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BIOSYNTHESIS OF SILVER NANOPARTICLES USING *BIFIDOBACTERIUM BIFIDUM* NCDC 229 AND EVALUATION OF SYNERGISTIC EFFECT WITH PENICILLIN AGAINST PATHOGENIC BACTERIA

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ABSTRACT
Silver nanoparticles were synthesized using *Bifidobacterium bifidum* NCDC 229 by an economical and environment friendly process. Synthesis of silver nanoparticles was confirmed by change in color of supernatant from pale yellow to brown. The silver nanoparticles were characterized by UV-Vis spectroscopy at 200-600 nm. FTIR spectrum showed broad O-H stretching vibration, -C≡CH stretching vibration, C=C stretching vibration, C-H deformation vibration which confirmed different functional groups involved in reduction of Ag^+ to Ag^0 nanoparticles. X-Ray diffractogram confirmed the crystalline nature of silver nanoparticles with face centered cubic structure. Scanning electron microscopy showed rough, spherical shaped and agglomerated silver nanoparticles. Antibacterial activity of silver nanoparticles, penicillin and silver nanoparticles with penicillin has been tested against pathogenic bacteria i.e Gram Positive *Staphylococcus aureus* (MTCC 6908), *Staphylococcus epidermidis* (MTCC 3382), *Bacillus cereus* (MTCC 6728) and Gram negative *Escherichia coli* (MTCC 41), *Vibrio cholerae* (MTCC 3906), *Klebsiella pneumoniae* (MTCC 3384). Results showed that silver nanoparticles have antibacterial activity but lower than penicillin. When silver nanoparticles were used in combination with penicillin, antibacterial activity increased as compared to peniciliin. So synergistic effect of silver nanoparticles with penicillin against pathogenic bacteria can be used to treat harmful bacteria, difficult to treat with penicillin.

KEY WORDS: *Bifidobacterium bifidum*, Silver nanoparticles, Scanning Electron Microscopy, UV-Vis spectroscopy.

INTRODUCTION

Nanoscience has been established as a new interdisciplinary science which deals with wide knowledge on fundamental properties of nano-size objects (Sergeev, 2003, 2006; Sergeev and Shabatina, 2008). Nanoparticles have new or improved properties such as size, distribution and morphology based on specific characteristics (Murphy, 2008). Nanoparticles are produced by different methods and have been widely utilized in different industrial applications (Loo *et al*., 2013). Among nanoparticles, the metallic nanoparticles such as silver, gold, zinc oxide, platinum etc. have shown applications in medicine, electronics, water treatment, anti cancer and textile engineering (Wong and Liu, 2010; Nithya and Rangunathan, 2012). But silver is being used from ancient times as antimicrobial agent due to its broad spectrum antimicrobial activity against bacteria, fungi and viruses. Moreover, silver compounds are non-toxic to the human body at low concentration compared to other metals. In recent years, silver nanoparticles have been used to treat pathogenic bacteria. Metallic nanoparticles can be synthesized by physical, chemical and biological methods. Physical approach involve evaporation, condensation etc. Chemical based approach uses toxic chemicals for reduction of metal into metal nanoparticles or aggregates (Khomutov and Gubin, 2002; Egorova and Revina, 2000). Due to increasing environmental concern over chemical synthesis, this route has resulted in development of biological methods being cost-effective, eco-friendly and can be scaled for large scale synthesis. Biological methods also remove the need for temperature, high pressure, energy and toxic chemicals (Sathyavathi *et al*., 2010). Biosynthesis of silver nanoparticles involves synthesis from bacteria, fungi, actinomycetes *etc*. The term 'probiotic' is derived from the Greek word 'pro bios' which means 'for life'. WHO/FAO defined probiotics as the "Live microorganisms which when administered in adequate amount confer a health benefit to the host" (Kaenhammer, 2000; Sanders, 2003; Guaner *et al*., 2005). Probiotics consists of microorganisms which are similar to found in human guts naturally such as breast fed infants. Various species of *Lactobacillus, Bifidobacterium, Escherichia* and *Bacillus* are widely used as probiotics (Haukioja, 2010). Several antibiotics viz. penicillin, tetracycline, cephalosporin etc. are used for the treatment of infectious agents but these suffers from many limitations such as few antibiotics do not kill the pathogenic bacteria completely (Butaye *et al*., 2003). Also these antibiotics harmfully affect the gut microbiota, may lead to an imbalance, resulting in diarrhea etc. Resistance to antibiotics is a major clinical problem which occur due to lack of new therapeutic agents that block resistance mechanisms (Murugan and Paulpandian, 2013). Synergistic action of silver nanoparticles and antibiotics could result in enhanced antibacterial effects; therefore, the simultaneous action of antibiotics and silver nanoparticles could hamper the resistance development by pathogenic bacteria, also in view of the reduced amount of antibiotic administered (Singh *et al*., 2013). Keeping in view the importance of silver nanoparticles and probiotics

in healthcare industries, the present research work was completed with the synthesis and characterization of silver nanoparticles using a probiotic *Bifidobacterium bifidum* NCDC 229, Gram-positive, non motile and branched anaerobic bacteria. Different strains of *Bifidobacteria* might exert a range of beneficial health effects including regulation of intestinal microbial homeostasis, pathogen inhibition and the local and systemic immune responses, vitamin production and the bioconversion of a number of dietary compounds into bioactive molecules (Mayo and van Sinderen, 2010). Synergistic effect of silver nanoparticles and antibiotic against pathogenic bacteria was also investigated.

MATERIALS & METHODS

Silver nitrate was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Nutrient broth and agar powder were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Hydrochloric acid (HCl) was purchased from Fisher Scientific. *Bifidobacterium bifidum* NCDC 229 strain was procured from National Collection of Dairy Cultures (NCDC), NDRI, Karnal, Haryana, India. The pathogenic strains i.e. Gram positive bacteria *Staphylococcus aureus* MTCC 6908, *Staphylococcus epidermidis* MTCC 3382, *Bacillus cereus* MTCC 6728 and Gram negative bacteria *Escherichia coli* MTCC 41, *Vibrio cholerae* MTCC 3906, *Klebsiella pneumoniae* (MTCC 3384) were obtained from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India..

Biosynthesis of silver nanoparticles

A loop of pure culture of *Bifidobacterium bifidum* NCDC 229 was inoculated in 100 ml sterile nutrient broth and incubated in shaker at 37° C for 24 h at 160 rpm. After 24 h of incubation, culture suspension was centrifuged at 10000 x g rpm for 10 min. and supernatant was collected in a separate vial. The culture supernatant was filtered using Whatman No. 40. The pale yellow filtrate with pH between 7-8 was adjusted to 6 using 0.1 N HCl. To 100 ml of this filtrate added 0.0169 g (1 mM) silver nitrate in a flask. The flasks containing filtrate (with silver nitrate) and control flask (without silver nitrate) were agitated at 160 rpm under dark conditions at 37° C for 24 h, until the pale yellow color change to brown. The culture was centrifuged at 10000 x g rpm for 10 minutes and supernatant was discarded. The pellet containing silver nanoparticles was washed with distilled water, dried and stored for further use.

Characterization of silver nanoparticles

Silver nanoparticles were characterized using UV-visible spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM). The UV-Vis spectra of silver nanoparticles was characterized in the 200-600 nm range with Nanodrop 2000C (Thermo Scientific). Fourier Transform Infra-Red (FTIR) spectrum was recorded using Perkin Elmer - Spectrum RX-IFTIR spectrophotometer within range 4000 to 400 cm^{-1}. X-Ray Diffractogram of silver nanoparticles was analyzed using Panalytical's X'Pert Pro. Scanning Electron Microscopy helps to reveal morphology and topography of silver nanoparticles and were analyzed with JEOL Model JSM - 6390LV.

Antibacterial assay

The antibacterial activity of biosynthesized silver nanoparticles from *Bifidobacterium bifidium* NCDC 229 and their synergistic effect with penicillin was tested against pathogenic bacteria by agar well diffusion method (Thomas *et al*., 2014). Twenty five ml sterilized nutrient agar medium was transferred to sterilized petri plates and allowed to solidify at room temperature. Bacterial cultures were transferred to these plates with the help of inoculating loop to obtain fresh culture growth and incubated for 24 h at 37° C. Bacterial growth was observed for purity checking. A loop of bacteria from the plates were transferred to 50 ml nutrient broth medium and incubated in an incubator shaker for 18 h. The test bacteria were grown in nutrient broth and incubated for 18-20 h at 37° C. One ml of the suspension was plated with 20 ml of melted and cooled nutrient agar media and plates were kept undisturbed for 15 min. for solidification media. Three wells were made on the solidified media plates, seeded with bacteria using sterile hole borer (8 mm diameter). Different concentration of following samples (60 µl) were transferred into the respective wells with the help of micropipette:

1. Penicillin – 1 mg/ml
2. Silver nanoparticles – 1, 2 and 3 mg/ml
3. Combined formulation (penicillin and silver nanoparticles) - Silver nanoparticles in various concentrations from 1, 2 and 3 mg/ml and penicillin l mg/ml.

The plates were allowed to remain undisturbed for 2 h to ensure even diffusion of samples into agar. The plates were incubated at 37° C for 18-24 h without inversion. After incubation, zone of inhibition around the wells was measured with the help of scale and expressed in mm.

RESULTS & DISCUSSION

Biosynthesis of silver nanoparticles

The silver nanoparticles might be synthesized due to the presence of nitrate reductase enzymes in *Bifidobacterium bifidum*, causing reduction of Ag^{+} from $AgNO_3$ to Ag^{0}. There occurred a change in color of supernatant from pale yellow to brown, which confirmed the synthesis of silver nanoparticles as shown in Figure 1. Kumar *et al*. (2015) observed brownish color while synthesizing silver nanoparticles from Streptomyces sp. 09 PBT 005. Khatami *et al.* (2015) synthesized silver nanoparticles from silver nitrate using *Sinapis arvensis.* The synthesis of silver nanoparticles was confirmed by the change in mixtures color from light yellow to brown.

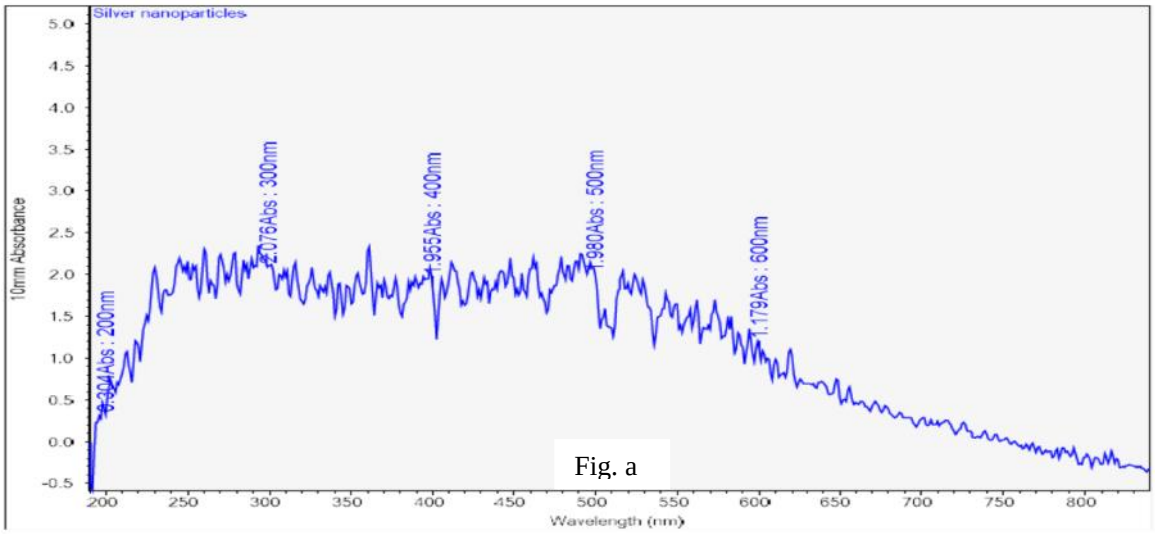
Characterization of nanoparticles

UV-Visible spectroscopy

The reduction of silver ions present in the aqueous solution of silver nitrate during the reaction with the ingredients of *Bifidobbacterium bifidum* has been characterized with UV-Vis spectroscopy from 200-600 nm. The number of peaks were observed initially (0 min) in the UV-Vis region in 200-600 nm range, which showed fast synthesis of nanoparticles as shown in Figure 2 a. Similar results were obtained by Khatami *et al*. (2015) for silver nanoparticles which showed maximum absorption at 412 nm due to surface plasmon resonance of silver nanoparticles. Extracellular synthesis of silver

nanoparticles from *Escherichia coli* S30, S78, *Bacillus megaterium* S52, *Acinetobacter* sp. S7 and *Stenotrophomonas maltophilia* S54 strain and UV–Visible spectrum of the aqueous medium silver ion with a peak at 420 nm have been resported (Zaki *et al*., 2011). After 24 h, a decrease in absorption after 400 nm was observed in UV-Vis spectrum as shown in Figure 2 b. This showed there occured a decrease in the synthesis of nanoparticles with passage of time. Rajeshkumar and Malarkodi (2014) also observed decrease in the synthesis of silver nanoparticles from *Planomicrobium* sp. after 24 h. Dhiman *et al*. (2014) monitored reduction in silver nanoparticles synthesis after 24 h by measuring the UV-Vis spectrum in the range 300-750 nm.

FIGURE 1: Biosynthesis of silver nanoparticles as visualized by color change from pale yellow to brown



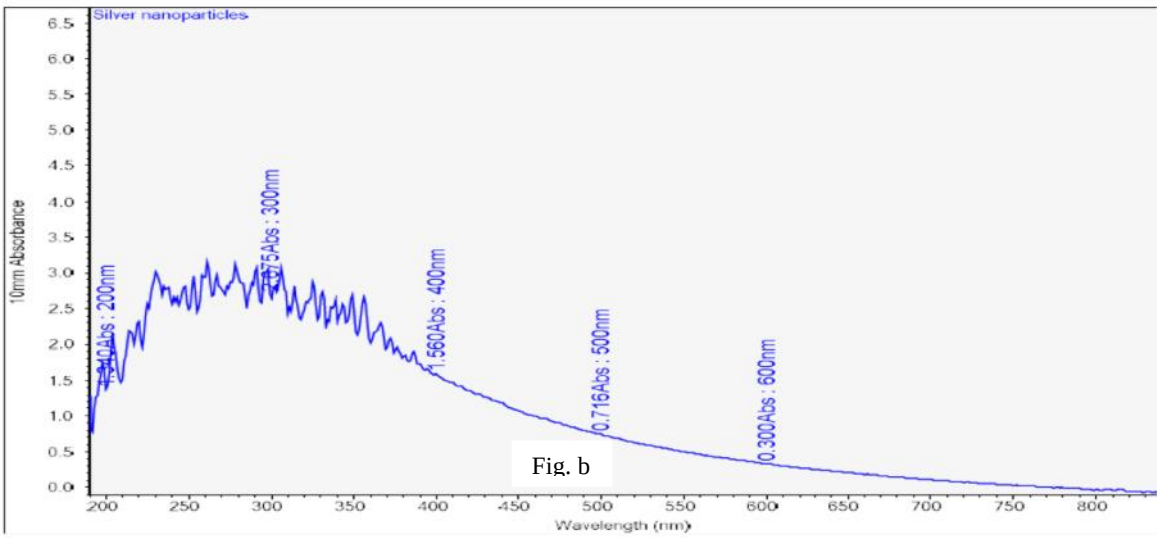


FIGURE 2: UV-vis spectra of silver nanoparticles showing different peaks (a) 0 min. b) after 24 h

Fourier Transform Infra Red (FTIR) spectroscopy

FTIR spectrum of the silver nanoparticles was carried out to identify the possible interactions between silver and bioactive molecules present in cell free extract of *Bifidobacterium bifidum*, responsible for the synthesis and stabilization (capping material) of silver nanoparticles. The peaks at 3398.5 cm^{-1} was assigned to broad O-H stretching vibration of quinone oximes, 2134.17 cm^{-1} (-C≡CH stretching vibration of monosubstitued alkynes), 1632.10 cm^{-1}, 1563.13 cm^{-1} (C=C stretching vibration of alkenes), 1388.15 cm^{-1}, 1226.16 cm^{-1} (C-H deformation vibration of secondary alcohols), 1085.16 cm^{-1} (C-N stretching vibrations of primary aliphatic amines), 710.15 cm^{-1} (broad, NH_2 deformation vibration of primary amide) and 462.18 cm^{-1} (C-C skeleton vibration of branched alkanes) as shown in Figure 3. Elbeshehy *et al*. (2015) observed different peaks in FTIR spectrum of silver nanoparticles synthesized from *Bacillus* spp. i.e. 3373 cm^{-1}, a strong peak at 3300–3500 cm^{-1}, 2359 cm^{-1}, 1650 cm^{-1}, 1600–1000 cm^{-1}. FTIR spectrum of silver nanoparticles suggested that proteins might act as capping agent of silver nanoparticles and different biological molecules were responsible for synthesis of silver nanoparticles.

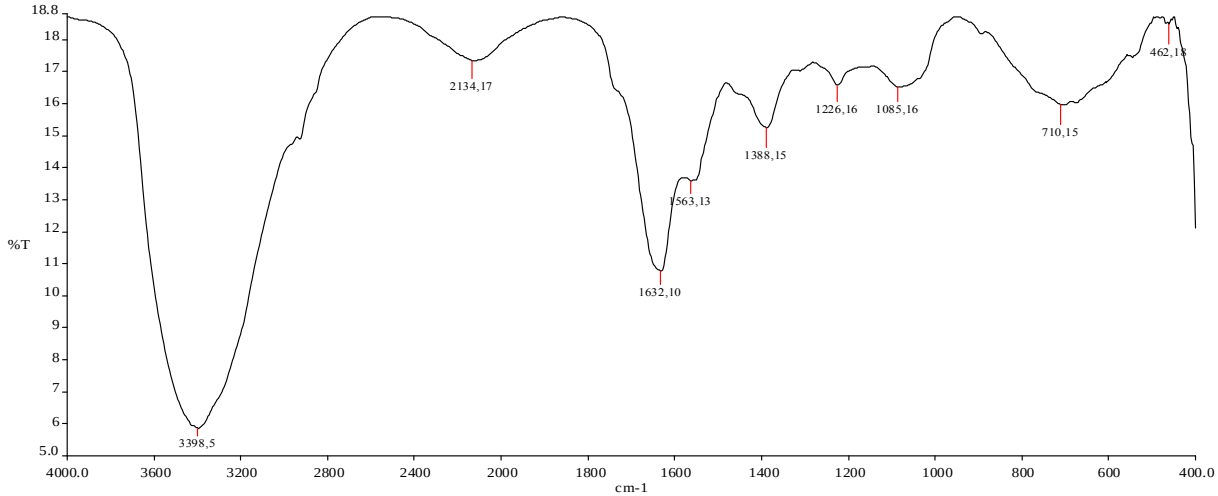


FIGURE 3: FTIR spectrum of silver nanoparticles showing functional groups

X-ray Diffraction (XRD) measurement

XRD diffractogram of silver nanoparticles is shown in Figure 4. Three strong peaks in diffractogram of silver nanoparticles at 2θ = 27.7849°, 32.1969°, 46.2153° were observed. These peaks showed miller indices values (h, k, l) at 100, 100 and 111, respectively and confirmed face-centered cubic structure and crystalline nature of silver nanoparticles. Otari *et al*. (2012) reported XRD diffractogram of silver nanoparticles synthesized from *Rhodococcus* sp. and peaks at (111), (200) and (311) which also confirmed face centered cubic and crystalline nature of silver nanoparticles.

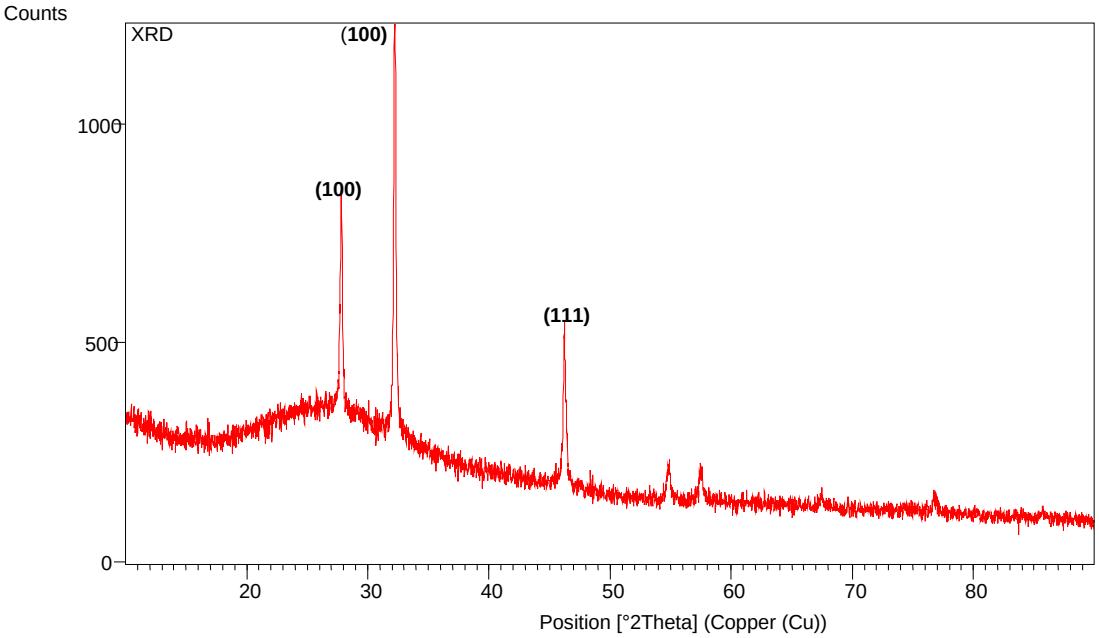


FIGURE 4: XRD diffractogram of silver nanoparticles showing crystalline nature

Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopy at different magnifications showed spherical shaped silver nanoparticles with roughness and agglomerated form as shown in Figure 5. Uniform and spherical shaped silver nanoparticles synthesized from *Brevibacterium linens* have been observed (Ranganathan and Ramachandran, 2015). Malarkodi *et al.* (2013) synthesized silver nanoparticles using *Bacillus* sp. and also observed spherical shape.

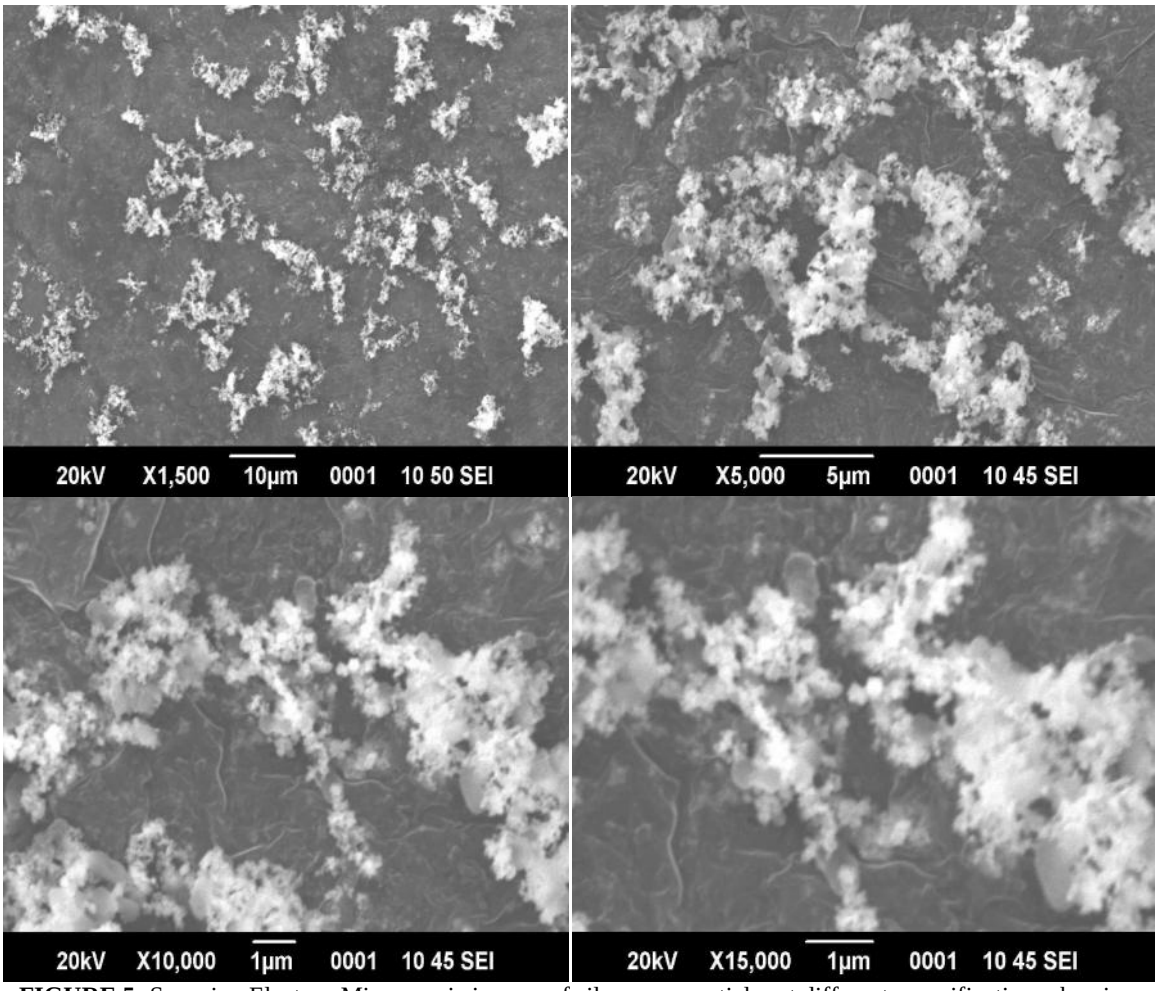


FIGURE 5: Scanning Electron Microscopic images of silver nanoparticles at different magnifications showing agglomeration

Antibacterial assay

Different concentrations of silver nanoparticles, penicillin and penicillin with silver nanoparticles were assessed on Gram-positive and Gram-negative bacteria as shown in Table 1, Figure 6 and 7. Among Gram-positive bacteria i.e. *Staphylococcus aureus* (MTCC 6908), *Staphylococcus epidermidis* (MTCC 3382) and *Bacillus cereus* (MTCC 6728), silver nanoparticles showed concentration dependant antibacterial effect with 3 mg/ml has maximum effect against *Staphylococcus epidermidis* (MTCC 3382). But this effect was lower than that of penicillin which showed maximum effect against *Staphylococcus aureus* (MTCC 6908). By keeping concentration of penicillin constant (1 mg/ml), synergistic effect of penicillin and silver nanoparticles also showed concentration dependant inhibition with maximum against *Staphylococcus aureus* (MTCC 6908) at 3 mg/ml for silver nanoparticles. Among Gram-negative bacteria i.e. *Escherichia coli* (MTCC 41), *Vibrio cholerae* (MTCC 3906), *Klebsiella pneumoniae* (MTCC 3384), silver nanoparticles also showed concentration dependant antibacterial effect with 3 mg/ml has maximum effect against *Klebsiella pneumoniae* (MTCC 3384) but was lower than that of Gram-positive bacteria. Penicliin at 1 mg/ml concentration showed maximum effect against *Vibrio cholerae* (MTCC 3906). Synergistic effect of penicillin and silver nanoparticles also showed concentration dependant inhibition with maximum against *Vibrio cholerae* (MTCC 3906) at 3 mg/ml for silver nanoparticles. These results clearly showed that silver nanoparticles had antibacterial activity against Gram-positive and Gram-negative bacteria. But their antibacterial activity can be enhanced in combination with penicillin. Thomas *et al.* (2014) synthesized silver nanoparticles by soil *Bacillus* sp. and observed increase in antimicrobial activity of gentamycin against *Staphylococcus epidermidis* strain73 to 0.15 fold.

Penicillin which does not inhibit the growth of *Staphylococcus aureus*, when mixed with silver nanoparticles showed 4.4 fold increase in zone of inhibition. Salar *et al*. (2015) also observed increase in synergistic antibacterial effect of silver nanoparticles synthesized from various leaf extracts of *Ficus virens* with streptomycin against *Bacillus subtilis*, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Vibrio vulnificus*.

TABLE 1: Inhibitory activity of silver nanoparticles, penicillin and penicillin with silver nanoparticles against pathogenic bacteria

Bacteria	Zone of inhibition (mm)						
	Silver nanoparticles			Penicillin	Penicillin + Ag-NPs		
	1 mg/ml	2 mg/ml	3 mg/ml	1 mg/ml	1 mg/ml + 1 mg/ml	1mg/ml + 2 mg/ml	1mg/ml + 3 mg/ml
Staphylococcus aureus (MTCC 6908)	8	8	8	33	34	36	37
Staphylococcus epidermidis (MTCC 3382)	8	13	16	25	30	31	32
Bacillus cereus (MTCC 6728)	12	8	14	15	18	20	25
Escherichia coli (MTCC 41)	8	8	9	13	14	15	17
Vibrio cholerae (MTCC 3906)	8	9	9	25	26	27	30
Klebsiella pneumoniae (MTCC 3384)	8	12	16	14	15	17	17

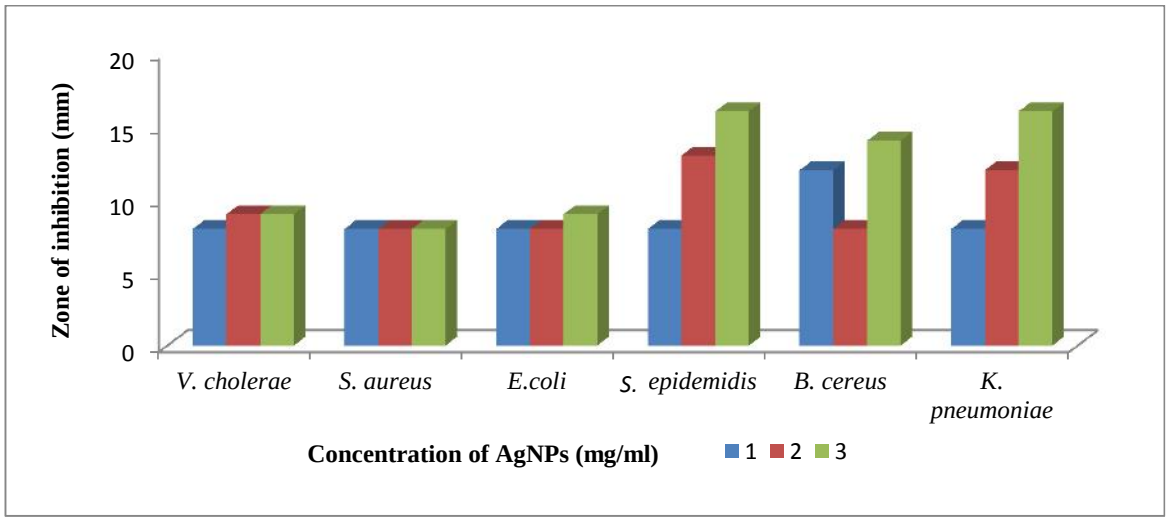


FIGURE 6: Antibacterial activity of silver nanoparticles

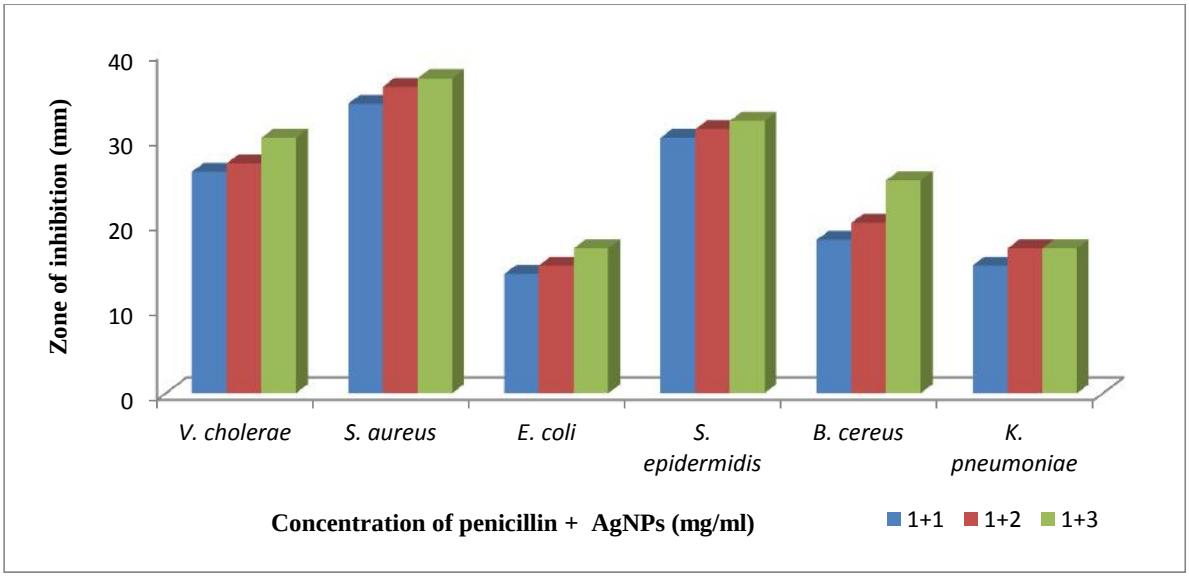


FIGURE 7: Synergistic effect of penicillin and silver nanoparticles against pathogenic bacteria

CONCLUSION

The nanotechnology has established as the most active areas of research in material science today. Nanoparticles exhibit unique properties such as size, distribution and morphology which can be used in different fields. Silver nanoparticles were synthesized using probiotic *Bifidobacterium bifidum*, confirmed by change in color of supernatant from pale yellow to brown. Silver nanoparticles shows peaks from 200-600 nm with UV-Vis spectrophotometer. FTIR spectrum showed broad O-H stretching vibration, -C≡CH stretching vibration, C=C stretching vibration, C-H deformation vibration, C-H symmetrical deformation vibration revealed the presence of protein in samples of silver nanoparticles along with amino acid for its stability. XRD diffractogram confirmed the crystalline nature of silver nanoparticles with face centered cubic structure. Scanning Electron Microscopy images showed that silver nanoparticles were rough, spherical in shape and agglomerated. The synthesized silver nanoparticles have shown antibacterial activity against pathogenic Gram positive and Gram negative bacteria less than that of penicillin. But the developed formulation of silver nanoparticles with penicillin showed higher antibacterial activity as compared to the silver nanoparticles and penicillin, when used alone. So it can be suggested that probiotics can be used for synthesis of silver nanoparticles and *Bifidobacterium bifidum* NCDC 229 is an ideal candidate for synthesis of silver nanoparticles. Further research is required to study different combination of silver nanoparticles using *Bifidobacterium bifidum* NCDC 229 against resistant pathogenic microorganisms.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest

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