, 52]**. The UV-vis spectroscopy analysis shows a strong absorbance peak centered at 405 nm **(Fig. 1)**, a characteristic feature of AgNPs **[25, 47, 53]**. Increased synthesis of AgNPs with progression of time correlated with color change and gradual raise of absorbance maxima **(Fig. 1)**. The UV-vis spectra recorded after 120 min showed slight red shift and decrease of absorption maxima perhaps due to aggregation of AgNPs, which indicates completion of reaction in 120 min **[19]**.

The FTIR spectroscopy is used to determine the possible participation of supernatant biomolecules for synthesis and stabilizing AgNPs, showed intense absorbance peaks at 3712 cm^{-1}, 3174 cm^{-1}, 2902 cm^{-1} , 2414 cm^{-1}, 2154 cm^{-1}, 1629 cm^{-1}, 1517 cm^{-1}, 1365 cm^{-1} and 1020 cm^{-1} **(Fig. 2a)**. A weak band at 3712 cm^{-1} observed might be due to the frequency of O-H group from carbohydrate or protein molecules in the sample **[54]**. The peaks revealed at 3174 cm^{-1} and 2902 cm^{-1} corresponds to vibration of O-H and C-H stretching of alkaline compounds **[25, 51]**. The band observed at 2154 cm^{-1}, 1629 cm^{-1} and 1515 cm^{-1}, 1020 cm^{-1} and 1365 cm^{-1} could be due to vibration of C-C group **[55]**, stretch vibration of N-H groups of amide I and II in protein linkage **[5, 56]** and C-N stretching of peptides linkage **[21, 47]** respectively. Further, FTIR peaks revealed the association of proteins with synthesized AgNPs by amide linkage. This data suggests that bacterial isolate *Aeromonas dhakensis* AS3 culture supernatant contain protein molecules which could have possible role for the formation as well as stabilizing AgNPs **(2, 18, 27, 47)**.

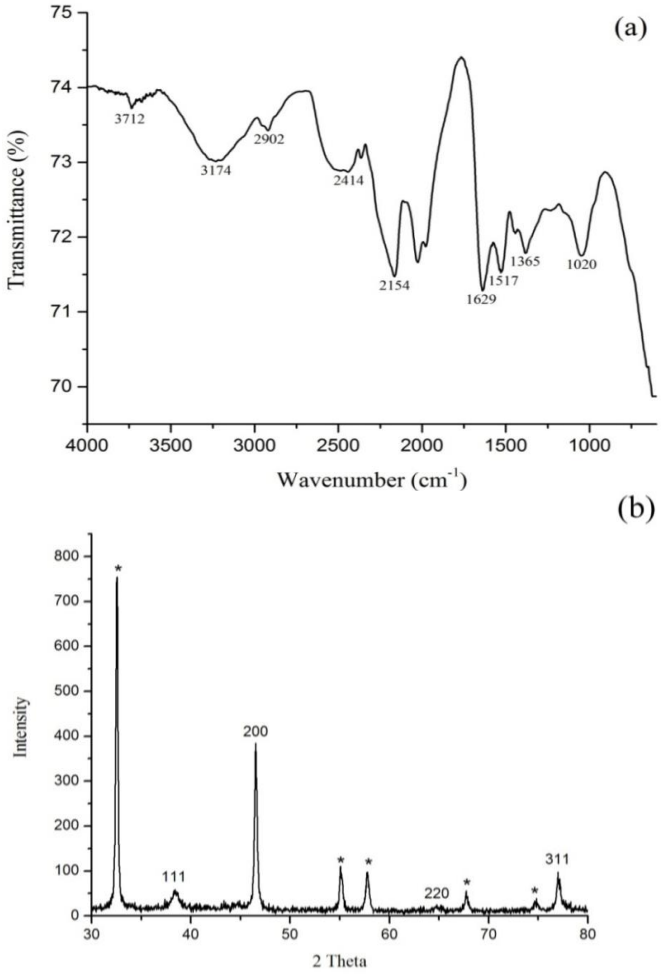

Fig. 2. (a) FTIR spectrum of synthesized AgNPs and (b) XRD pattern of synthesized AgNPs, asterisks indicate unassigned peaks may be due to the present of proteins in culture supernatant.

AgNPs synthesized in this study showed distinct XRD peaks at 2-theta values 38.39, 46.51, 64.76 and 77 corresponding to the intensities of [111], [200], [220] and [311] for metallic silver **(Fig. 2b)**. These values are in close agreement with the database of joint committee on powder diffraction standards (JCPDS) file no: 04-0783. Some unassigned peaks were also observed in this XRD spectrum, it may be due to the association of bacterial extracellular protein molecules with AgNPs during synthesis **[47, 57]**. The similar kind of XRD pattern have been reported for AgNPs, synthesized using *B. licheniformis* **[57]**, *K. pneumoniae* **[47]** and *S. nematodiphila* **[58]**.

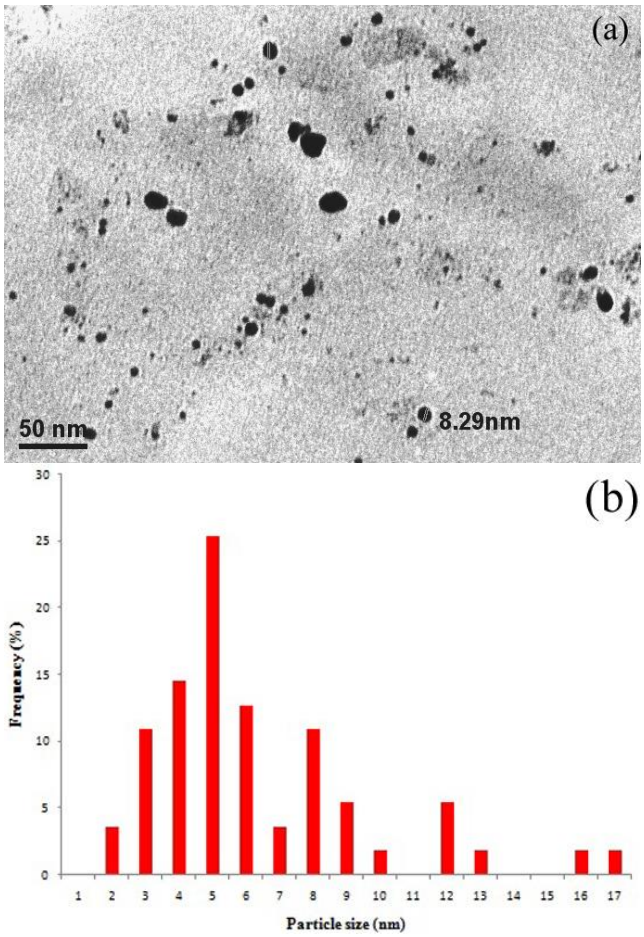

Fig. 3. (a) HR-TEM micrograph of synthesized AgNPs and (b) Particles size histogram.

The HR-TEM analysis of AgNPs revealed that particles have spherical shape **(Fig. 3a)** and their size range between 2-17 nm, with an average particles size of 5 nm **(Fig. 3b)**. The morphological characteristic of AgNPs was observed by FE-SEM **(Fig. 4a)** is in good agreement with TEM data. Further, EDX spectroscopy analysis was carried to determine the elemental composition of synthesized particles along with FE-SEM. The EDX spectrum showed a strong peak of silver at 3 keV confirms the presence of AgNPs **(Fig. 4b)**. The morphology of particles was further studied by AFM with intermittent mode that depicts 2D and 3D image of the sample spin coated on glass substrate **(Fig. 5a & 5c)**. The particles are spherical in shape with almost uniform distribution **(Fig. 5b)**. The agglomerations of the particles on the surface are due to multilayer coating of the particle leading to the formation of a few huge clusters on the surface.

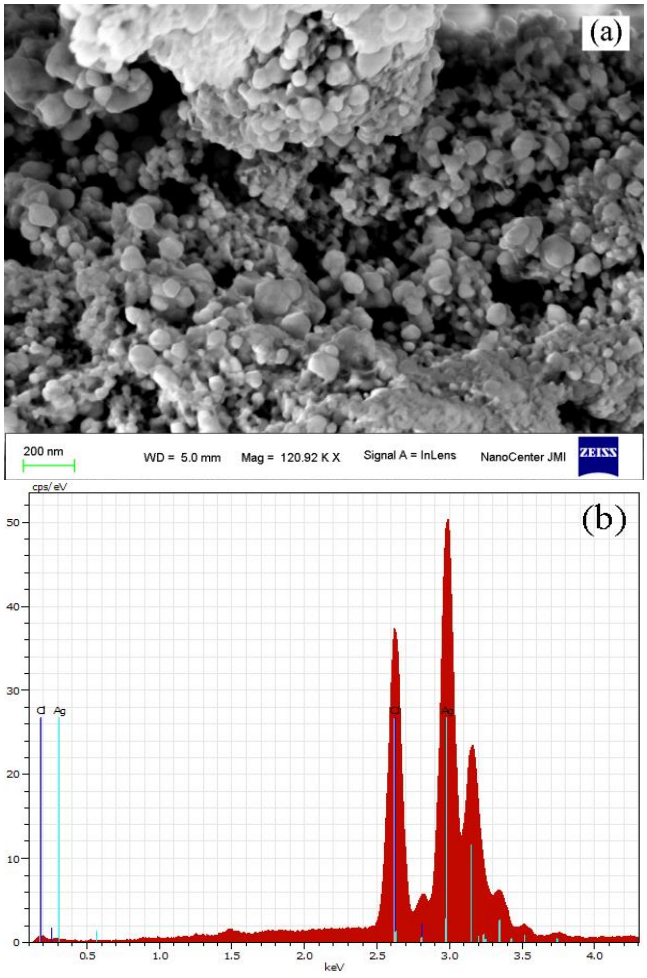

Fig. 4. (a) FE-SEM micrograph and (b) EDX spectrum of synthesized AgNPs.

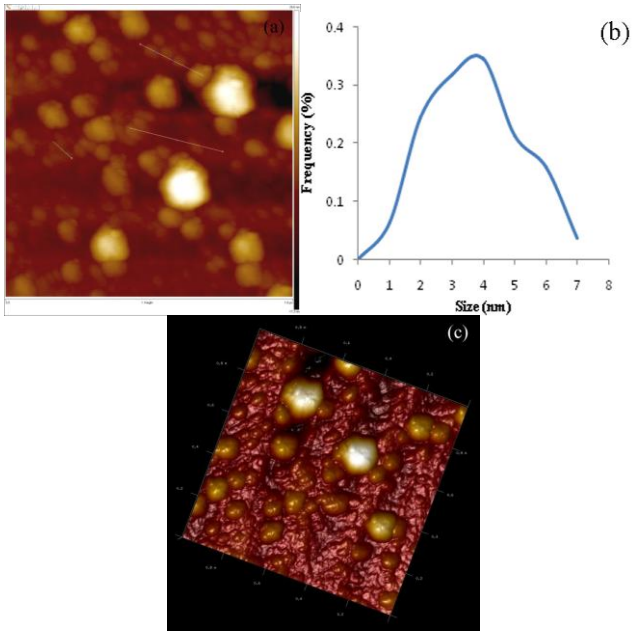

Fig. 5. (a) AFM images of synthesized AgNPs in 2D form, (b) Particles size distribution and (c) 3D form of particles.

Isolation and identification of ESBLs producing bacteria

The ESBL producing isolates of *E. coli*, *Klebsiella pneumonia*, *Klebsiella oxytoca* and *Acinetobacter junii* were isolated from river Yamuna (Delhi stretch). Above bacterial isolates were characterized based on their 16 S rRNA gene sequences. Nucleotide sequence data have been submitted to NCBI with accession Nos. KJ923017, KJ906614, KJ957161 and KC963028. The successful PCR amplification of specific ESBLs gene confirmed that *E. coli and A. junii* contained both bla_{TEM} and bla_{CTXM} genes, where as bla_{TEM} gene was harbored by all isolates.

Table 1. Antibacterial activity of synthesized AgNPs against ESBLs producing isolates.

ESBLs producing Isolates	ZOI (mm) for different concentration of AgNPs							MIC (µg/ml)	MBC (µg/ml)
	(+ ve) control	(- ve) control	10µg	15 µg	20 µg	25 µg	30 µg		
E. coli	10	0	8	10	15	16	17	6	8
K. pneumoniae	8	0	7	11	12	13	15	6	8
K. oxytoca	27	0	8	10	12	13	14	4	5
A. junii	23	0	0	9	10	11	11	9	10

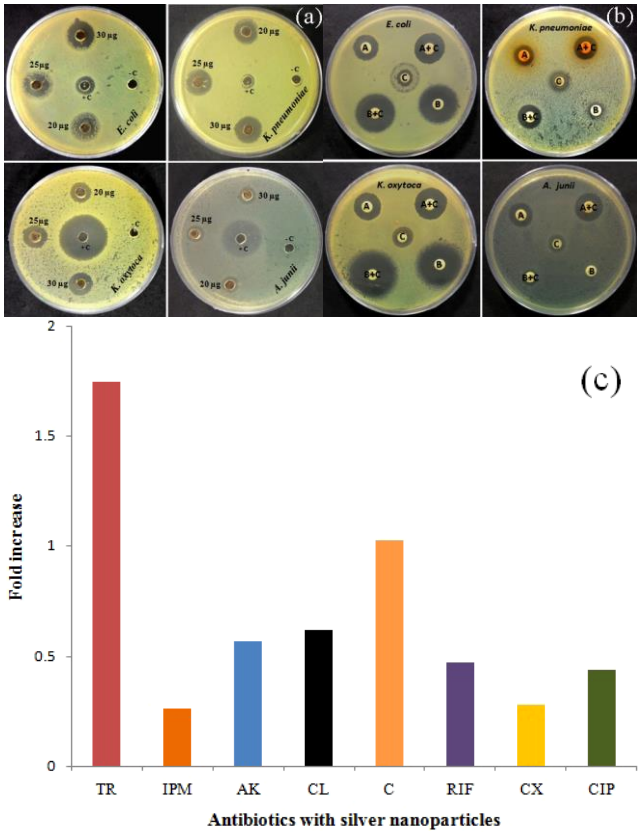

Fig. 6. Antibacterial activity of synthesized AgNPs against ESBL producing isolates by (a) Well diffusion method, (b) Combined antibacterial activity of AgNPs with different antibiotics A, zone of inhibition only for antibiotics, C, only for AgNPs, and AgNPs with antibiotics A+C respectively, (c) Fold increase of antibiotics with AgNPs against test isolates (where TR-Trimethoprim, IPM-Imipenem, AK-Amikacin, CL-Colistin, C-Chloramphenicol, RIF-Rifampicin, CX- Cefoxitin and CIP- Ciprofloxacin).

Antibacterial activity of AgNPs

Antibacterial activity of biosynthesized AgNPs was determined by agar well diffusion test. A clear zone of inhibition (ZOI) was observed across wells in all ESBLs producing isolates **(Fig. 6a)**. Concentration dependent increase in diameter of ZOI was recorded up to 30 µg. **(Table 1)**. No antibacterial activity was observed for the culture supernatant (used as negative control). Our results are in line with previous study on ESBL producing strains of *Enterobacteriaceae* by Banu *et al.*, 2011 **[44]**. The MIC and MBC values for tested isolates varied from 4-9 µg/ml and 5-10 µg/ml respectively **(Table 1)**. The antibacterial activities of antibiotics with AgNPs combinations were more than antibiotic alone for all test isolates **(Fig. 6b)**. The diameter of ZOI for different antibiotic discs with and without AgNPs is shown in **Table 2**. The highest fold increase was observed for AgNPs in combination with the antibiotic Trimethoprim followed by Chloramphenicol, Colistin, Amikacin, Rifampicin, Ciprofloxacin, Cefoxitin and Imipenem **(Fig. 6c)**.

Table 2. The mean zone of inhibition (mm) of different antibiotics (alone and combined AgNPs) against the test isolates.

Antibiotics	*K. oxytoca*	*E. coli*	*A. junii*	*K. pneumoniae*	Over all synergistic effect
A. TR (5 µg)	6	10	30	25	1.75
AgNPs	12	15	10	11	
B. TR + AgNPs	16	12	33	28	
Increase in fold area*	6.11	0.44	0.21	0.25	
A. IPM (10 µg)	40	30	34	31	0.26
AgNPs	12	15	10	11	
B. IPM + AgNPs	43	35	36	37	
Increase in fold area	0.15	0.36	0.12	0.42	
A. AK (30 µg)	22	18	12	20	0.57
AgNPs	12	15	10	11	
B. AK + AgNPs	25	20	16	25	
Increase in fold area	0.29	0.23	0.77	0.56	
A. CL (10 µg)	16	13	6	13	0.62
AgNPs	12	15	10	11	
B. CL + AgNPs	19	15	9	16	
Increase in fold area	0.41	0.33	1.25	0.51	
A. C (30 µg)	27	30	20	6	1.03
AgNPs	12	15	10	11	
B. C + AgNPs	30	35	25	12	
Increase in fold area	0.23	0.36	0.56	3	
A. RIF (30 µg)	20	16	30	11	0.47
AgNPs	12	15	10	11	
B. RIF + AgNPs	21	19	37	15	
Increase in fold area	0.10	0.41	0.52	0.86	
A. CX (30 µg)	20	17	12	6	0.28
AgNPs	12	15	10	11	
B. CX + AgNPs	24	18	15	6	
Increase in fold area	0.44	0.12	0.56	0	
A. CIP (5 µg)	23	7	20	9	0.44
AgNPs	12	15	10	11	
B. CIP + AgNPs	25	9	24	11	
Increase in fold area	0.18	0.65	0.44	0.49	

* Fold increases for different antibiotics were calculated by the formula $(B^2-A^2)/A^2$, where A and B are the zone of inhibition for only antibiotic and antibiotic with AgNPs, respectively.
In case of no zone of inhibition, diameter of the disc (6 mm) was considered for the calculation.

Typically, maximum fold increase was observed in *E. coli* for CIP+AgNPs, *K. pneumoniae* with C+AgNPs, *K. oxytoca* with TR+AgNPs and *A. junii* with CL+AgNPs. In contrast, the highest and lowest synergistic effect was observed against *K. oxytoca* for antibiotic Trimethoprim with AgNPs and Rifampicin with AgNPs respectively. The similar result of high fold increase for Trimethoprim in combination with AgNPs against multidrug resistant bacteria *E. faecalis* has been reported by Naqvi *et al*., 2013

[59]. The mode of action of Trimethoprim is to prevent folic acid biosynthesis by inhibiting dihydrofolate reductase (DHFR) enzymes. While in our case, bacterial isolate *K. oxytoca* showed high level of resistance against the antibiotic Trimethoprim. This resistance may be due to the permeability barrier in bacteria **[60]**. In combination, the functional group of antibiotic bind with AgNPs by chelation **[18]**. Therefore, AgNPs increases the permeability of cell membrane thus acting as drug carrier to facilitate the transport of antibiotic in to the cell **[61]**. As a result, antibiotic concentration increased at target site which causes to enhanced antibacterial activity. Beside, chelation can also act on bacterial DNA and prevents DNA unwinding, which result in more serious damaged to bacterial cells **[62].** This may be the possible explanations for enhancement of synergistic activity of antibiotic in combination with AgNPs. Synergistic antibacterial activities of AgNPs in combination with different antibiotics against the test isolates were evaluated by FICI calculation. The FICI values of combination assay are shown in **Table 3**. All the antibiotics in combination with AgNPs showed synergistic or partial synergistic effect. This observation is in line with the previous study on synergistic effects between AgNPs and antibiotics against pathogenic bacteria **[48]**. Typically, synergistic interaction was observed for AK+AgNPs in *E. coli*; TR+AgNPs, AK+AgNPs, C+AgNPs and RIF+AgNPs in *K. pneumoniae*, combinations of TR+AgNPs, AK+AgNPs, CL+AgNPs, C+AgNPs, RIF+AgNPs in *K. oxytoca*, and TR+AgNPs, AK+AgNPs, CL+AgNPs, RIF+AgNPs and CX+AgNPs in *A. juni*. Other combinations of AgNPs and antibiotics showed partial synergistic interaction against all the tested isolates.

Table 3. Fractional inhibitory concentration index (FICI) values of AgNPs in combination with different antibiotics.

	FICI							
Bacterial isolates	**TR+AgNPs**	**IPM+AgNPS**	**AK+AgNPs**	**CL+AgNPs**	**C+AgNPs**	**RIF+AgNPs**	**CX+AgNPs**	**CIP+AgNPs**
E. coli	0.515 (PS)	0.562 (PS)	0.375 (S)	0.75 (PS)	0.5 (PS)	0.5 (PS)	0.75 (PS)	0.531 (PS)
K. pneumoniae	0.312 (S)	0.625 (PS)	0.312 (S)	0.5 (PS)	0.312 (S)	0.375 (S)	0.75 (PS)	0.531 (PS)
K. oxytoca	0.281 (S)	0.75 (PS)	0.312 (S)	0.375 (S)	0.375 (S)	0.312 (S)	0.515 (PS)	0.531 (PS)
A. junii	0.312 (S)	0.625 (PS)	0.312 (S)	0.312 (S)	0.531 (PS)	0.375 (S)	0.375 (S)	0.562 (PS)

Antibacterial activities of AgNPs have been widely reported but their exact mechanisms of action are poorly understood **[4, 44, 45]**. Therefore, multiple mechanisms have been hypothesized to explain its antibacterial activity. One of the hypothesis proposed state that AgNPs are attracted towards bacterial outer lipopolysaccharides layer and disrupt it, cell membrane and block the transport channels **[15, 63]**. The other possibility is that they may be penetrating cell membrane and disrupt the cellular activities like nucleic acid biosynthesis **[64]**. It has been also reported that silver have strong affinity to bind thiol groups of vital enzyme thereby inactivating them **[64, 65]**. Synergistic effect of silver nanoparticles with different classes of antibiotic may be due to bonding reaction between functional groups of antibiotic with AgNPs by chelation **[18]**. This considerable efficacy of AgNPs need further study to find out exact mode of action in order to develop new combination therapy against ESBLs producing pathogens.

Conclusion

This is the first report on extracellular biosynthesis of AgNPs using *Aeromonas dhakensis* AS3, an environmental isolate. The present study describes an eco-friendly, rapid and efficient method for synthesis of AgNPs. The involvement of active supernatant biomolecules during synthesis of AgNPs has demonstrated by FTIR. Synthesized AgNPs are crystalline, spherical with an average size of 5 nm confirmed by XRD, EDX, TEM, FE-SEM and AFM analysis. Synthesis of AgNPs was achieved in a short time period, which may be applied for large scale production at industrial level. Further, biosynthesized AgNPs shows promising synergistic and independent antibacterial activity against ESBLs positive isolates of *Acinetobacter junii*, *E. coli* and *Klebsiella* spp. Biosynthesized AgNPs can be used to develop new drug to treat ESBLs producing pathogens.

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