Digest Journal of Nanomaterials and Biostructures Vol. 10, No. 4, October - December 2015, p. 1189 - 1199

ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES SINGLY AND IN COMBINATION WITH THIRD GENERATION ANTIBIOTICS AGAINST BACTERIA CAUSING HOSPITAL ACQUIRED INFECTIONS BIOSYNTHESISED BY ISOLATED *BACILLUS MARISFLAVI* YCIS MN 5

N. Y. NADAF, S. S. KANASE*
Department of Microbiology and Biochemistry, Yashvantrao Chavan Institute of Science, Satara, Maharashtra, India – 415001.

We report eco-friendly, extracellular and cost effective synthesis of Silver nanoparticles using *Bacillus marisflavi* YCIS MN 5, isolated from an estuarine source and their antibacterial activity singly and in combination with third generation antibiotics against bacteria causing hospital acquired infections. The biosynthesised AgNPs were characterised using UV-visible spectroscopy, X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), Field emission scanning electron microscopy (FE-SEM), Energy dispersive spectroscopy (EDS) and Dynamic light scattering (DLS). The UV-visible spectrophotometric analysis showed strong Surface Plasmon Resonance (SPR) peak at 434 nm. XRD analysis revealed the crystalline nature of AgNPs with FCC lattice. The FTIR analysis confirmed the role of biomolecules in the formation of AgNPs. FE-SEM revealed the size of AgNPs in the range 20-30nm. EDS confirmed the formation of metallic AgNPs. The average particle size calculated using DLS measurements was found to be ~27 nm.

(Received August 4, 2015; Accepted October 21, 2015)

Keywords: Bacillus marisflavi YCIS MN 5, silver nanoparticles, XRD, FTIR, FE-SEM, DLS, hospital acquired infections, antibacterial activity.

1. Introduction

Nanotechnology has become one of the most dynamic research fields in science and technology and is emerging as a newest technology interdisciplinary with physics, chemistry, biology, material science and medicine. Nano research starts with the synthesis of stable metal nanoparticles of different chemical compositions and sizes. A wide variety of chemical, physical and biological methods are employed for the synthesis of such nanoparticles. Use of high temperature, hazardous and expensive chemicals as well as generation of toxic by-products make the physical and chemical methods less suitable for production of nanoparticles, where biological applications are concerned. Biological synthesis of nanoparticles has been proved to be superior to chemical and physical methods [1] as they are eco-friendly, facile and reproducible [2].

Many micro-organisms such as bacteria, fungi, algae have been used for biosynthesis of different nanoparticles. Bacteria have an innate ability to reduce metallic ions to their respective metallic nanoparticles [3]. Few bacterial strains *Brevibacterium casei* [4], *Bacillus megaterium* [5], *B. stratosphericus* [6], *Geobacillus stearothemophilus* [7], *Aeromonas hydrophila* [8], *Idiomarina* sp. [9], *Stenotrophomonas maltophila* [10], *Acinetobacter* sp. [11] and *Klebsiella pneumoniae* [12] have been explored for their ability to reduce metals to nanoscale.

Hospital acquired infections is a major problem that doctors and patients have to face in day to day life. *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* are most common bacteria causing nosocomial infections. *P. aeruginosa* is well known for urinary tract infections, acute purulent meningitis following lumbar puncture, wound and burn infections etc.

*Corresponding author: srkanase@rediffmail.com

S. aureus causes pyogenic infections, food poisoning and toxic shock syndrome and virulent strains of *E.coli*, are known to produce urinary tract infections and diarrhoea. The problem becomes more severe when these organisms show drug resistance. Growing resistance of different pathogenic organisms to various antibiotics has opened a broad area of research to find alternative treatment strategies. Research now is focused on use of nanoparticles as antimicrobial agents in nano drug formulations to combat with the resistant organisms and treatment of various diseases.

The antimicrobial properties of silver have been known for many centuries. AgNPs have received more importance than metallic silver due to their increased bactericidal and antifungal action at a nanoscale reflecting their potential use in biomedical applications including preparation of topical aids for wound healing, coatings for medical devices, water purification, antimicrobial textile products etc. [13-16]. Taking into consideration of wide applicability of AgNPS, the present work was focused on biosynthesis of AgNPs and assessment of their antibacterial activity against bacteria causing hospital acquired infections.

In this study, AgNPs synthesising bacterium was isolated from Vashisti estuary, situated at Dabhol, Konkan, west Coast of Maharashtra, India. Industrial development along the riverine and estuarine system is one of the main causes for pollution of these natural water sources. The content of heavy metals in Vashisti estuary is reported to be higher [17]. This site was therefore selected as a sampling site assuring greater probability of finding organisms having a capacity to grow in presence of heavy metals due to acclimatisation. Here we report for the first time, biosynthesis of AgNPs by *Bacillus marisflavi* YCIS MN 5 isolated from Vashisti estuary in Dabhol.

In this study we further report, antibacterial activity against *P. aeruginosa, S. aureus* and *E.coli*, the most common bacteria causing hospital acquired infections by using biosynthesised AgNPs, singly and in combination with third generation cephalosporin antibiotics.

2. Materials and methods

2.1 Materials

Silver Nitrate ($AgNO_3$) was purchased from Merck, India. Nutrient agar and nutrient broth was procured from Himedia, India. Antibiotics- cefixime and cefoperazone were purchased from local medical store.

2.2 Methods

2.2.1 Sample collection and isolation of bacteria

Water samples were collected from the Vashisti estuary, Konkan, Maharashtra, India. Sterile bottles were used for collection of water samples, which were proceeded for isolation of bacteria by plating onto nutrient agar. Culture plates were incubated at 27°C for 24 h. Pure isolates were transferred and maintained on nutrient agar slants and sub cultured at regular intervals.

2.2.2 Screening of isolate for extracellular synthesis of AgNPs

Out of twenty bacterial isolates, obtained from Vashisti estuary, ten bacterial isolates were randomly selected for testing the extracellular synthesis of AgNPs. The bacterial isolate showing potential to synthesise extracellular AgNPs was selected on the basis of visual observation of colour change.

2.2.3 Extracellular Synthesis of AgNPs

Bacterial culture of O.D 1.0 was inoculated in 250ml Erlenmeyer flask containing 100ml of sterilised nutrient broth and kept in orbital shaker incubator at 27°C for 24 h at 120 rpm. The biomass was separated from cultured broth by centrifugation (at 8000 rpm for 10 min) using cooling centrifuge (Remi). Further, bacterial biomass was collected and washed thrice with sterile distilled water. Approximately 2 g biomass was suspended in 20ml sterile distilled water and agitated at 27°C, 120 rpm for 24 h. After centrifuging, supernatant was collected (cell free extract) and challenged with $AgNO_3$ to make the final concentration 1mM and kept in shaker incubator at 120 rpm at 27°C under dark condition. Controls of heat inactivated cell free extract with $AgNO_3$ and only $AgNO_3$ solution were also run simultaneously along with the experimental flask under

same conditions. The flasks were periodically monitored for colour change and UV-Visible absorption study.

2.2.4 Molecular Identification

Molecular identification of the bacterial isolate was carried out by 16S rRNA sequencing. The 16S rRNA sequences obtained were aligned using EZ-Taxon Database and subjected to Basic Local Alignment Search Tool (BLAST) and analysed at National Centre for Biotechnology Information (NCBI). The phylogenetic analysis of the 16S rRNA sequence of the isolate was inferred using the Neighbour - Joining method [18] in MEGA 5 [19]. The 16S rRNA sequence was deposited in the NCBI Gene Bankit nucleotide sequence database (accession number KP163987).

2.2.5 Characterization of AgNPs

The formation of AgNPs was monitored by visual inspection of the reaction mixture as well as by periodical analysis of UV spectra using the UV-Visible spectrophotometer (SYSTRONICS 119) in the 350 to 750nm range. For XRD studies the biosynthesised AgNPs solution was drop coated on glass substrate and X ray diffractogram was recorded on X-Ray diffractometer (Bruker AXS Analytical Instruments Pvt. Ltd, Germany) at an operating voltage of 30kV using Cu Kα radiation (k_λ=1.54060 Å) in the 2θ range from 0° to 80°. FTIR analysis was carried out using FTIR spectrophotometer (Bruker, TENSOR 37) to confirm the role of biomolecules in the formation of AgNPs. FE-SEM analysis of air dried sample was executed for morphological study of AgNPs under FE-SEM (Hitachi S-4800, Japan). EDS was obtained using JEOL JSM-6360A (Analytical scanning electron microscope, Japan) at an accelerating voltage of 20 keV for elemental analysis. The hydrodynamic size of nanoparticles in the solution was examined using DLS (PSS- Nicomp 388-ZLS Particle sizing systems, Inc., Santa Barbara, California, USA).

2.2.6Antibacterial activity of AgNPs with and without antibiotics

Antibacterial activity of AgNps with and without antibiotic was studied against *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E.coli* ATCC 25922. In this study, third generation antibiotics, cefixime was used as positive control against *E. coli* and cefoperazone against *S. aureus* and *P. aeruginosa* for comparison with AgNPs. The overnight culture of each bacteria of 10^6 CFU/ml was spread inoculated onto nutrient agar. Wells of 6mm diameter were bored on nutrient agar and loaded with AgNPs, antibiotic, combination of antibiotic and AgNPs and bacterial cell free extract (Control) each containing 20µl. Plates were incubated at 37°C for 24 h. After incubation period, zone of inhibition was observed and measured (in mm). The experiment was carried out in triplicates.

Percent fold increase for different antibiotics against bacterial pathogens was calculated using the formula [20],

$$\frac{(B^2 - A^2)}{A^2}$$

Where, A – inhibition zone for antibiotic and B – inhibition zone for antibiotic in combination with AgNPs.

3. Results

3.1 Isolation and identification of bacteria

Out of twenty bacterial isolates obtained from Vashisti estuary, ten bacterial isolates were used for screening of biosynthesis of AgNPs. One bacterial isolate showing positive results was proceeded for molecular identification on the basis of 16S rRNA sequencing. The identification details were obtained using EZ-Taxon Database. Based on phylogenetic analysis the bacterium

was identified to be *Bacillus marisflavi*. The phylogenetic analysis showed the evolutionary relationship with 100% similarity to *Bacillus marisflavi* TF-11T.

3.2 Extracellular Synthesis of AgNPs

Visual observation of colour change from pale yellow to brown after addition of $AgNO_3$ in cell free extract is the primary indication of formation of AgNPs (Fig. 1). The reaction mixture when kept at 27°C under dark and shaking conditions showed a colour change from pale yellow to light brown within 24 h which turned increasingly to dark brown up to 96 h of reaction time. After 96 h of reaction, the intensity of the peak remained unaltered indicating that complete reduction of $AgNO_3$. No colour change was observed in control tubes containing, i) Heat inactivated cell free extract with $AgNO_3$, and ii) $AgNO_3$ solution (Data not shown). Thus, it can be said that cell free extract of *B. marisflavi* effectively acts as a reducing agent transforming $AgNO_3$ to metallic AgNPs. .

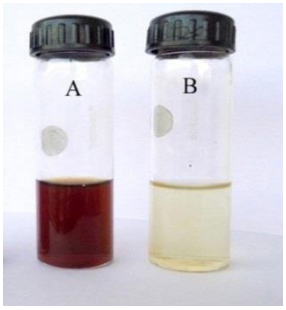

Fig. 1 Colour change observed within 24 h at 27°C. (A) Test- Cell free extract with $AgNO_3$, (B) Control- Heat inactivated Cell free extract with $AgNO_3$.

3.3 Characterization of synthesised AgNPs

3.3.1 UV-Visible Spectroscopy

UV-Visible spectroscopy is used to characterise the kinetics of formation of AgNPs. AgNPs absorb radiation in the visible region of the electromagnetic spectrum 380- 450nm due to SPR. Hence UV-visible spectra of biosynthesised AgNPs was recorded over the function of time of reaction (Fig. 2). The strong surface plasmon resonance (SPR) peak was observed at 434 nm. The intensity and broadness was increased with the function of time of reaction up to 96 h, without any shift in peak centre which may be due to the size distribution of nanoparticles. The AgNPs solution remained stable for five months without settling down which indicate that the cell free extract has effectively acted as a capping agent of AgNPs preventing their extensive agglomeration.

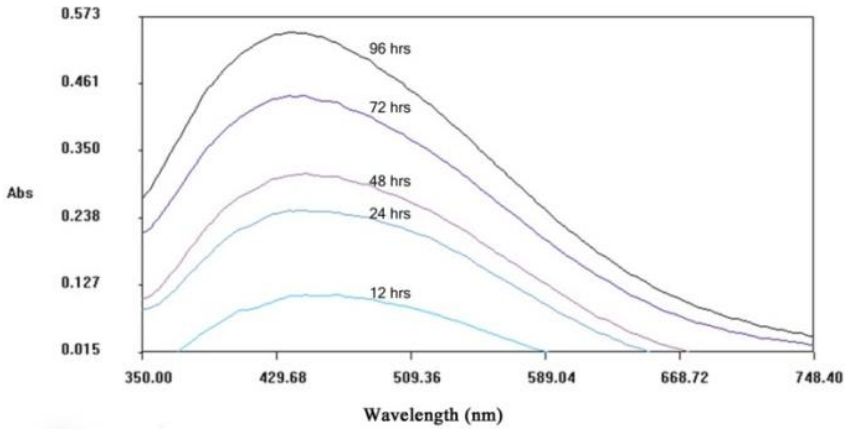

Fig.2 UV-Vis spectra of colloidal solution of AgNPs synthesised using B. marisflavi showing strong peak at 434 nm.

3.3.2 X-Ray Diffraction (XRD)

XRD is used to affirm the crystalline nature of AgNPs. The presence of four major Bragg peaks at 2θ are 38.21°, 44.34°, 64.70° and 77.57° confirm typical diffraction pattern of silver (Fig. 3). The strong diffraction peak at 38.21° corresponds to (111) whereas moderate peaks at 44.34°, 64.70°, 77.57° respectively correspond to (200), (220), (311) planes of silver with FCC lattice (JCPDS card No. 04-0783). Other peaks at 28.04° and 34.35° have also been observed. The peak broadening is a clear indication of nanocrystalline nature of AgNPs. The average crystallite size of AgNPs calculated using Debye-Scherrer formula is found to be ~23 nm corresponding to the highest intensity peak of 38.21°.

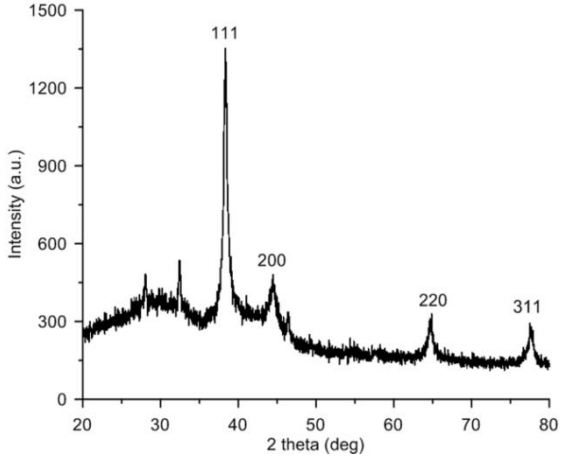

Fig. 3 X-ray diffraction patterns of AgNPs film deposited on glass substrate.

3.3.3 Fourier Transform Infra Red Spectroscopy (FTIR)

FTIR spectroscopy is an analytical technique, use to identify organic, polymeric materials present in the sample. Here FTIR analysis was performed to understand the possible involvement of biomolecules in the formation of AgNPs (Fig.4). The intense bands were observed at 3217cm^{-1}, 1596cm^{-1}, 1374cm^{-1}, 1034cm^{-1} and 530cm^{-1}. The band at 3217cm^{-1} and 1596cm^{-1} is assigned to N-H stretching and bending vibrations of amides respectively. The band at 1374cm^{-1} is due to C-H bending in alkane group. The peak at 1034cm^{-1} indicates the involvement of S=O stretching vibration. The peak at 530cm^{-1} is assigned to carboxylic groups.

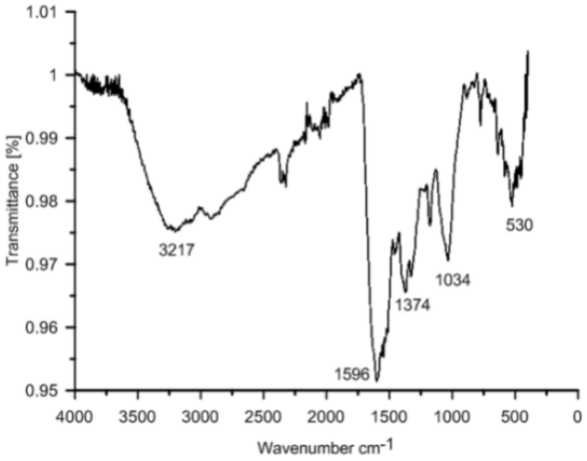

Fig. 4 FTIR spectra of AgNPs synthesised using cell free extract.

3.3.4 Field Emission Scanning Electron Microscopy (FE-SEM) and Energy Dispersive X-Ray Spectroscopy (EDS)

FE-SEM provides topographical information of the materials at higher magnification. The AgNPs synthesised using *B. marisflavi* were scanned using FE-SEM. Spherical AgNPs with size range 20-30 nm and these nanoparticles were clearly visible in the FE-SEM micrograph (Fig. 5 a, b). The elemental analysis performed using EDS confirmed synthesis of AgNPs. Presence of carbon and other signals is likely due to the presence of proteins capped on the surface of the AgNPs (Fig. 5 c).

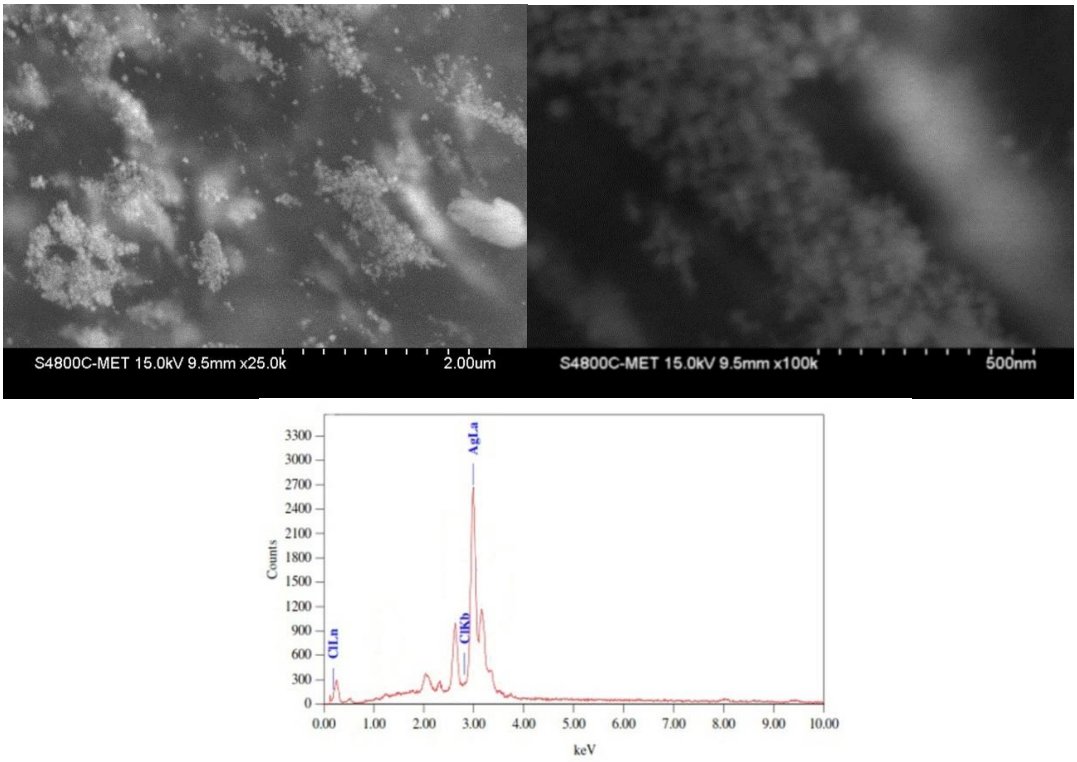

Fig. 5 FE-SEM image of AgNPs at a) ×25000 magnifications and b) ×100000 magnification, c) EDS spectrum showing the strong peak at ~3keV indicates the presence of elemental nanosilver

3.3.5 Dynamic Light Scattering (DLS)

DLS is a technique use to determine the size distribution profile of nanoparticles. The hydrodynamic particle size of the sample reveals bi-modal particle size distribution (Fig.6). Majority of the particles lay in the range of 20-50 nm with average particle size ~ 27 nm. A very small quantity of nanoparticles has been also seen in the range of 100-200 nm which can be attributed to agglomeration of few nanoparticles. Interestingly, hydrodynamic size matches relatively well with the particle size obtained from FE-SEM analysis, though obtaining a higher nanoparticle size using this technique is a regular feature due to agglomeration.

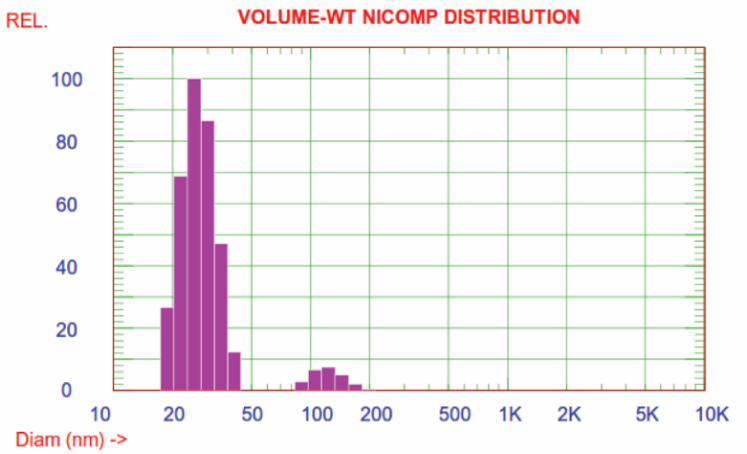

Fig. 6 Hydrodynamic particle size measurement using DLS (Volume weighted) technique.

3.4 Antibacterial activity of AgNPs with and without antibiotics

The antibacterial activity was studied by agar well diffusion method. Organisms selected for the study were representative pathogenic organisms showing antibiotic resistance. They were *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 and *E.coli* ATCC 25922.

The study revealed antibacterial activity of AgNPs, standard antibiotics as well as combination of antibiotics with AgNPs against *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Fig. 7).

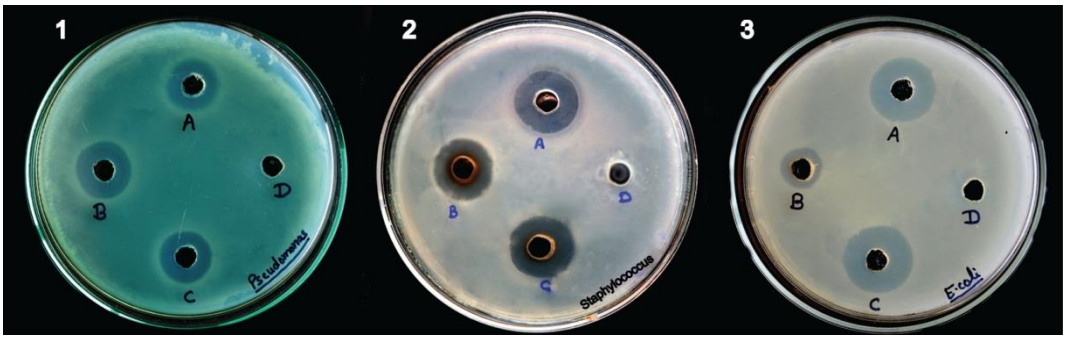

Fig.7 Zone of inhibition of A) Antibiotic, B) AgNPs, C) Antibiotic + AgNPs and *D) Control against 1] P. aeruginosa, 2] S. aureus, 3] E. coli*

It was observed that AgNPs showed good antibacterial activity against *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (Table 1). AgNPs, showed greater zone of inhibition than standard antibiotic against *P. aeruginosa* ATCC 27853 (19mm) and *S. aureus* ATCC 25923(20mm), whereas zone of inhibition against *E.coli* ATCC 25922 was found to be less than standard antibiotic (15mm) (Fig. 8 and Table 1).

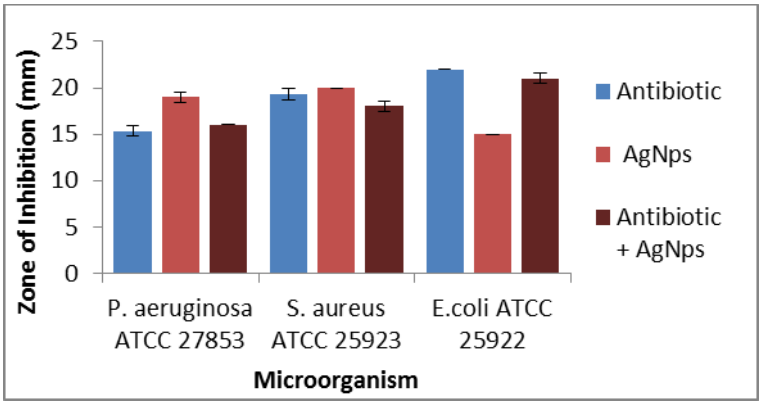

Fig. 8 Graphical representation of inhibitory effect of antibiotic, AgNPs, antibiotic + AgNPs against bacterial human pathogens

The combination of AgNPs and cefoperazone showed enhanced antibacterial activity against *P. aeruginosa* ATCC 27853 as compared to standard antibiotic and zone of inhibition was found to be increased by 0.13 fold ($P \leq 0.05$). On the other hand, no enhanced activity of AgNPs plus cefoperazone and AgNPs plus cefixime was observed against *S. aureus* ATCC 25923, *E. coli* ATCC 25922, respectively (Table 1).

Table 1 Comparison of inhibitory activity of antibiotic, AgNPs, combination of antibiotic and AgNPs, control against test pathogens.

Microorganism	**Mean Zone of Inhibition (in mm)**			
	Antibiotic	**AgNPs**	**Antibiotic + AgNPs**	**Control**
P. aeruginosa ATCC 27853	(a)15 ± 0.58	19 ± 0.58	16	-
S. aureus ATCC 25923	(a)19 ± 0.58	20	18 ± 0.58	-
E. coli ATCC 25922	(b) 22	15	21 ± 0.58	-

Note: Mean±SD, Antibiotics (a) Cefoperazone and (b) Cefixime.

4. Discussion

This study represents the extracellular biosynthesis of AgNPs using cell free extract of *Bacillus marisflavi* YCIS MN 5, isolated from estuarine water. The use of bacterial cell free extract resulted in the formation of clear solution of AgNPs.

The first indication of formation of AgNPs is colour change from pale yellow to brown, as reported previously [21, 22, 23]. Our culture exhibits the same colour change within 24 h with increase in intensity up to 96 h. Further confirmation and characterization of AgNPs is performed by UV-Vis spectroscopy, XRD, FTIR spectroscopy, FE-SEM, EDS and DLS.

The absorption band in the range 380-440 nm is due to excitation of surface plasmon resonance of nanoparticles, which is typical for nanoscale silver [24]. The strong surface plasmon resonance (SPR) peak was observed at 434 nm which is very close to the reported SPR peak for AgNPs [25]. The position and shape of plasmon absorption of nanosilver are strongly dependent on the particle size, dielectric nature of the medium and surface adsorbed species [26]. In comparison with the above observations, UV-Visible spectroscopy of our sample, revealed the strong absorption peak at 434nm which is an indication of the formation of AgNPs while peak broadening indicates polydispersed particle size.

The crystallography of synthesised AgNPs was investigated by XRD. The presence of four major Bragg peaks at 2θ ~ 38.21°, 44. 34°, 64.70° and 77.57° confirmed the typical diffraction

pattern of silver, which indicates presence of highly crystalline AgNPs. These Bragg peaks could be indexed to (111), (200), (220) and (311) planes of silver. The obtained data matched perfectly with the database of Joint Committee on Powder Diffraction Standards (JCPDS) file no. 04-0783. These results are in agreement with the previous reports [4, 27, 28]. Few other minor peaks have also been observed which could be due to interaction of organic biological compounds present in bacterial cell free extract with AgNPs which is in accordance with previous studies [29, 30].

FTIR spectroscopy is an excellent tool for comprehending the involvement of bacteria originated chemical functional groups in the reduction of silver ions. FTIR spectroscopic analysis revealed the involvement of biomolecules like proteins released by bacteria in the synthesis and stabilisation of the AgNPs. It can be speculated that these proteins bind to the AgNPs via amine groups or alkanes providing the stability to AgNPs [31].

Biologically synthesised AgNPs can be of various shapes such as hexagonal, pentagonal, spherical, nanobowls [29, 32]. The shape of the biosynthesised AgNPs can be variable depending upon the culture used as well as other physical conditions. In present work, *B. marisflavi* is found to synthesise spherical AgNPs as clearly seen in FE-SEM images. Further evidence for the formation of AgNPs is provided by energy dispersive spectroscopy of the sample. In EDS spectrum, optical absorption band peaking at ~3 keV indicated the presence of elemental silver, which is typical for AgNPs [33]. DLS, also known as Photon Correlation Spectroscopy is an important tool used to realise the hydrodynamic diameter of very small particles present in suspension or solution. The particle size of the AgNPs as determined from DLS analysis is in the range of 20-50nm with average particle size ~ 27±5.7nm (Vol. 92.98%). The DLS analysis also showed the presence of larger AgNPs of size range 100-200nm with average particle size ~123±20.8nm (Vol. 7.02%) which may be due to particle aggregation in aqueous solution. The hydrodynamic particle size of phytosynthesised AgNPs was reported to be 40-100 nm [34] and the particle size of mycosynthesised AgNPs was reported to be 17±5.9 nm [35].

Silver and its compounds are known to have antimicrobial properties for centuries. Recent studies have showed the significance of AgNPs as effective antimicrobial and antifungal agents [10, 25]. In present work of comparative antibacterial activity of AgNPs with and without the third generation antibiotics, against *P. aeruginosa, S. aureus* and *E. coli* showed that, AgNPs alone were effective against *P. aeruginosa, S. aureus* and *E. coli*, whereas enhanced antibacterial activity of AgNPs combined with antibiotics was observed against *P. aeruginosa.* The enhanced antimicrobial activity may be due to the bonding reaction between antibiotic and AgNPs [36, 37]. The active groups such as hydroxyl and amido groups of antibiotics react easily with AgNPs by chelation and may enhance the antibacterial activity [37]. The enhanced effect and extent of efficacy of AgNPs with various antibiotics is also depends on the type of antibiotics and bacterial strain used for study [38].

5. Conclusion

Here we report, for the first time, isolation of *Bacillus marisflavi* from Vashisti estuary, Dabhol, Konkan, Maharashtra and eco-friendly, extracellular and cost effective synthesis of AgNPs using its cell free extract. Further, synthesised AgNPs were characterised using UV-Vis spectroscopy, XRD, FE-SEM, EDS and DLS and found to be of crystalline nature with FCC lattice, spherical with size in the range of 20-50 nm. FTIR spectra suggested the role of biomolecules in the reduction of silver nitrate to AgNPs. The antibacterial activity of AgNPs with and without cephalosporin antibiotics, against bacteria causing nosocomial infections, viz. *P. aeruginosa, S. aureus* and *E. coli* was studied. It was observed that AgNPs were effective against *P. aeruginosa, S. aureus* and *E. coli*, whereas enhanced antibacterial activity of AgNPs in combination with antibiotic was observed against *P. aeruginosa*. This result seems to be significant in designing the strategies for curing *Pseudomonas* infections. Future studies can be directed towards assessment of interaction between AgNPs and antibiotics and their role as antibacterial agents against pathogens under study.

Acknowledgment

The authors are thankful to Dr. K.G. Kanade (Yashvantrao Chavan Institute of science, Maharashtra, India), Dr. S. D. Sartale (Savitribai Phule Pune University, Pune, India) for their valuable guidance. We also extend our sincere thanks to Dr. Manish Shinde (C-MET, Pune, India) for assistance with the DLS analysis of the AgNPs. NYN thanks University Grants Commission-Ministry of Minority Affairs (UGC-MOMA) for awarding Maulana Azad National Fellowship (MANF).

References

[1] M Rai, N Duran Metal nanoparticles in microbiology. Springer, Verlag Berlin Heidelberg. (2011)
[2] VC Verma, RN Kharwar, AC Gange, Nanomedicine, **5**, 33 (2010).
[3] SK Srivastava, M Constanti, J Nanopart Res, **14**, 1(2012).
[4] K Kalishwaralal, V Deepak, SRK Pandian, M Kottaisamy, S BarathmaniKanth, B Kartikeyan, S Gurunathan, Colloids surf B, **77**, 257 (2010).
[5] M Saravanan, AK Vemu, SK Barik, Colloid surface B, **88**, 325(2011).
[6] A Hosseini-Abari, G Emtiazi, SH Lee, BG Kim, JH Kim, Appl Biochem Biotechnol, **174**, 270 (2014).
[7] AM Fayaz, M Girilal, M Rahman, R Venkatesan, PT Kalaichelvan, Process Biochem, **46**, 1958 (2011).
[8] C Jayaseelan, A Abdul Rahuman, A Vishnu Kirthi, S Marimuthu, T Santhoshkumar, A Bagavan, K Gaurav, Karthik L, BhaskaraRao KV, Spectrochim Acta A,**90**, 78(2012).
[9] S Seshadri, A Prakash, M Kowshik,) B Mater Sci, **35**, 1201(2012).
[10] Oves M, Khan MS, Zaidi A, Ahmed AS, Ahmed F, Ahmad E, Sherwani A, Owais M, Azam A, PLoS One, **8**, 1(2013).
[11] SA Wadhwani, UU Shedbalkar, R Singh, MS Karve, BA Chopde, World J Microb Biot, **30**, 2723 (2014).
[12] C Malarkodi, S Rajeshkumar, M Vanaja, K Paulkumar, G Gnanajobitha, G Annadurai, J Nanostruct Chem, **3**, 1(2013).
[13] C Dowsett, Nursing Standard, **19**, 56 (2004).
[14] I I Raad, H A Hanna, Arch Intern Med, **162**, 871(2002).
[15] Q Li, S Mahendra, DY Lyon, L Brunet, MV Liga, D Li, Alvarez PJJ, Water Res, **42** 4591(2008).
[16] N Duran, PD Marcato, GIH De Souza, OL Alves , E Esposito, J Biomed Nanotechnol, **32**, 203 (2007).
[17] P Dandekar, National river conservation plan ministry of environment and forest, 1, (2010).
[18] N Saitou, M Nei, Mol Biol Evol, **4**, 406 (1987).
[19] K Tamura, J Dudley, M Nei, S Kumar,Mol Biol Evol, **24**, 1596 (2007).
[20] M Gajbhiye, J Kesharwani , A Ingle, A Gade, M Rai, Nanomed Nanotechn Biol Med, **5**, 382 (2009).
[21] M Sastry, A Ahmad, MI Khan, R Kumar , Curr Sci,**85**, 162(2003).
[22] S Sunker, Nachiyar CV, Global J of Medical Research, **12**, 43(2012).
[23] F Alani, M Moo-Young, W Anderson, World J MicrobBiot, **28**, 1081(2011).
[24] R Varshney, AN Mishra, S Bhadauria, MS Gaur, Dig J Nanomater Biostruct, **4**, 349 (2009).
[25] N F Zonooz, M. Salouti, Scientia Iranica, **18**, 1631 (2011).
[26] V Gopinath, P Velusamy, Spectrochim Acta A, **106**, 170 (2013).
[27] HJ Bai, BS Yang, CJ Chai, GE Yang, WL Jia, ZB Yi, World J Microb Biot, **27**, 2723 (2011).
[28] K Shameli, MB Ahmad, A Zamanian, P Sangpour, P Shabanzadeh, Y Abdollahi, M Zargar, Int J Nanomed, **7**, 5603 (2011).
[29] K Chitra, G Annadurai, J Nanostruct Chem, **3,** 1(2013).
[30] S Hamedi, SA Shojaosadati, S Shokrollahzadeh, S Hashemi-Najafabadi, World J Microb Biot,**30**, 693(2014).

[31] P Manivasagan, J Venkatesan, K Senthilkumar, K Sivakumar, SK Kim, BioMed Res Int, 1(2013).
[32] J Baharara, F Namvar, T Ramezani, N Hosseini, R Mohamad, Molecules, **19** 4624 (2014).
[33] FU Mouxing, Q Li, D Sun, Y Lu, N He, X Deng, H Wang, J Huang, Chinese J Chem Eng, **14**, 114 (2006).
[34] SV Otari, RM Patil, SJ Ghosh, SH Pawar, Mater lett, **116**, 367 (2014).
[35] N Jain, A Bhargava, S Majumdar, J Tarafdar, Panwar J, Nanoscale, **3**, 635 (2011).
[36] AJ Kora, L Rastogi, Bioinorg Chem Appl, (2013), 1(2013).
[37] AM Fayaz, K Balaji, M Girilal, R Yadav, PT Kalaichelvan, R Venketesan, Nanomed Nanotech Biol Med, **6**,103(2010).
[38] L Rastogi, A J Kora, R. B Sashidhar, Appl Nanosci, **5**, 535, (2014)