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Full Length Research Paper

Synthesis of silver nanoparticles using a probiotic microbe and its antibacterial effect against multidrug resistant bacteria

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In this paper, a probiotic microbe mediated biosynthesis of silver nanoparticles (AgNPs) is reported. The synthesis of AgNPs was monitored by ultraviolet (UV)-visible spectroscopy. The particles thereby obtained were characterized by UV spectra, Fourier transform infrared (FTIR), scanning electron microscopy (SEM) with energy dispersive spectroscopy (EDS) and using particle size analysis. The mechanism leading to nanoparticle formation was studied by using nitrate reductase assay. The bactericidal effect against common gram-positive and gram-negative bacteria has also been investigated. The results indicate a high antimicrobial activity for gram-negative organism so that this material can be a promising antimicrobial compound.

Key words: *Brevibacterium linens*, biosynthesis, scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS), antimicrobial activity.

INTRODUCTION

All biological and man-made systems have the first level of organization at the nanoscale (such as a nanocrystals, nanotubes or nanobiomotors) where their fundamental properties and functions are defined. The goal of nanotechnology might be described by the ability to assemble molecules into objects, hierarchically along several length scales, and to disassemble objects into molecules. This is what nature already does in living systems and in the environment. Biosystems are governed by nanoscale processes that have been optimized over millions of years; examples of biostrategies have been surveyed (Ball, 2002).

A new branch of nanotechnology is nanobiotechnology. Nanobiotechnology represents an economic alternative for chemical and physical methods of nanoparticles formation (Ahmad et al., 2003). Nature has devised various processes for the synthesis of nano- and micro-length scaled inorganic materials which have contributed to the development of relatively new and largely unexplored area of research based on the biosynthesis of nanomaterials (Mohanpuria et al., 2008). Biosynthesis of nanoparticles is a kind of bottom up approach where the main reaction occurring is reduction/oxidation. Silver is a naturally occurring precious metal, most often as a mineral ore in association with other elements. It has been used in a wide variety of applications as it has special properties like high electrical and thermal conductivity (Nordberg et al., 1998).

Over the last decades, silver nanoparticles (AgNPs) have found applications in catalysis, optics, electronics and other areas due to their unique size-dependent optical, electrical and magnetic properties. Currently, most of the applications of AgNPs are in antibacterial/ antifungal agents in biotechnology and bioengineering, textile engineering, water treatment and silver-based consumer products. The biologically synthesized AgNPs could be of immense use in medical textiles for their

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Abbreviations: AgNPs, Silver nanoparticles; **FTIR,** Fourier transform infrared; **SEM,** scanning electron microscopy; **EDS,** energy dispersive spectroscopy.

efficient antimicrobial function (Vigneshwaran et al., 2006). The sterile cloth and materials play an important role in hospitals, where often wounds are contaminated with microorganisms, in particular fungi and bacteria, like *Staphylococcus aureus* (Lee et al., 2003).

Both bacteria and fungi make such an existing category of microorganisms having naturally bestowed property of reducing/oxidizing metal ions into metallic/oxide nanoparticle thereby functioning as mini nanofactories (Jha et al., 2009).

Vighneshwaran et al. (2007), Sadowski et al. (2008) and several authors have reported the extracellular synthesis of AgNPs using several bacteria. Sintubin et al. (2009) reported that both gram-positive and gram-negative bacteria have been used to synthesize AgNPs. Saiffudin et al. (2009) explained the synthesis of AgNPs using microwave-irradiated culture supernatant of *Bacillus subtilis*. Kumar et al. (2010) reported that purified rhamnolipids is produced by a newly isolated *Pseudomonas aeruginosa* strain BS-161R for the synthesis of biosurfactant-based monodispersed AgNPs. Synthesis of protein capped AgNPs by *Calotes versicolor* was reported by Rashmi and Preeti (2009). Jain et al. (2011) has reported the synthesis of AgNPs using *Aspergillus flavus* NJ: 08 and the role of fungal proteins involved in the reduction.

Extracts from bio-organisms may act both as reducing and capping agents in AgNPs synthesis. The reduction of silver ions by combinations of biomolecules found in these extracts such as enzymes/proteins, amino acids, polysaccharides and vitamins (Collera et al., 2005) is environmentally benign, yet chemically complex. The microbial enzymes or the plant phytochemicals with anti oxidant or reducing properties are usually responsible for reduction of metal compounds into their respective nanoparticles.

Duran et al. (2005) have indicated that nicotinamide adenine dinucleotide (NADH-) and NADH-dependent enzymes are important factors in the biosynthesis of metal nanoparticles. The reduction seems to be initiated by electron transfer from the NADH by NADH-dependent reductase as electron carrier.

Several salts of silver and their derivatives are commercially employed as antimicrobial agents (Holladay et al., 2006). Nanoparticles of silver have been studied as medium for antibiotic delivery (Li et al., 2005) and to synthesize composites for use as disinfecting filters (Jain, 2005) and coating materials. Saravanan (2010) reported the antimicrobial activity of *Aspergillus niger* silver nanoparticle against multidrug resistant (MDR) *S. aureus*. The antibacterial study of AgNP was carried out on human pathogenic *Escherichia coli* by Ghodake et al. (2011).

In the present investigation, we report the synthesis of AgNPs using probiotic organism *Brevibacterium linens* whose enzymes are used widely in cheese production endowed with significant antibacterial properties.

MATERIALS AND METHODS

Culture and culture maintenance

The bacterial strain *B. linens* (NCIM 2149) were obtained from the National Collection of the Industrial Microorganisms (NCIM), Pune, India. The Luria Bertani (LB) broths, Nutrient agar, Mueller Hinton Agar used were obtained from Hi-media Laboratories and it was ready to use. Silver nitrate and other chemicals used in this study were also obtained from Hi-Media Laboratories, India. The strain was maintained at 4°C on nutrient agar plates.

Biosynthesis of silver nanoparticle synthesis

B. linens was cultivated in LB broth. Erlenmeyer flasks of the capacity 150 ml were used for cultivating the bacterium. It consists of 50 ml of the growth medium. The strain was inoculated in the autoclaved media under sterilized and static conditions. This was allowed to grow for 24 h at 37°C. The culture *B. linens* after incubation was centrifuged at speed of 10,000 rpm for 10 min and the supernatants with pH of 6 were used for further experiments. The supernatant obtained after centrifugation was brought in contact with 10^{-3} M silver nitrate final concentration and agitated at 150 rpm in dark conditions. Simultaneously, control without silver ions was also run along with the experimental flasks.

Characterization of silver nanoparticles

The formation of AgNPs was monitored by visual inspection of the solution, as well as by periodical recording of the ultraviolet (UV)-vis spectra of the reaction mixture. The UV-vis spectroscopy measurements were recorded on a UV visible ELICO SL 191 double beam spectrophotometer. The aqueous filtrate containing AgNPs and their controls was subjected to Fourier transform infrared (FTIR) spectrum using a Thermo Nicolet, Avatar 370. The aqueous solution of AgNPs synthesized was freeze dried and used for scanning electron microscopy (SEM). The sample was examined under JEOL 6390 LV SEM equipped with energy dispersive spectroscopy (EDS). The aqueous dispersions of the synthesized particles were subjected to particle size analysis using a 90 plus nanoparticle size analyzer. The mechanism leading to the formation of silver nanoparticle was determined by using nitrate reductase assay and by determining the presence of tyrosine and tryptophan residues. The protein profile of the organism was obtained by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Antimicrobial activity analysis

The AgNPs were tested for their antibacterial activity by disc diffusion and by viable counting method.

MDR Gram-positive bacteria represented by *S. aureus* and *Bacillus* sp. Gram-negative represented by *E. coli* and *P. aeruginosa* were used for the antibacterial study. The bacteria were grown in LB broth. 10 and 50 µl concentration discs of *B. linens* silver nanoaprticles were prepared and then placed in Mueller Hinton agar plates swabbed with the bacterial culture. The zone of inhibition was compared with standard antibiotic amikacin.

The formation of a clear zone around the discs is an indication of antibacterial activity. The viable counting was done by inoculating the MDR pathogens in LB broth and to this the nanoparticles of *B. linens* was added and at each hour the growth delay by the nanoparticles was measured spectrophotometrically at 600 nm.

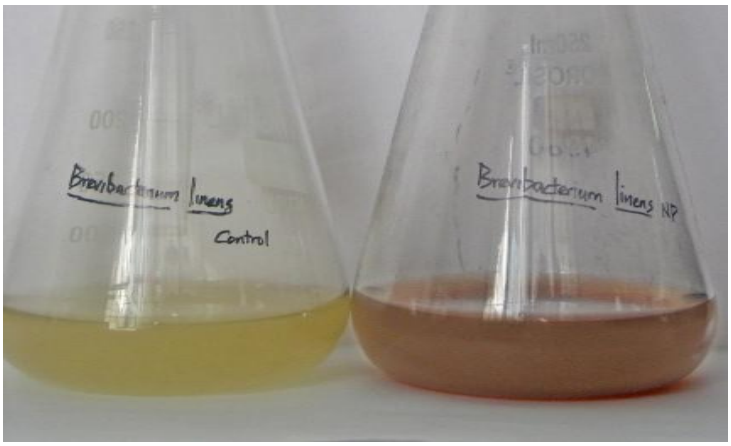

Figure 1. Color change of the filtrates.

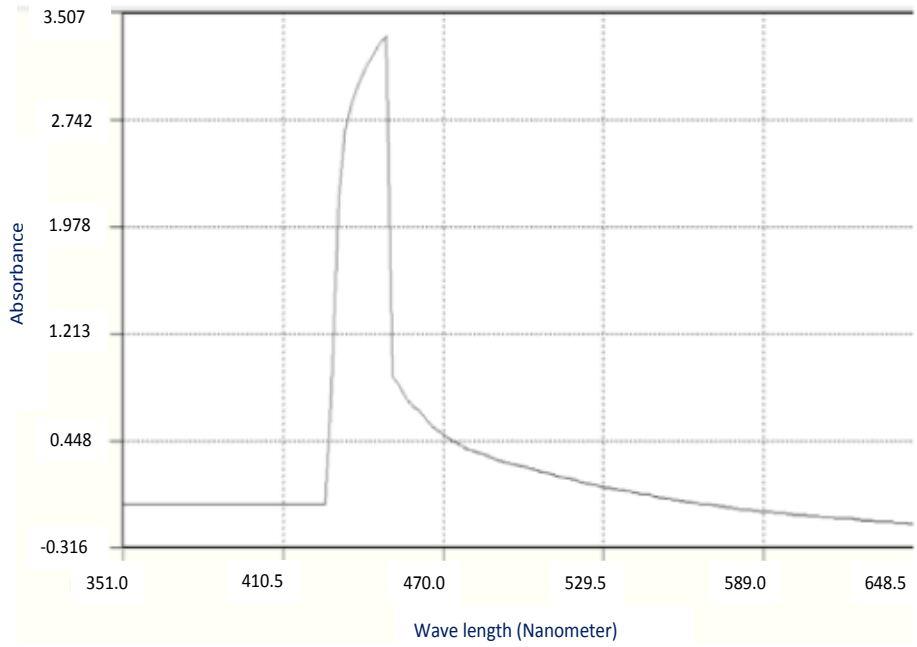

Figure 2. UV-visible graph of the *B. linens* AgNP.

RESULTS AND DISCUSSION

This work explores the biosynthesis of AgNPs using *B. linens* NCIM 2149. The synthesis of AgNPs was validated by visually monitoring the flasks containing only the aqueous filtrate prepared from *B. linens* NCIM 2149 and the reaction mixture of aqueous filtrate with silver nitrate. The aqueous filtrate was observed to retain its original color, whereas the silver nitrate mixed with supernatant turned to brown after 12 h incubation (Figure 1). The appearance of dark brown in solution is a clear indication of the formation of AgNPs in the reaction mixture. Underwood (1994) has stated that the change in the color of the solution is the excitation of surface plasmon vibrations in the AgNPs, which is characteristic property of the nanoparticles.

Zadowski et al. (2008) has reported that upon addition of silver ions into cell free filtrates in dark changed in color from almost colorless to brown with intensity increasing during the period of incubation. Reshmi and Preeti (2009) reported the change of color from pale yellow to brown.

The UV-Vis spectra of the aqueous reaction mixture were recorded (Figure 2). Aliquots of the reaction mixture were withdrawn at 12 h time interval and scanned on a UV-visible spectrophotometer. The absorbance band was observed at 448 nm in our study. Shankar et al. (2004) suggested that the shoulder at 370 nm corresponded to

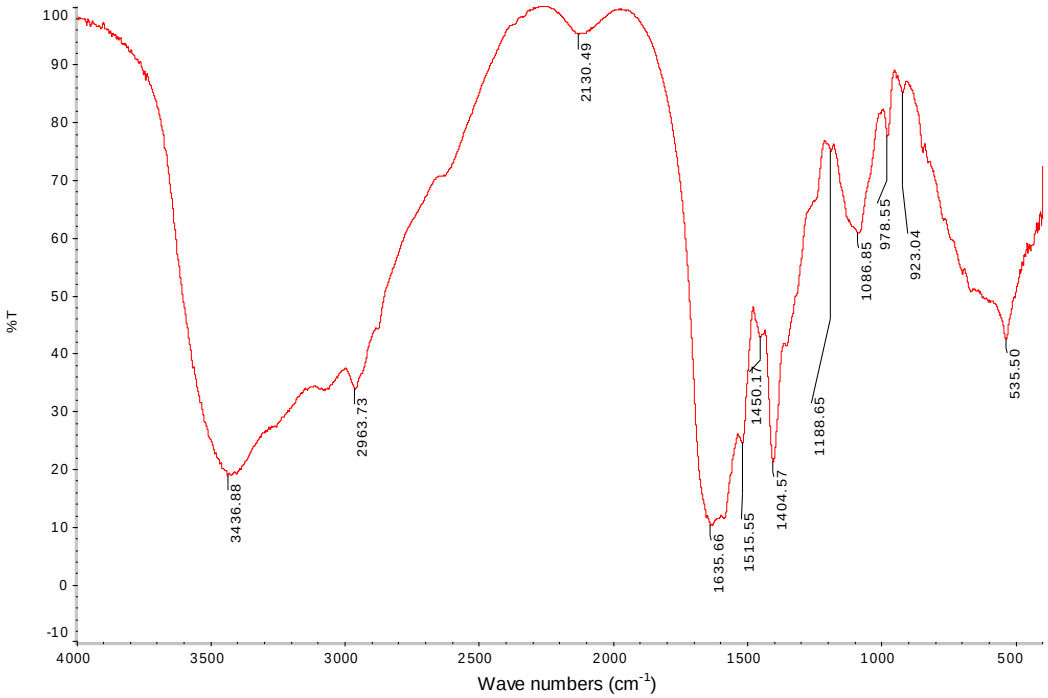

Figure 3. FTIR spectra showing possible groups involved in the reduction process.

the transverse plasmon vibration in silver nanoparticle, whereas the peak at 440 nm is due to excitation of longitudinal plasmon vibrations. Shahverdi et al. (2007) stated that as the particle size increases, the peak becomes narrower with a decreased bandwidth and increased band intensity.

Karthik Raja et al. (2010) reported silver plasmon peak for *Lactobacillus acidophilus* 01 strain at 430 nm. Jain et al. (2010) reported their absorption peak for *Bacillus thuringiensis* spore crystal silver nanoparticle at 450 nm. Our peak was obtained at 448 nm which is the excitation peak of silver nanoparticle.

The amide linkages between amino acid residues in proteins give rise to well-known characteristic bands in the infrared region of the electromagnetic spectrum. Figure 3 shows the FTIR spectrum of the AgNPs obtained from the bacteria. The FTIR measurement indicated that the structure of the protein was not affected because of its interaction with Ag^+ ions or nanoparticles. The band at 1450 cm^{-1} can be assigned to methylene scissoring vibrations from the proteins in the solution. A distinctively new bending was observed at positions 1086.85, 978.5 and 923 cm^{-1} clearly indicating the presence of aliphatic amines, carboxylic acids involved in the reduction of $AgNO_3$ to Ag^+. These bending peaks were entirely different from its corresponding control samples (control not shown). The study clearly explains the presence of protein molecules involved in the reduction of $AgNO_3$.

Stacchiola et al. (2007) and Calaza et al. (2007) mentioned the peak at 970 cm^{-1} which is the indication of formation of C=C double bond after the reduction process. The C=C double bond is in conjugation with the lone pair of electrons on the sulfur atom, thereby increasing the acidity of the S-H moiety. Reshmi and Preeti (2009) reported that peak at 1456 cm^{-1} is indicative of the role of aromatic groups in the reduction of Ag^+ ions in the filtrate. The FTIR spectral analysis revealed the presence of -C-O-C- and -C=C- functional groups, which may be present between amino acid residues and protein synthesized during silver bionanoparticles (Ag-BNPs) (Saravan, 2010).

SEM determination of the freeze dried sample showed formation of AgNPs (Figure 4). The morphology of the nanoparticles was highly variable. The morphology of the nanoparticles was uniform and spherical. The particles are nanosized and well dispersed with the size range of 90 nm.

Sadowski et al. (2008) reported the nanoparticles synthesized in the size range of 100 nm and the shape of the particle can be affected due to drying. Mona et al. (2009) has reported the nanoparticle in the size range of 50 to 100 nm using Enterobacteriaceae family and geranial, respectively.

The *B. linens* AgNP has strong signals of silver at 3 keV. The other signals of K and Na might be due to the excitation of enzymes which are left unreduced (Figure 5). Varshney et al. (2009) reported strong signals from the silver atoms in the nanoparticles of their studied fungi while weaker signals from C, O, S, P, Na, Mg and Ca

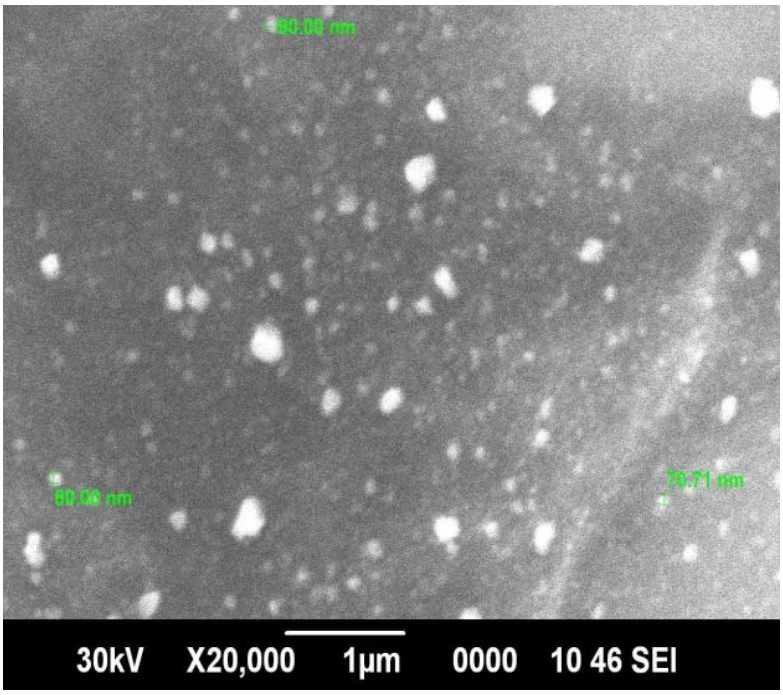

Figure 4. SEM image of the *B. linens* AgNP.

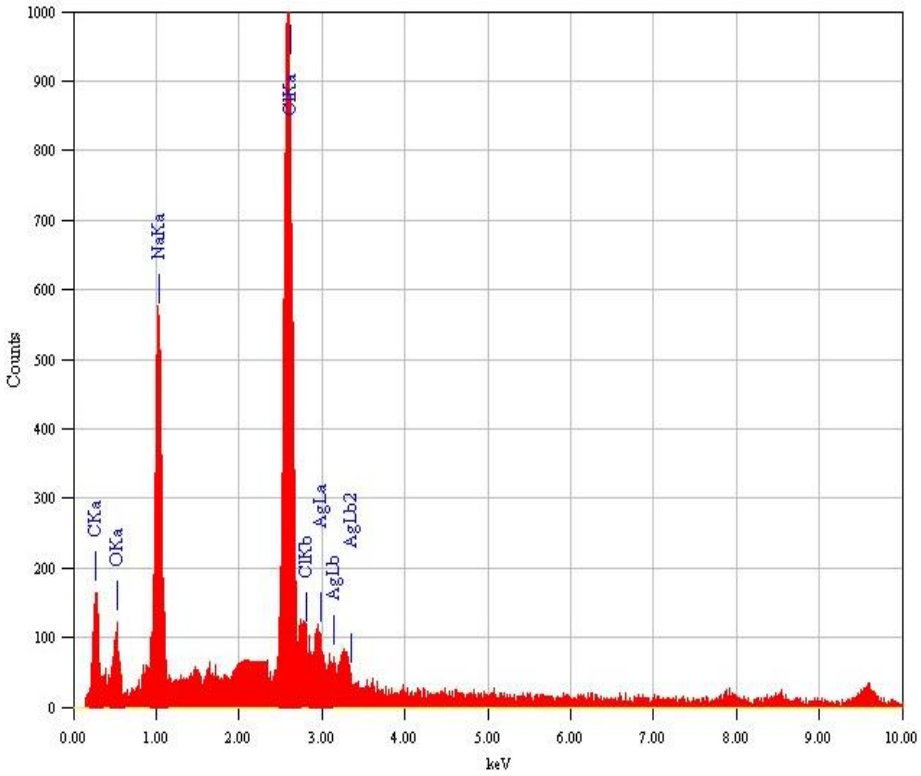

Figure 5. EDS of the *B. linens* AgNP.

atoms were also recorded. Mona et al. (2009) reported the presence of elemental silver in their study using geraniol as substrate. The average particle size of the *B. linens* nanoparticle in our study was found to be 238 nm. Natarajan et al. (2010) reported particle size obtained in size range of 40 to 60 nm for *E. coli* silver nanoparticle.

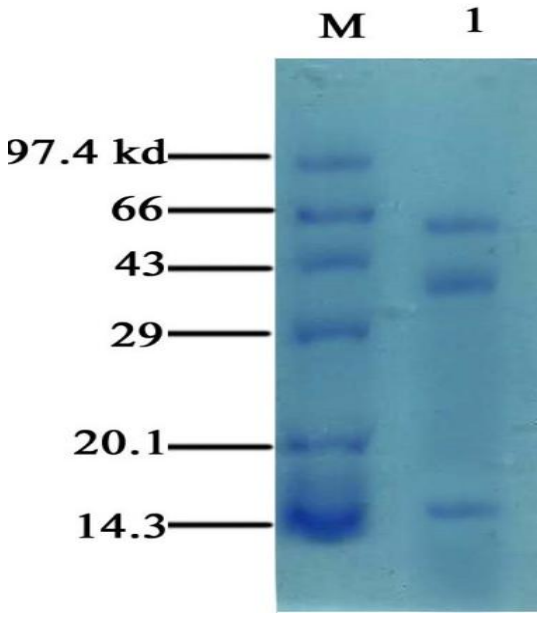

Figure 6. Protein bands of *B. linens*. M, Marker; 1, *B. linens*

The amount of nitrate reductase enzyme was found to be 300 nmol/hr/mL. This enzyme would have played a major role in the reduction of silver nitrate to silver. Bhaskara rao et al. (2010) reported 270 nmol/h/ml of nitrate reductase activity in the culture supernatant of *Pencillium* sp.

Saiffudin et al. (2009) stated that the average protein contains 3.5% phenylalanine, 3.5% tyrosine and 1.1% tryptophan: these three amino acids contribute the majority of the proteins absorbance and fluorescence properties in the range of 250 to 300 nm wavelength, although if the protein contains significant numbers of disulphide bonds, these too will contribute to the absorption properties in the wavelength range of 250 to 280 nm. The cell free filtrates of *B. linens* contain significant amount of disulphide amounts and hence, the band was observed in the range of 275 nm clearly indicating the presence of excitations of tryptophan and tyrosine residues. Two protein bands of molecular weight 66 and 42 kDa was obtained for *B. linens* which is similar to reductase enzyme suggesting the presence of protein molecules involved in the reduction of silver nitrate to silver (Figure 6). Manivannan et al. (2010) reported the molecular weight estimated of the protein by SDS-PAGE of the ammonium sulfate-precipitated protein sample of culture filtrate of marine yeast which revealed a single protein band, with a molecular weight of 55 kDa which is similar to that of NADH-dependent nitrate reductase.

Antimicrobial activity analysis

The antibacterial activity of silver species has been well known since ancient times (Catauro, 2004). The studied AgNPs exhibited excellent antibacterial activity against MDR pathogens. The number of viable colonies of *E. coli* after incubation with *B. linens* AgNP was found to be very low in case of liquid media. The nanoparticle was able to inhibit 80% of the pathogens. SEM image recorded for *E. coli* cells incubated with *B. linens* AgNP after 3 h shows a gradual decrease in number of cells when recorded along with its corresponding control (Figures 7, 8 and 9). The *B. linens* silver nanoparticle showed maximum zone of inhibition for *S. aureus*. The result of the nanoparticle almost was similar to the standard antibiotic amikacin (Figure 10 and Table1).

Several studies have investigated the interaction of monosilver with bacteria. Morones et al. (2005) revealed that the majority of nanosilvers were localized on the membrane of treated *E. coli*. Baker et al. (2005) reported that nanoparticles of low concentration in solution were completely cytotoxic to *E. coli*. Lok et al. (2005) demonstrated that treatment of *E. coli* cells with nanomolar concentration of nanosilver results in the immediate dissipation of the proton motive force, killing the cells. Such a biological mode of action is similar to that found for silver nitrate.

Wang et al. (2007) demonstrated that the changes in morphology presented in the membrane of the bacteria in conjunction with the possible damage caused by the AgNPs reacting with sulfur containing proteins in the interior of the cell as well as with phosphorous containing compounds such as DNA, will affect the bacteria in the processes such as respiratory chain and cell division, finally causing the death of the cell. Jain et al. (2010) reported that silver nanoparticle synthesized by *B. thuringiensis* spore exhibited antibacterial activity against MDR human pathogenic *E. coli*, *P. aeruginosa*, *S. aureus* as it showed a clear inhibition zone whereas the standard antibiotics (piperacillin, doxycycillne, cefixime, cefepime, chorampenicol, tetracycline, ampicillin, cefoperazone, gatifloxacin and gentamycin) did not show any inhibition.

Conclusion

We have reported the simple biological way for synthesizing the AgNPs. The synthesis process was quite fast and extracellular mode of synthesis is presented. The presence of elemental silver was confirmed using EDS analysis. The results of FTIR and SDS-PAGE have suggested that protein might have played an important role in the reduction and stabilization of AgNPs. The 50 µl concentration nanosilver was found to have wider antimicrobial activity. Thus, these particles can be used in minimum amount and inhibit the growth of the pathogens without any harmful side effects replacing the conventional antibiotics. This process of the nanoparticles production is eco-friendly as it is free from any solvent or toxic chemicals, and also easily amenable on large scale production.

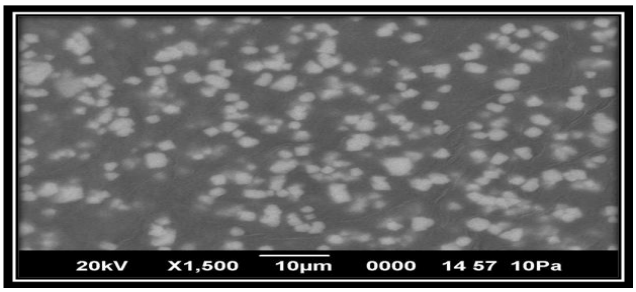

Figure 7. SEM image of *E. coli* treated initially after addition of *B. linens* AgNP.

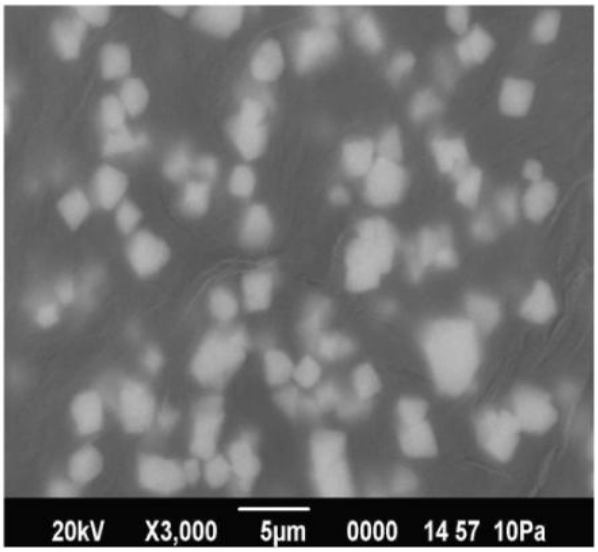

Figure 8. SEM image of *E. coli* after 3 h incubation with AgNP.

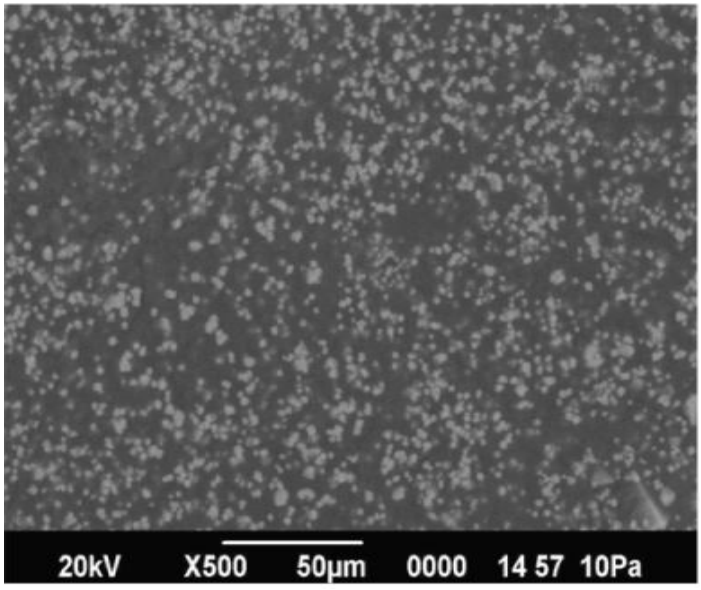

Figure 9. Control of *E. coli cells* without silver nanoparticle.

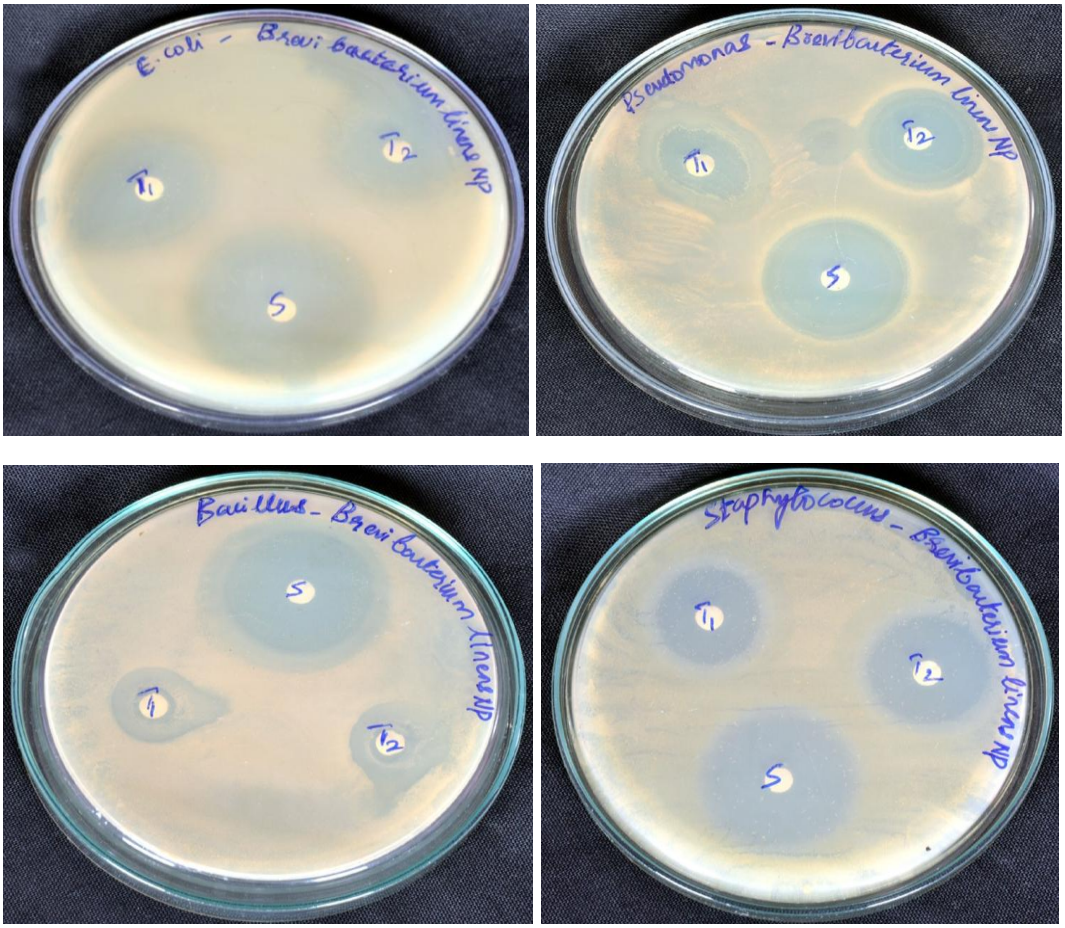

Figure 10. List of plates showing zone of inhibition by *B. linens* against MDR pathogens. T_1, *B. linens* AgNP 10 µl concentration; T_2, *B. linens* AgNP 10 µl concentration; S, standard antibiotic disc amikacin.

Table 1. The antimicrobial action of *B. linens* AgNP zone of inhibition in mm.

MDR pathogen	Standard antibiotic amikacin	*B. linens* AgNP	
		10 µl	50 µl
E. coli	33	25	29
P. aeruginosa	26	23	25
Bacillus sp.	30	25	23
S. aureus	30	20	30

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