SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL EFFICACY OF CYANOBACTERIAL (POLYMER) SILVER NANO PARTICLE CONJUGATES

1RITIKA CHANAN, 2MINAKSHI LALIT, 3NAVEEN, 4 NAMITA SINGH

1Research Scholar, Microbial Biotechnology Laboratory, Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar-125 001, Haryana, India, Email: chananriti16@gmail.com
2Research Scholar, Microbial Biotechnology Laboratory, Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar-125 001, Haryana, India, Email: pr.minal@gmail.com
3M. Tech. Student, Microbial Biotechnology Laboratory, Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar-125 001, Haryana, India
4Associate Professor, Microbial Biotechnology Laboratory, Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar-125 001, Haryana, India, Email: namitasingh71@gmail.com

*corresponding address: Email: namitasingh71@gmail.com

ABSTRACT

Emergence of microbial resistance is the one of the major problem nowadays; thus there have been tremendous efforts towards finding new metabolites for the development of new antimicrobial drugs. Cyanobacteria have been identified as one of the most promising group of micro-organisms from which novel and biochemically active natural products can be isolated. Synthesis of nanoparticles using plant extracts has gained utmost importance in research world but biosynthesis of nanoparticles using microbes is still unexplored and underexploited. In the case of micro-algal species, Cyanobacteria have been studied in great detail for in-vitro silver nano-particle generation and stabilization. Treatment of Cyanobacterial cell extracts with silver nitrate solution in proper concentration and conditions may cap the microbial metabolic proteins and may cause the reduction of silver ions leading to synthesis of silver nanoparticles. In the present study, chemically synthesized silver nanoparticles (particle size ~ 85nm) were stabilized using different Cyanobacterial polymeric particles (biological) and were evaluated in terms of antibacterial activity and characterized using UV-Visible Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) techniques. The particle size of nanoparticles was determined using particle size analyzer. With this study, these nanoparticles bearing antimicrobial activity thus, can be used as a novel source for commercial applications in the different fields: pharmaceutical, cosmetics, industrial, Environmental, food, Agricultural, chemical, medical sector etc.

EY WORDS: Silver nanoparticles, Cyanobacterial polymeric particles, Antimicrobial activity.

1. INTRODUCTION

Due to unique electronic, mechanical, magnetic and chemical properties of metal nanoparticles that are significantly different from those of bulk materials [1] attributed to their small sizes and large specific surface area, scientists found that silver nanoparticles exhibit interesting antibacterial activities [2, 3]. Antibacterial activity of the silver-containing materials can be used, for example, in commercial sectors- medical, pharmaceutical, agricultural, textile etc [4-10]. Different methods to synthesize metal nanoparticles include: electrochemical method, thermal decomposition, laser ablation, microwave irradiation and sono-chemical synthesis [11-17]. The simplest and the most commonly used synthetic method for metal nanoparticles is the chemical reduction of metal salts [18, 19]. This chemical method involves reduction of an ionic salt in an appropriate medium in the presence of surfactant using various reducing agents [20, 21]. Stability and toxicity of nanoparticles is the main problem that affects the commercial use of silver nanoparticles [22]. In this study, we have tried using biological (Cyanobacterial) polymer to minimize toxicity and stability problem. Long term stability of the silver nanoparticles is not desirable, due to this reason; we have used cyanobacteria to stabilize the silver nanoparticles by their secreted proteins [23]. Stress tolerance during adverse conditions by Cyanobacterial nanoparticles is because they possess highly heterogeneous polymers containing a number of distinct polysaccharides and non-carbohydrate constituents including proteins, phospholipids and nucleic acids. Studies have shown that polysaccharides play a crucial role in bio-sorption and binding of toxic heavy metals (silver particles). These properties of bio-nanoparticles find special applications in bioremediation, food, and pharmaceutical industries [24]. Our study signifies on the usage of cyanobacteria as a stable carrier for silver nanoparticles and calcium alginate as a cross linker between silver nanoparticles and cyanobacteria. Alginate and its composites have been used in many biomedical applications including drug delivery and wound dressings. It can also be effectively used for the immobilization of silver nanoparticles [25]. Cyanobacterial silver nano-particle conjugates were bio-evaluated against E.coli and S.aureus.

2. MATERIALS AND METHODS

2.1 CHEMICAL SYNTHESIS OF SILVER NANOPARTICLES
8.5 mg of silver nitrate was dissolved in 50 ml distilled water (1M). Tri-sodium citrate was used as a reducing agent. 5ml of 1% tri-sodium citrate was added drop-wise in the silver nitrate solution at 85°C on continuous stirring at 50-70 rpm [26]. The samples were analysed with visual inspection and UV-Vis spectroscopy. Pale yellowish color was observed. The time scale of visual evolution depends on the silver nitrate solution. In general, nano-particle formation is visually appreciable after 4min of the beginning of the reaction.

2.2 CULTIVATION OF CYANOBACTERIA

The cultivation of cyanobacteria was carried out using BG-11 media at 27±2°C, under continuous illumination of 2000lux maintained by white fluorescence lamps. The cultures were regularly sub-cultured and maintained under continuous shaking conditions for few weeks. The cells were separated from the medium by centrifugation (4000rpm/10min) followed by filtration with Whatman filter paper. Finally, the Cyanobacterial pellets were lyophilized and stored at – 20°C. Three Cyanobacterial samples depicting high amounts of protein/lipids were separated from the medium by centrifugation under continuous shaking conditions for few weeks. The bacterial strains were maintained on nutrient agar slants and incubated at 30°C.

2.3 PREPARATION OF LINKERS WITH CYANOBACTERIAL SAMPLES (CYANOBACTERIA-LINKER SOLUTION)

70mg of Sodium alginate was dissolved in 20ml distilled water (3mM) and calcium chloride solution (45mg in 10ml, 0.03M) was added drop wise on continuous stirring to sodium alginate solution. To the above mixture, after 10 minutes, PBS dissolved three different Cyanobacterial samples were added drop wise in three different beakers. These solutions were used in 5:1:4 respectively.

2.4 CONJUGATION OF SILVER NANOPARTICLES WITH CYANOBACTERIAL - LINKER SOLUTION

Mix 5ml of each cyanobacteria-linker sample to 5ml of silver nano-particle solution on continuous stirring for 3 to 4 hours. The samples were further subjected for UV-visible spectroscopic analysis.

The micro organisms including gram positive and gram negative bacteria were used in this study. Staphylococcus aureus NCIM 5021 (gram positive), E.coli MTCC-723 (gram negative) were provided from NCIM (National Collection of Industrial Microorganisms) culture collection, Pune and Institute of Microbial Technology, Chandigarh, India respectively. The bacterial strains were maintained on nutrient agar slants and incubated at 30°C.

2.6 ANTIBACTERIAL ASSAY

The antimicrobial susceptibility of Silver Nanoparticles (Ag NPs) was evaluated using well diffusion method. Zones of inhibition were measured after overnight incubation at 30°C. The comparative study was made between cyanobacteria linked silver nanoparticles and silver nanoparticles alone.

3. RESULTS AND DISCUSSION

Silver nanoparticles were synthesized according to the chemical reduction method.

Figure 1: Silver Nanoparticle solution
Surface Plasmon absorption maximum was shifted to lower wavelength with addition of citrate of sodium as reducing agent [30]. UV-Visible Spectroscopy for Cyanobacteria-Microcystis aeruginosa showed the absorption maxima at 300-700 nm with the presence of chlorophyll a, phycoerythrin and phycocyanine and absorption maxima at 700-800 nm showed the presence of chlorophyll a [31]. Fig 2 describes the UV-Vis photo spectra of the mixture sample of (a) Silver Nanoparticles (b) Microcystis aeruginosa linked silver nanoparticles (c) Frischerella linked silver nanoparticles and (d) B6 Cyanobacteria linked silver nanoparticles recorded in the range of 200-800 nm.

![Graphs showing UV-Visible Spectra](image)

**Figure 2**: UV-Visible Spectra of Ag NPs and Cyanobacteria linked Ag NPs

### 3.2 PARTICLE SIZE ANALYSIS

Particle size of Ag NPs formed was 85 nm in diameter. Shape of the particles and spherical structure of the particles can be controlled experimentally. The result indicated that the average particle size of the synthesized silver nanoparticles was highly influenced by the reaction. The nano size of material results in specific physicochemical characteristics different than those of their bulk materials or larger particles. This effect is mainly credited to high surface-area-to-volume ratio, which results in increased reactivity; hence, the nano scale materials are more advantageous than their bulk counterparts. Average size of silver nano-particles was recorded to be 85 nm. After conjugation, Microcystis linked silver nano particles conjugate showed 144 nm size of particles. Frischerella linked silver nanoparticles conjugate showed 503 nm size of the particle. Cyanobacteria B6 linked silver nanoparticles showed 216 nm size of particles. This means these particles, give high-surface-to-volume ratio, which results in increased reactivity.

**Table-1**: Peak values depicting Average Size (r. nm), Intensity (%), and Width (r. nm) of three different Cyanobacterial samples inked with Ag NPs

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>PEAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silver Nano-particles (Ag NPs)</td>
<td>Average Size (r. nm)</td>
</tr>
<tr>
<td></td>
<td>85.0</td>
</tr>
<tr>
<td>Microcystis aeruginosa linked Ag NPs</td>
<td>144</td>
</tr>
<tr>
<td>Frischerella linked Ag NPs</td>
<td>503</td>
</tr>
<tr>
<td>Cyanobacteria B6 linked Ag NPs</td>
<td>216</td>
</tr>
</tbody>
</table>
3.3 FTIR ANALYSIS

The FTIR spectrum indicates various functional groups present at different positions. IR spectroscopy study has confirmed that the carbonyl group of amino acid residues and peptides of proteins has a stronger ability to bind metal, so that the proteins may form a coat, covering the metal nanoparticles (i.e. capping of Ag NPs) to prevent the agglomeration of the particles and to stabilize the nanoparticles in the medium. In the case of Microcystis aeruginosa, Frischerella, B6 Isolate linked silver nano particle conjugates, the peaks in the region between 3859.55 to 3422.27 are assigned to O-H stretching of alcohol and phenol compounds and aldehyde–C–H– stretching of alkanes. The peaks in the region 1615.76 to 1407.02 and 1300 to 650 corresponds to N–H(bond) of primary and secondary amides and –C–N– stretching vibration of amines or C–O stretching of alcohols, ether and carboxylic acid.

**Figure 3: FTIR Spectra of Silver Nanoparticles linked with three different Cyanobacteria**

The overall peaks from FTIR observation confirm the presence of protein in the samples of silver nanoparticles. It can be assumed that there is the presence of nitrate reducing bacteria to produce nitrate reductase enzyme (protein). This reduces to silver nitrate solution from silver nano particles [32, 33]. The
bands at 1622 cm\(^{-1}\) and 1527 cm\(^{-1}\) correspond to the stretch molecule vibration. The two bands existing at 1412 cm\(^{-1}\) and 1029 cm\(^{-1}\) can be assigned to the C-N stretching vibrations of aromatic and aliphatic amines. The corresponding bending vibrations were seen at 1651 cm\(^{-1}\) and 1548 cm\(^{-1}\), respectively. The two bands observed at 1379 cm\(^{-1}\) and 1033 cm\(^{-1}\) can be assigned to the C–N stretching vibrations of aromatic and aliphatic amines, respectively [34]. 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 agent [35]. The presence of protein acts as a stabilizing agent and surrounds silver nanoparticles [36]. The clarity of the peak showed the presence of protein in the conjugation. The shifting of some peak at 528, 805, 1127 and 1320 showed the presence of disulphide, S-OR, thio-carbonyl, phosphonate groups also.

Table-2: Stretching and vibration of functional group of Nano formulations

<table>
<thead>
<tr>
<th>Frequency [cm(^{-1})]</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3200-3400</td>
<td>Alcohol/phenol stretching</td>
</tr>
<tr>
<td>2300-2320</td>
<td>C-H structure</td>
</tr>
<tr>
<td>1615</td>
<td>Alkene stretching</td>
</tr>
<tr>
<td>1450</td>
<td>-CH(_3) (bend)</td>
</tr>
<tr>
<td>1000-1300</td>
<td>Alcohol, ether esters, carboxylic acid, anhydrides</td>
</tr>
<tr>
<td>650-1000</td>
<td>Alkene (out-of-plane bend)</td>
</tr>
</tbody>
</table>

3.4 ANTIMICROBIAL ACTIVITY

It is well known that silver ion nanoparticles are highly toxic to microorganisms. Silver nanoparticles have been known to have inhibitory and bactericidal effects and thus we extend its application as an antibacterial agent. The biological activity of silver based materials, depending on their structure and physicochemical properties, affects the interaction with the cytoplasmic membrane of bacteria and influences cell metabolism. In our study, the antimicrobial activity of nano-silver-containing Cyanobacterial films was investigated against *E. coli* and *Staphylococcus aureus*. The Antibacterial activity is estimated by the zone of inhibition [37]. The differences in the composition of the cell wall of Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacteria and hydrophilic and hydrophobic character of *E. coli* and *S. aureus* respectively induce different antimicrobial activity. The mechanism of the bactericidal effect of silver and silver nanoparticles remains to be understood. Several studies propose that silver nanoparticles may attach to the surface of the cell membrane disturbing permeability and respiratory function of the cell. It is also possible that silver nanoparticles not only interact with the surface of membrane, but can also penetrate inside the bacteria [38]. It may be observed that silver nanoparticles have comparatively higher anti-bacterial activity against gram negative organism than gram positive, probably due to thinner peptidoglycan layer and presence of porins [39].

Two aspects are to be considered:

- Mean Width of Zone of inhibition (Diameter, cm)
- Microorganism used:

  *Escherichia coli* MTCC 723

  *Staphylococcus aureus* NCIM 5021

![Figure 4: Antimicrobial efficacy of (A)AgNP(Positive Control),(B)Microcystis linked AgNP, (C)Frischerella linked AgNP, (D)B6 linked AgNP (E) Negative control (Only PBS Buffer instead of the test extract) against *E.coli.*](image-url)
The antibacterial activity of the test extracts (Cyanobacteria Linked Silver Nanoparticles) and positive control (Silver Nanoparticles alone) maintained at 4°C, were again measured after one month against test pathogens in terms of zone of inhibition. It was observed that the zone of inhibition in cms for the silver nanoparticles decreased in diameter. The possible reason for this decrease could be due to the agglomeration of the silver nanoparticles with time whereas the zone of inhibition of cyanobacteria linked silver nanoparticles remained same in diameter even after one month. This indicated that the nanoparticles linked with cyanobacteria did not agglomerate and were stabilized for a longer period of time due to the secretion of proteins from cyanobacteria which acted as a capping agent to help these conjugates to not to lose their effective bioactivity whereas non-conjugated silver nanoparticles showed a decrease in size of their zone of inhibition and bioactivity with time.

4. CONCLUSION

In our study, silver nanoparticles with mean diameter of 85nm were synthesized using tri-sodium citrate as a reducing agent. The nanoparticles were characterized by UV/Vis Spectroscopy, FTIR. UV/Vis spectra showed the characteristic Plasmon absorption peak for the silver nanoparticles ranging from 200-230 nm. Additionally, the antibacterial activity of the nanoparticulate dispersion was measured. The results of this study clearly demonstrated that the colloidal silver nanoparticles inhibited the growth and multiplication of the tested bacteria, including highly multi resistant bacteria such as S. aureus and Escherichia coli. The silver nanoparticles linked cyanobacteria help to reduce the toxicity of the silver nanoparticles and improve the stability of the silver nanoparticles by the secretion of proteins (capping agent) after conjugation with cyanobacteria. The Bio-Nano-formulation of cyanobacteria and silver nano-particles conjugate were more stable than the silver nanoparticles alone against pathogenic bacteria and thus these conjugates find application in commercial fields such as biomedical, pharmaceutical, cosmetics, health, food and agriculture sectors.

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