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Screening of silver nanoparticles producing cyanobacteria and its characterization

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ABSTRACT

Biosynthesis of nanoparticles is the major division in the field of applicable nanoscience and nanotechnology. Nanoparticles can be synthesized using plant extracts, enzymes, bacteria, fungi and algae. Nanoparticles can be made of materials of diverse chemical nature, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon and biomolecules. Silver nanoparticles are widely used particularly in textiles, plastics and medical industries, changing the pattern of silver emission as these technologies and products diffuse through the global economy. In the present investigation, twenty cyanobacterial isolates were screened for the synthesis of silver nanoparticles. Out of that *Oscillatoria* spp. was shown to be capable to biosynthesize Ag-NPs. Silver nano particles synthesis of has been shown from filamentous *Oscillatoria* sp. and it was confirmed using yellowish-brown color in aqueous solution along with the uv-vis spectroscopy. It was confirmed by scanning electron microscopy (SEM) which showed that Ag-NPs were present and evenly distributed throughout the cell free liquid culture of the AgNO3-incubated culture. XRD X-ray diffraction (XRD) was carried out to confirm crystalline nature of the particles and it is concluded that the mix phase of AgNO3 and silver nanoparticles is observed. FTIR was used to identify the biomolecules in *Oscillatoria* sp. responsible for the silver ions reduction and stabilization of reduced silver ions. The antibacterial activity of silver nanoparticles produced by *Oscillatoria* sp. was observed against pathogens, viz., *E. coli, Klebsiella* sp., *Salmonella* sp., *Pseudomonas* sp. using disc diffusion method. The bioactive-ty of the synthesized silver nanoparticles had inhibitory effect on important human pathogens. It would be desirable to develop a technology in which the specific size and shape of the particles could be obtained by the use of a specific strain of cyanobacteria.

Keywords: Silver nanoparticles, Cyanobacteria, Scanning electron microscopy, X-ray diffraction Antibacterial activity.

INTRODUCTION

Nanotechnology has a very important modern field concerns with the growth of new processes for the synthesis of nanoparticles of different sizes, shape and proscribed dispersity [1]. These particles can be made up with diverse chemical nature materials, the most common being metals, metal oxides, silicates, non-oxide ceramics, polymers, organics, carbon and biomolecules. Nanoparticles exist in several different morphologies [2]. Biosynthesis of nanoparticles is the major division in the field of applicable nanoscience and nanotechnology therefore there is a need for microbe mediated synthesis that includes a clean, nontoxic and ecofriendly method of nanoparticles synthesis [3].

Nanotechnology is enabling technology that deals with nanometer sized objects. The biosynthesis of silver nanoparticles of different sizes, ranging from 1-70nm, and shapes including spherical, triangular & hexagonal [4]. The mechanism for the bioreduction of silver by bacteria involves reducing & other proteins in which sulphur and carboxylate group from cell wall. The silver nitrate caused the reduction of silver through nitrate dependant reductase. Biosynthesis of nanoparticles is a kind of bottom up approach where the main reaction occurring is reduction/oxidation [5]. Silver nanoparticles are of interest for antimicrobial applications, biosensor materials, composite fibers, cryogenic superconducting materials, cosmetic products, and electronic components because of the unique properties and morphologies [7]. There is however various theories on the action of silver nanoparticles on microbes to cause the microbicidal effect [8].

The need for biosynthesis of nanoparticles rose as the physical and chemical processes were costly [9]. Microorganisms including bacteria, fungi and algae have been proposed as potential eco-friendly nano-factories for the synthesis of metal including silver [10,12]. A two step mechanism for synthesis of silver nanoparticles suing microbes is, the first step involved trapping of Ag+ ions at the surface of biological cells & in the second step enzymes nanosilver particles. Silver ion, Ag+ has been reported to have high bio concentration factors (>105), for freshwater green algae and marine algae [13; 14]. Mubarak et al., [15] reported that the marine cyanobacterium *Oscillatoria willei* synthesized silver nanoparticles. They screened cyanobacteria & green algae as model biological system for their ability to form AgNPs. Nowadays, silver nanoparticles gain more attention by researchers not only because of their wide application and antimicrobial effects but also having potential risk in environmental and human health [16]. Against these backdrops the present study aimed at the synthesis of silver nanoparticles through biological methods using blue green algae and their application in inhibition of pathogenic bacteria and fungi [15,17].

The aim of the study is to isolate cyanobacteria from different locations & test the ability of synthesizing silver nanoparticles from cellular extracts of cyanobacterial isolates, the positive strain used for nanoconversion & also characterize the size of synthesized silver nanoparticles & study their antimicrobial property against various pathogenic bacteria.

METHODOLOGY

1. Collection of cyanobacterial samples:

Samples were collected from fresh water ponds, rivers, ditches and soils from Baramati region. Samples were collected in plastic bags or in bottles. The collected samples were brought in laboratory and used for isolation. The Cyanobacterial cultures were enriched in sterile BG-11 medium.

2. Isolation and identification of isolate:

The enriched samples showing blue-green, green coloration are inoculated in liquid medium for isolation of cultures. For unicellular cyanobacterial enriched samples were streaked on respective agar plates of growth medium. Visibly distinct cyanobacterial colonies are reinoculated for further purification. In case of filamentous cultures, selective inoculation of single filaments under aseptic conditions is the methods of isolation used. On the basis of morphological characteristics cyanobacterial cultures were identified as described by Rippka *et al*. [18] and Desikachary [19].

3. Screening of cyanobacteria for synthesis of silver nanoparticles:
Cyanobacterial isolates are inoculated in fresh BG-11 medium (100ml) and incubated at for 21 days under 1800-2000 lux light intensity. After the incubation, 20 ml each culture centrifuged at 10,000 rpm for 10 min at 4°C (Make- Remi Cooling centrifuge) and biomass were homogenized and again centrifuged. The supernatant and homogenized solution is used for the synthesis of the silver nanoparticles. The supernatant (5% of total volume) of cyanobacterial cultures are separately added to the flasks containing silver nitrate at a concentration of 0.1 g/L. The reaction between these supernatants and silver ions is carried out for 72 hrs. The bioreduction of silver ions in the solution is monitored by sampling the aqueous solution (2ml) and measuring the absorption spectrum of the solution using UV-Visible spectrophotometer (Shimdzu UV-1800) at a resolution of 1 nm [20].

3. Production of silver nanoparticles:
On the basis of preliminary detection using UV-Vis spectra the silver nanoparticles producing cyanobacterial cultures were selected for the further production. The supernatant (5% of total volume) of cyanobacterial cultures are separately added to the flasks containing silver nitrate at a concentration of 0.1 g/L. The reaction between these supernatants and silver ions is carried out for 72 hrs. The solution was centrifuged and the particles were separate out. These particles were used for further characterization.

5. Characterization of silver nanoparticles
***5.1* UV–Visible spectra analysis:** After 4 hrs of incubation of the above mixture, the preliminary detection of silver nanoparticles were carried out by visual observation of color change (white to brown) of the culture filtrate. The reduction of silver ions measured by using a UV-Visible spectrophotometer (Shimdzu UV-1800) at varying intervals (4hrs, 24 hrs, 48 hrs and 72 hrs) and scanning the spectra between 200-800 nm at the resolution of 1 nm[8].

5.2 Fourier Transform Infrared (FTIR) analysis: The silver nanoparticles analyzed by Fourier transform infrared (FTIR) analysis. The dried silver nanoparticles are analyzed using FTIR spectroscopy (Perkin Elmer Spectrum Version 10.03.06) between frequency range 450 to 4000 cm-1 with resolution 4 cm-1 [20].

5.3 Scanning Electron Microscopy
A scanning electron microscope was used to record the micrograph images of synthesized silver nanoparticles [21].

5.4 X-Ray Diffraction (XRD) Analysis
The formation of silver nanoparticles was checked by X-ray diffraction (XRD) using an X-ray diffractometer. The supernatant treated with silver nitrate is evaporated to dryness under sunlight. The air dried biomass is analyzed. The full widths at half maximum (FWHM) values of X-ray diffractions were used to calculate particles size[20].

6. Antibacterial activity of silver nanoparticles: The silver nanoparticles synthesized from cyanobacterial isolates tested for antimicrobial activity by disc diffusion method against pathogenic microorganisms like *E. coli*, *Staphylococcus* sp., *Salmonella* sp., *Klebsiella* sp. and *Pseudomonas* sp. The pure cultures of these organisms were sub cultured on nutrient agar slants and incubated at 37°C for 24 hrs. Each strain having cell number 10^6/ml was spread uniformly on the nutrient agar plates and sterile paper discs dipped into the samples of nanoparticles solution were placed onto the nutrient agar plates. After incubation at 37°C for 24 hrs, the zones of inhibition of bacteria were measured.

RESULTS AND DISCUSSION

3.1. Isolation and identification of cyanobacteria:
Cyanobacteria were inoculated in BG11 medium. The flasks were incubated at 25±2°C at 1800-2000 lux light intensity for 21 days. After 21 days incubation, enriched samples showing blue-green, coloration were inoculated in medium for isolation of cultures. The data of isolation and identification of cultures enriched in sterile BG_{11} medium are presented in Figure 1. It could be seen that a total 20 isolates were obtained and identified from enriched flasks. The isolated and laboratory set of cyanobacterial cultures were identified as per the morphological characters given by Desikachary [19] and Rippka *et. al*., [18].

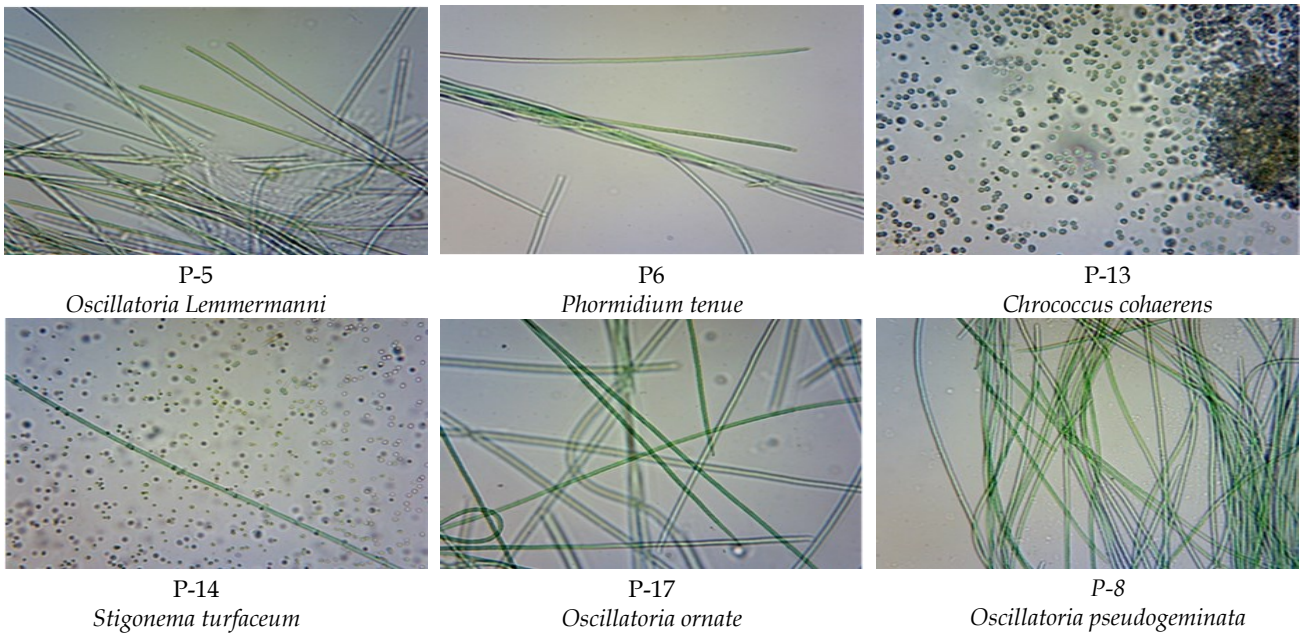

Figure 1 Microscopic observation of cyanobacterial cultures

This morphological form of cyanobacteria were observed under Lynx microscope. All cultures were maintained in BG-11 (pH 7.4) under 1800-2000 lux light intensity at temperature of 25±2°C.

3.2. Screening of cyanobacteria for synthesis of silver nanoparticles:

Cyanobacterial cultures were screened based on the preliminary visual color observation, which was changed from white to yellowish brown and based on UV spectra. From the twenty different isolated cultures, one culture was found to be capable of producing silver nanoparticles showing a peak at wavelength in the range between 420-450 nm. That culture was further used for large scale production.

3.3. Production and extraction of silver nanoparticles:

In the present study, extracellular biosynthesis of silver nanoparticles of isolates was studied. It shows color changes from colorless to dark brown due to reduction of silver (Ag+) ions to silver nanoparticles (AgNPs) by the cyanobacterial reductase enzyme.

The exact mechanism leading to the extracellular formation of silver nanoparticles by the algal biomass is not fully understood; there are still several possible mechanisms involved in the process [11]. It is thought that the first step involves the trapping of metal ions on the surface of algal cells, possibly via electrostatic interaction between the ions and negatively charged carboxylate groups present in the cell surface. Thereafter, the ions are reduced by the enzymes, leading to the formation of nuclei, which subsequently grow through the further reduction of metal ions and accumulation of these nuclei [22]. Most probably, the reduction of SNPs occurs due to the presence of cellular reductase released by Spirulina platensis into the solution. Also, in cyanobacteria, localized reducing conditions may be produced by a bacterial electron transport chain, via energy generating reactions within the cells [24]. In this respect, secreted cofactor NADH plays an important role [23].

3.3. Characterization of silver nanoparticles

1. **UV- Visible spectra analysis:**

In this study, extracellular synthesis of SNPs has been shown from filamentous Oscillatoria spp. It is well known that SNPs exhibit a yellowish-brown color in aqueous solution, due to the excitation of Surface Plasmon Vibrations in SNPs [3]. Reduction of the silver ion to SNPs during exposure to the *Spirulina platensis* biomass could be followed by a color change, and thus, UV-Vis spectroscopy.

Figure 3 shows the UV-Vis spectrum of the nano silver formation and the change in the color of the reaction mixture to dark brown, indicating the biotransformation of ionic silver to reduced silver, and the subsequent formation of SNPs in an aqueous medium.

Figure: 2 Biosynthesized silver nanoparticles (formation of brown color) after 0, 24, 48 & 72 hrs incubation

It was observed that the maximum absorbance occurs at 430 nm (Figure 4 a & b). Mubarak *et al*. [15] reported that the extracellular biosynthesis of silver nanoparticles using marine cyanobacterium *Oscillatoria willei* NTDM01 which secreted protein. The silver nitrate solution incubated with washed marine cyanobacterium changed to yellow color indicating the formation of silver nanoparticles and the UV spectra was observed at 430 nm.

Figure: 3 Production and extraction of cyanobacterial silver nanoparticles

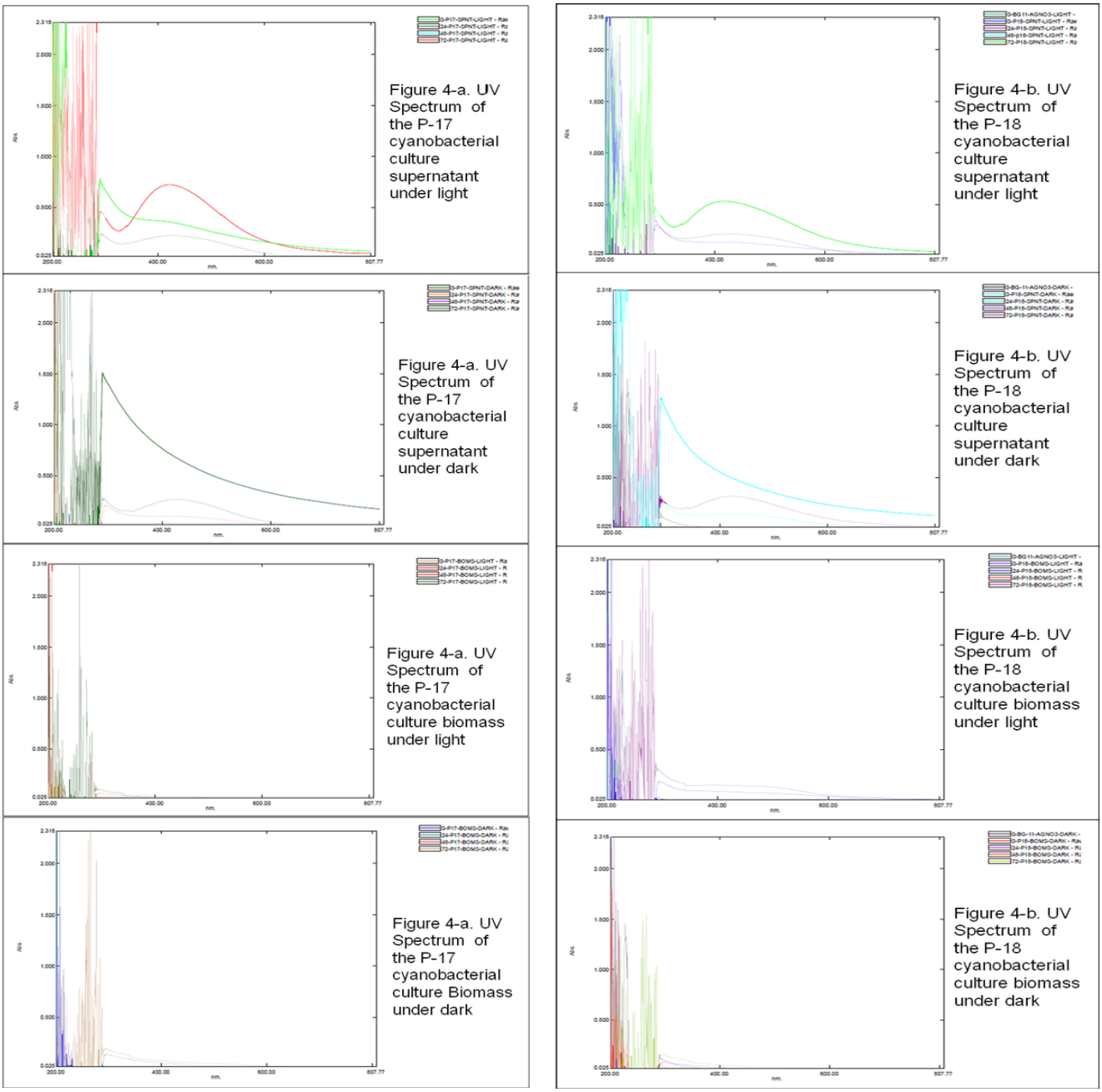

Figure 4 UV Spectrum of the P-17 & P-18 cyanobacterial culture

3.4.2. Fourier Transform Infrared (FTIR) Analysis:
FTIR was used to identify the biomolecules in *Oscillatoria* sp. responsible for the silver ions reduction and stabilization of reduced silver ions. The FTIR Spectrum of the cell free liquid media (Figure 5(A) shows strong absorption peaks at 3246.34, 1791.29, 1649.21, 1219.06, 1086.93, 78.67 cm-1 which represents that the band at 324.34 were assigned to the stretching vibration of amides, 1791.29 represent acyl chlorides,1649.21 represent alkenes, 1219.06 represent ethers group, 1086.93 represent ethers, 768.67 represent alky halides group.

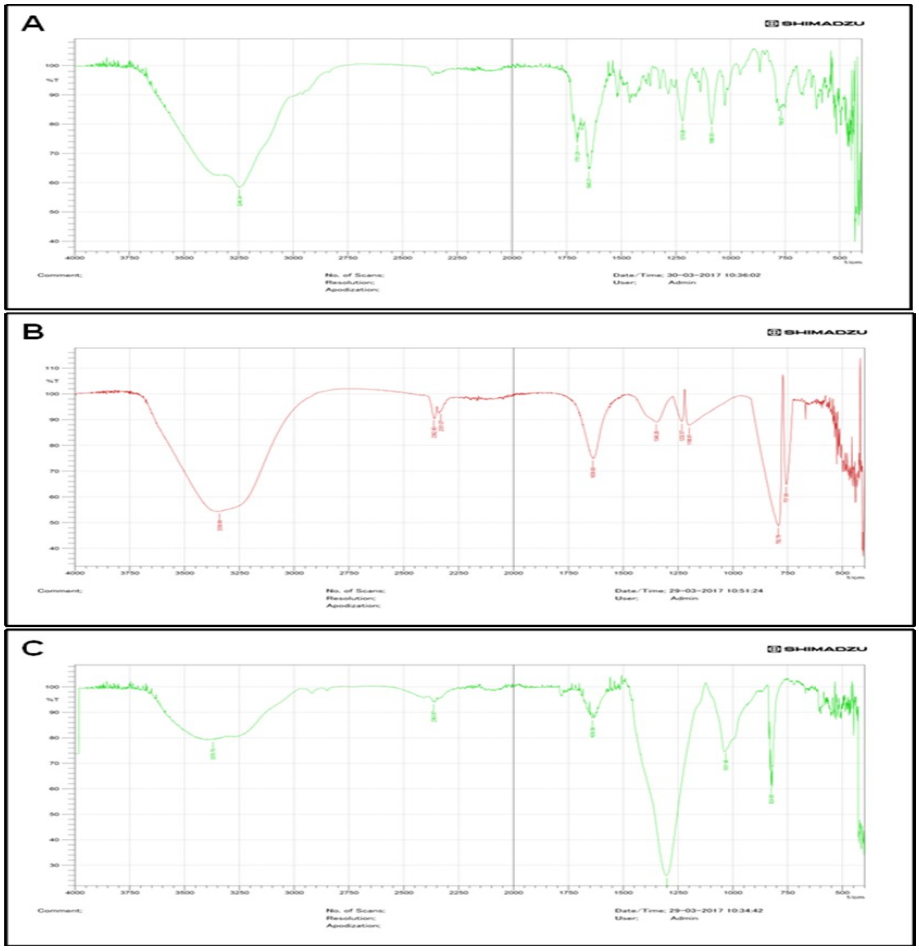

Figure 5 FTIR comparison of cell free liquid media(A), colloidal liquid AgNPs (B) and Powdered AgNPs (C).

Table 1. FTIR comparison of cell free liquid media, colloidal liquid AgNPs and Powdered AgNPs

Liquid media		Colloidal liquid AgNPs		Powdered AgNPs	
Vibration(cm^{-1}) Cell free liq. Media	Functional Group/Name of compound	Vibration(cm^{-1}) AgNPs (Liquid)	Functional Group/Name of compound	Vibration(cm^{-1}) AgNPs (Powder)	Functional Group/name of compound
768.67	R-Cl (Alkyl halides)	757.09	R-Cl (Alkyl halides)	624.60	C-Cl (Alkyl halides)
1086.93	R-O-R' (Ethers)	1198.81	RR'R"C-OH (3^{o}) (Ter. alcohols)	1031.96	Ar-O-R (Ethers)
1219.06	C-O (Carboxylic acids)	1232.57 1349.26	C-O (Acids) N-O(Nitro)	1310	C-O (Acids)
1649.21	R-CH=CH2 (Alkenes)	1638.60	C=O (Carboxylic acids)	1639.56	R-C(O)-NR'R" (Amide)
1791.29	Ar-C(O)-Cl (Acyl chlorides)	2331.07	R-C(O)-OH (Alcohols)	2363.87	R-C(O)-OH (Alcohols)
3246.34	R-C(O)-NH2 (Amides)	2362.90 3339.89	R-C≡N (Nitriles) O-H (Alcohols)	3770.75	O-H (Alcohols)

The FTIR spectrum was recorded from the colloidal silver nanoparticles (Figure 5(B), formed after 72 days of incubation with the cyanobacteria. The bands seen at 3339.89 cm−1 and 1198.81 cm−1 were assigned to the stretching vibrations of primary and tertiary alcohols. The corresponding bending vibrations were seen at 792.98 cm−1 respectively. The bands observed at 1349.26 cm−1 and 2362.90 and 1838.60 cm-1 can be assigned to the primary and secondary amides.

The FTIR spectrum was recorded from the freeze powder of colloidal silver nanoparticles (Figure 5(C), formed after 72 days of incubation with the cyanobacteria. The bands seen at 3770.75 cm−1 and 1639.56 cm−1 were assigned to the stretching vibrations of primary alcohols, and amides. Whereas frequencies 2363.87 and 1310, shows c=o and c-o stretch vibration of carboxylic acid and ether. The overall observation confirms that the presence of protein used in the synthesis of silver nanoparticles, in the cell free liquid culture the protein present contains the free residual groups of alkene and acyl chloride groups' catalyses reduction of silver (Ag+) ions to colloidal silver nanoparticles. Alkenes, Nitro and Nitrile groups were present in colloidal silver nanoparticles, which were absent in powdered AgNPs, so it was found that Alkenes, Nitro and Nitriles may get evaporated during the process of evaporation.

3.4.3 Scanning Electron Microscopy

Light microscopy showed that clusters of nanoparticles were attached to the surface of the cyanobacterial filaments. This was confirmed by scanning electron microscopy (SEM) which showed that AgNPs were present and evenly distributed throughout the cell free liquid culture of the AgNO3-incubated culture (Figure 6). Elemental analysis by EDS identified those particles indeed as silver not being present in the control culture. At room temperature, the addition of $AgNO_3$ to the cyanobacteria caused the precipitation of silver nanoparticles at cell surfaces. Small spherical silver nanoparticles with size ranging from 100 nm to 200 nm (extracellular) were also precipitated in solution silver nanoparticles were deposited at cell surfaces. EDS showed the occurrence of silver particle in higher amount with trace of magnesium, calcium and chloride. In this analysis silver nanoparticles was confirmed the presence of elemental silver signal (Figure 7).

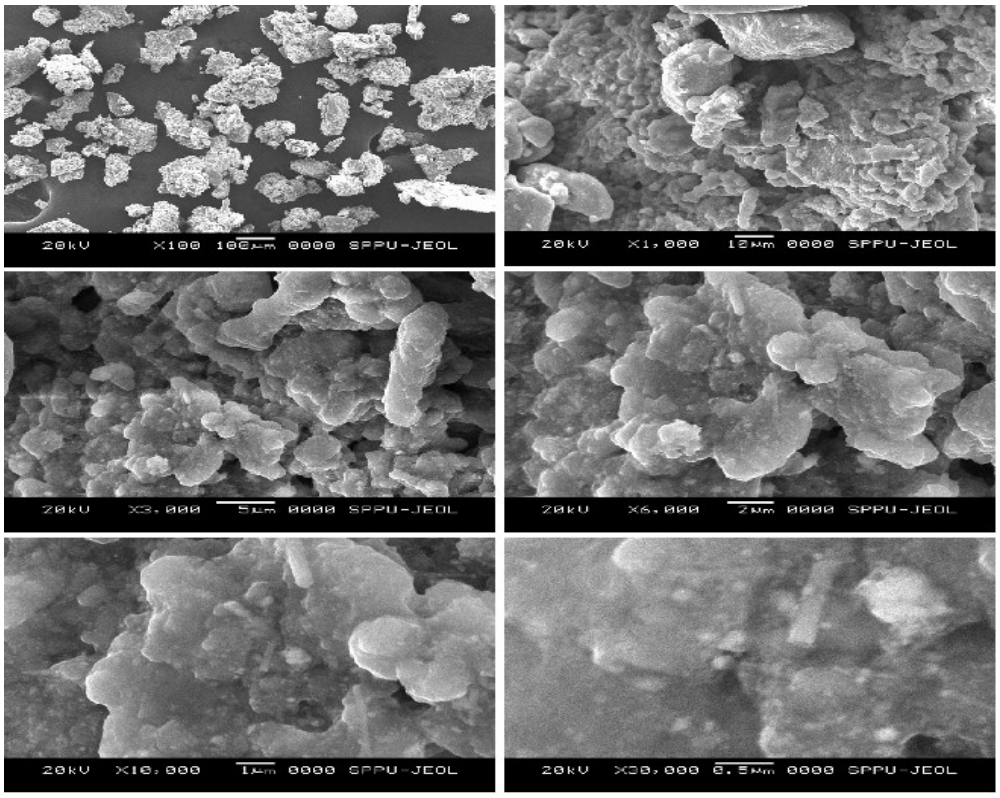

Figure 6 Scanning electron microscopic images of silver nanoparticles.

3.4.4. X-ray diffraction (XRD) analysis

X-ray diffraction (XRD) was carried out to confirm crystalline nature of the particles. X-RD pattern shown in Figure 7 and the XRD data analyzed using Expert Highscore plus software. From XRD data, it was concluded that the mix phase of AgNo3 and silver nanoparticles is observed. Also it was found that the intense peak was observed for that of silver nitrate. The data is matched to the JCPDS Card No.01-073-1411 for untreated (Residual) silver nitrate.

For silver nanoparticles the peaks are matched to JCPDS Card No.00-004-0783.The "Five" peaks are observed for that of silver nanoparticles at 38.1044, 42.2917, 64.5068, 77.29, 80.75 and the remaining peaks observed are of residual $AgNo_2$. It has been shown that biosynthesis of silver Nanoparticles process is not 100% completed due to incomplete oxidation of $AgNo_3$ and less incubation period. The silver ions were reduced in the presence of nitrate reductase, leading to the formation of a stable silver hydrosol 10-25 nm in diameter and stabilized by the capping peptide [11]. Most probably, the reduction of SNPs occurs due to the presence of cellular reductase released by *Spirulina platensis* into the solution [27]. Also, in cyanobacteria, localized reduce conditions may be produced by a bacterial electron transport chain, via energy generating reactions within the cells [29]. In this respect, secreted cofactor NADH plays an important role [23].

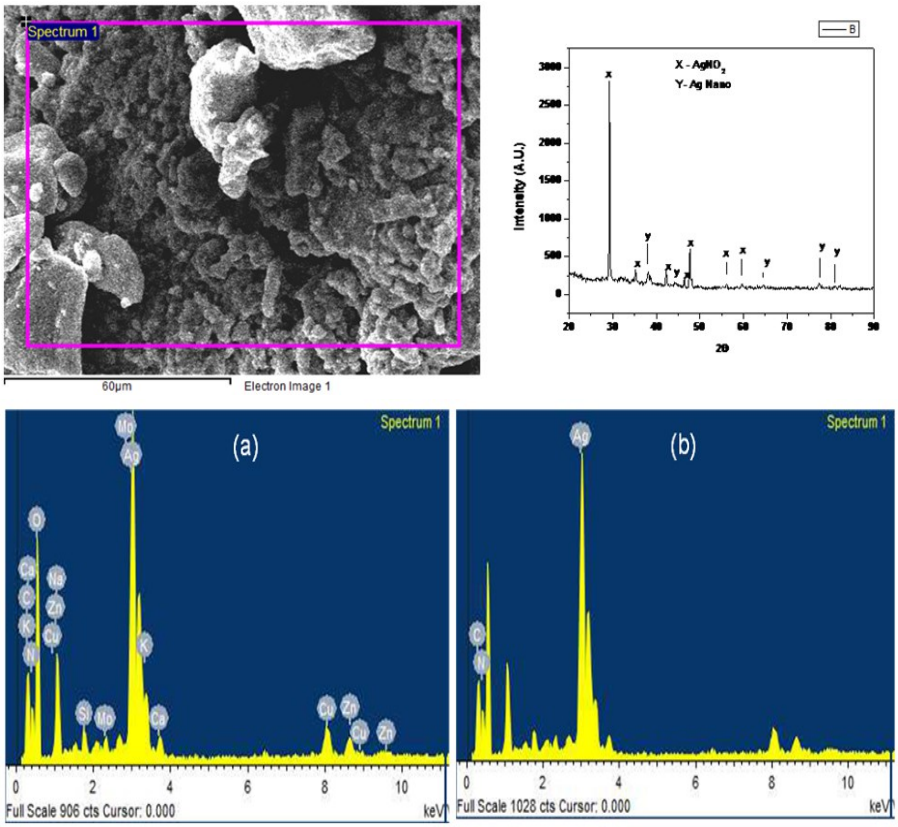

Figure 7 SEM image and EDS with of AgNPs with media (a) and only of AgNPs (b).

Table 2. XRD pattern of silver nanoparticles formed after reaction of culture supernatant with $AgNO_3$ solution

No.	Pos. [°2Th.]	d-spacing [A°]	FWHM [°2Th.]
1	29.279	3.05036	0.2952
2	35.2155	2.54856	0.2952
3	38.1044	2.36172	0.3936
4	42.2917	2.13708	0.2952
5	46.4019	1.95691	0.3936
6	47.6759	1.90755	0.2952
7	59.6749	1.5495	0.5904
8	77.3901	1.23213	0.72

Table 3. Antimicrobial activity of silver nanoparticles against test bacteria

Pathogen	Zone of inhibition (mm)	
	Standard antibiotics	Biosynthesized AgNPs
E. coli	10 (Ciprofloxacin)	10
Klebsiella sp	17 (Azithromycin)	13
Salmonella sp	19 (Amphicilin)	14
Pseudomonas sp	18 (Gentamycin)	15

Figure 8 Antimicrobial activity of biosynthesized silver nanoparticles against pathogenic bacteria and zone of inhibition compared with standard antibiotics

Antimicrobial activity of silver nanoparticles:

The antibacterial activity of silver nanoparticles produced by *Oscillatoria* sp. was observed against pathogens, viz., *E. coli*, *Klebsiella* sp, *Salmonella* sp, *Pseudomonas* sp. using disc diffusion method (Figure 8). The antimicrobial activity of silver nanoparticles produced by *Oscillatoria* spp. (P-18) presented in Table 3. The result shows maximum antibacterial effects against *Pseudomonas* sp. (15 mm), *Salmonella* sp. (14 mm), *Klebsiella* sp. (13 mm) and minor antibacterial effect was recorded against *E. coli*. (10 mm) & E. coli showed resistance to AgNPs. Sondi and Salopek-Sondi, [17] reported that the silver nanoparticles are used as antimicrobial agents against the gram negative bacteria. Antibacterial activity of biosynthesized AgNPs was evaluated against the *Pseudomonas aeruginosa* and *S. auerus* [26,25].

CONCLUSION

It was concluded that among 20 cyanobacterial isolates *Oscillatoria* sp. was shown to be able to synthesize Ag-NPs. The active factor involved in nanoparticles formation may be an extracellular molecule, activation of which requires light. In the analysis UV-visible spectrum and SEM the biosynthesis of silver nanoparticles was determined. XRD and FTIR analysis confirms the presence of functional groups of protein used in the synthesis of silver nanoparticles. The bioactivity of the synthesized silver nanoparticles had inhibitory effect on important human pathogens. More research is needed not only to identify the compounds responsible but also for better understanding the mechanism of nanoparticles formation by microalgae. It would be desirable to develop a technology in which the specific size and shape of the particles could be obtained by the use of a specific strain of cyanobacteria.

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