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Green Synthesis of Silver Nanoparticles from *Bacillus* Strains and their Antimicrobial Investigations against Plant Pathogens

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A B S T R A C T

The development of a reliable green chemistry process for the microbial fabrication of nanoparticles is an important aspect of current nanotechnology research. More interest has been generated in silver nanoparticles (AgNPs) due to their inhibitory and bactericidal effects. In the present study, extracellular syntheses of silver nanoparticles by bacterial strains isolated from the agricultural soils were reported. When silver ions are exposed to the cell free supernatant of the isolated bacterial strains, reduction of silver ions occurs which leads to the formation of AgNPs. The synthesized AgNPs were characterized by UV-Visible spectroscopy and scanning electron microscopy which also exhibited maximum absorbance at 420 nm. They were spherical in shape and size ranging between 75-98 nm. The antimicrobial activity of synthesized AgNPs was evaluated against plant pathogenic bacteria *Xanthomonas campestris, Agrobacterium tumifaciens, Arthrobacter sp.,* and *Erwinia chrysanthemi burkholder.*

***Key words*:** Silver nanoparticles, Microbial fabrication, Extracellular, Bactericidal, Antimicrobial activity

Nanoparticles are nanometer sized particles, which have a higher surface to volume ratio due to that it has higher reactivity and biochemical activity. In recent years, metal nanoparticles have manifold applications in electronics, agriculture, nanomedicine, energy, biosensors, catalysts, etc. [10]. Numerous physical and chemical methods were used for the synthesis of nanoparticles. But traditional methods are expensive, toxic, and require high energy and temperature [7], [17]. An alternative method that is environmentally benign and ecofriendly and also does not generate toxic byproducts needs to be developed. Thus, the biogenic approach called 'The Green Synthesis' is a suitable alternative due to the use of plants, microorganisms, fungi, and algae for the fabrication of nanoparticles [4], [12].

Among various metal nanoparticles, silver nanoparticles have gained enormous importance due to their physicochemical properties such as antimicrobial, anticancer, larvicidal, catalyst, biolabeling, and biosensors [3], [9]. Apart from these, AgNPs are also utilized in the food industry, sunscreen, clothing, cosmetics, and in wastewater treatment [13]. First time synthesis of AgNPs using *Pseudomonas stutzeri* AG 259 isolated from the silver mine [6], [11]. Microbial fabrication of metal nanoparticles can be done either extracellularly or intracellularly. The intracellular synthesis involved additional steps, ultrasonic wave treatment or the use of detergent for cell disruption. In contrast, the extracellular method is cheap and favorable for the large-scale production of nanoparticles [1], [2], [5], [8].

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MATERIALS AND METHODS

Media and chemicals

All media and chemicals used in this study were procured from the Hi-media laboratory. The plant pathogenic bacterial strains *Xanthomonas campestris, Agrobacterium tumifaciens, Arthrobacter sp.,* and *Erwinia chrysanthemi burkholder* were procured from NCIM, Pune.

Isolation and screening of microorganisms

Agricultural soil samples were collected from the Dang (20.8254° N, 73.7007° N) and Tapi (21.2789° N, 73.6065° N) districts of the South Gujarat region, India using the standard microbiological protocols. Three different places Samgahan, Lavchali, and Dodipada from Dang district and Dolvan, Karjan, and Vedchi from Tapi district were selected for soil sample collection. From the samples two tubes of soil suspension were prepared using sterile distilled water. One sample was heated at 80 °C in the water bath and one remained untreated. From each tube, one loop full of soil samples was streaked onto different media Nutrient agar, R2A medium, and Actinomycetes agar. The colonies were further subcultured on respective media to obtain a pure culture and stored for future use.

Identification

Identification and characterization of cultures were done by the morphological and biochemical tests. Molecular identification of the isolates was done through 16s rRNA based method.

Extracellular synthesis of AgNPs

For synthesis of AgNPs two bacterial strains SNH8 and EH1 were inoculated into a 250 ml flask containing 100 ml of sterile Luria-Bertani (LB) medium for 24 hours at 30°C. After 24 hours of incubation, the cell free supernatants were collected by centrifugation at 10,000 rpm for 10 min. The supernatants were treated with $AgNO_3$ to make a final volume of the reaction mixture to 10^{-3} M and incubated under the dark conditions to prevent any photochemical reaction during the experiment. The flask containing supernatant without $AgNO_3$ was also maintained as a control. After 24 hours of incubation, the initial yellow color changed to the brown color indicating AgNPs synthesis [14].

Characterization of AgNPs

UV-visible spectroscopy

The Plasmon- resonance property of fabricated AgNPs was studied by a UV-visible spectrophotometer. Thus, samples containing nanoparticles were subjected to absorption analysis at 350-800 nm range using UV-visible spectroscopy.

Scanning electron microscopy

Scanning electron microscopy was performed by S-3400N Type-III, Hitachi High Technologies, Singapore. SEM showed the size and shape of the synthesized AgNPs.

Optimization study

Effect of temperature and pH

Three different temperatures (30°C, 37°C, and 55°C) and pH values (5, 7, and 9) were selected. Cell free supernatant was treated with $AgNO_3$ and incubated for 24 hours in dark conditions. Cell free supernatant without $AgNO_3$ was kept as a control.

Effect of $AgNO_3$ concentration

Three different concentrations of $AgNO_3$ 0.5 mM, 1 mM, and 2 mM were selected for the study. 100 ml of Culture supernatants were taken in three different 250 ml flasks. To these, AgNO3 was added to achieve a final concentration of the reaction mixture of 0.5 mM, 1 mM and 2 mM respectively. Culture supernatant without $AgNO_3$ was kept as a control.

Evaluation of antimicrobial activity

The synthesized AgNPs from SNH8 and EH1 were evaluated for their antimicrobial activity by well diffusion method against plant pathogenic bacteria *Xanthomonas campestris, Agrobacterium tumifaciens, Arthrobacter sp.* and *Erwinia chrysanthemi burkholder*. Each organism was lawn cultured on the individual nutrient agar plate. Well of 6 mm size were made on the nutrient agar plate using sterile cup borer. 100 µL aliquot of synthesized nanoparticles were taken and added to the well and placed in a refrigerator for 30 min. The plates were then incubated at 37°C for 24 hours and determined the zone of inhibition.

RESULTS AND DISCUSSION

A total of 29 and 14 isolates were obtained from the Dang (20.8254° N, 73.7007° N) and Tapi (21.2789° N, 73.6065° N) districts of South Gujarat, India respectively. All were then screened for their ability to produce AgNPs [15]. Out of them SNH8 and EH1 were selected and optimized for the production of AgNPs on a large scale. The nanoparticles synthesized from these two isolates showed shortest λ max between the range 420-450 nm. The selected bacterial strains were further subjected to molecular identification by 16S rRNA sequencing method. The obtained sequence data were subjected to BLAST. The EH1 isolate showed a high 99.79% similarity with *Bacillus aerius* and SNH8 showed a high 100% similarity with *Bacillus paramycoides*. The formation of AgNPs was indicated by the visual observation of the color change of the reaction mixture from light yellow to brown color whereas no color change was observed in the control (Fig 2).

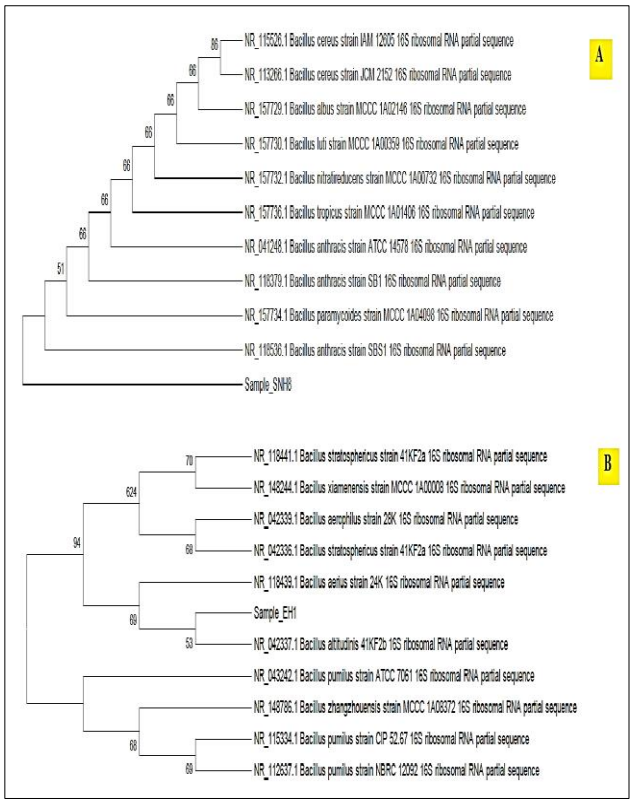

Fig 1 The phylogenetic tree of (A) SNH8 and (B) EH1

Fig 2 Results of visible color change in samples. (A) SNH8 culture supernatant without AgNO3 acted as a control (left side) and the brown color extract of AgNPs after 24 hours (right side) (B) EH1 culture supernatant without AgNO3 as a control (left side) and the brown color extract of AgNPs after 24 hours (right side)

The synthesis of silver nanoparticles by SNH8 and EH1 isolates were optimized using different parameters temperatures, pH, and $AgNO_3$ concentration.

Effect of temperature and pH

Three different temperatures (30°C, 37°C, and 55°C) and pH (5, 7, and 9) were selected. After incubation UV- visible spectra were obtained. The AgNPs synthesized from SNH8

isolate showed maximum absorbance of 0.529 at 30°C and EH1 showed 0.878 at 30°C respectively. No characteristic peaks were seen at pH 5 and 9 but maximum absorbance of 0.612 was recorded at pH7 for SNH8 and 0.369 for EH1.

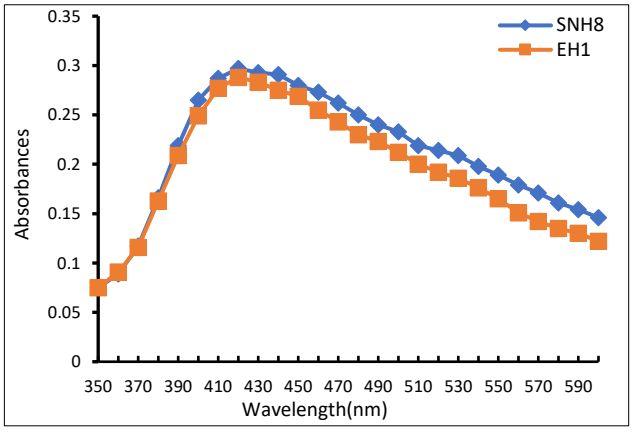

Fig 3 UV-Visible spectra of synthesized AgNPs

Effect of $AgNO_3$ concentration on the particle formation

0.5 mM, 1mM, and 2mM AgNO3 concentrations were added into SNH8 and EH1 cell free supernatants and incubated for 24 hours. UV- visible spectra were obtained after incubation. 0.5 mM concentration was not effective in the production of the particles by the isolates whereas 2 mM concentration showed color change but no characteristic peak was obtained in the range of 420-450 nm. 1mM concentration was considered favorable as isolate SNH8 showed maximum absorbance of 0.224 and EH1 shows 0.422 absorbance at 420 nm. Further, confirmations of AgNPs were done by UV-visible spectroscopy. For this purpose, the reaction mixture was scanned at the wavelength of 350-800 nm. The AgNPs synthesized from both isolates SNH8 and EH1 showed a surface Plasmon resonance peak at 420 nm (Fig 3).

Scanning electron microscopy was performed to understand the morphology of AgNPs. SEM images reveal that the synthesized AgNPs were generally spherical in shape and size ranging from 70-94 nm (Fig 4). The nanoparticle size range of 100 nm [15]. These AgNPs were synthesized from *Penicillium* fungi isolated from soil samples. The nanoparticles synthesized in the range of 50-100 nm from *Enterobacteriaceae* family and geranial [16]. Overall, we found that both isolates SNH8 and EH1 were capable of efficient synthesis of AgNPs.

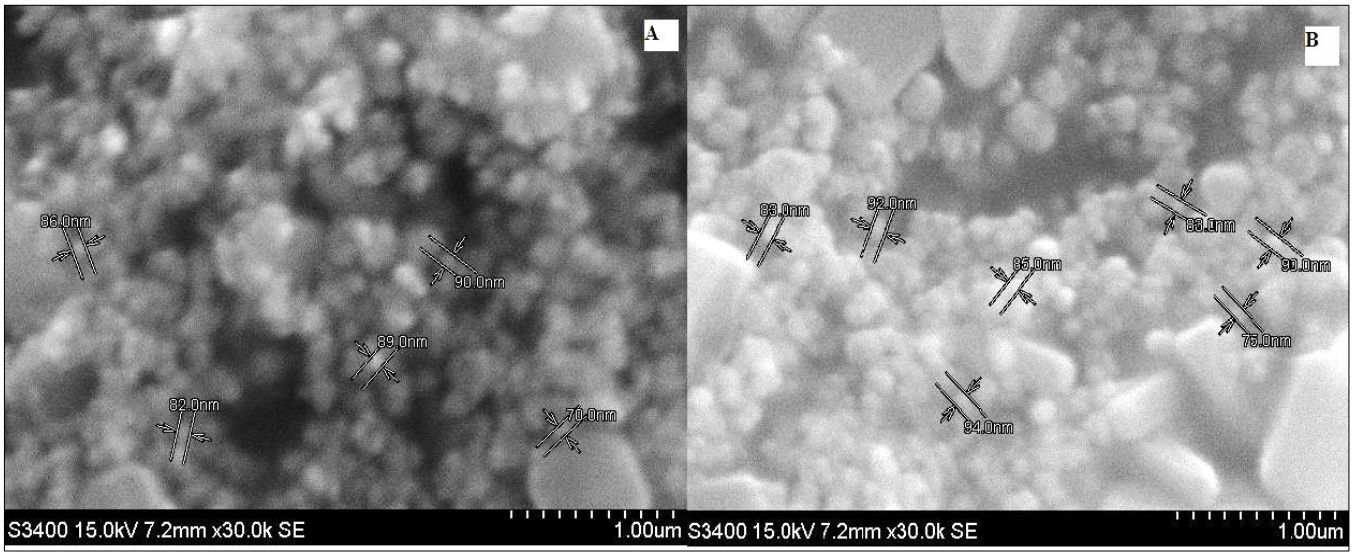

Fig 4 SEM images of AgNPs synthesized using (A) SNH8 isolate (B) EH1 isolate

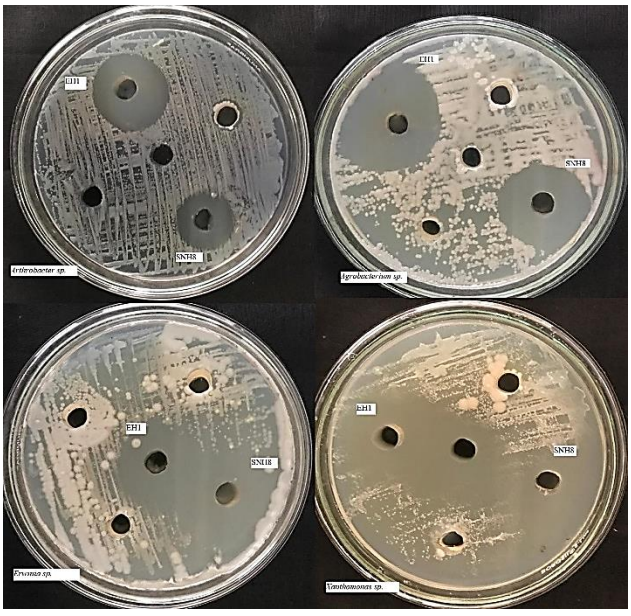

Fig 5 Antimicrobial activity of biosynthesized silver nanoparticles from SNH8 and EH1 bacterial strains

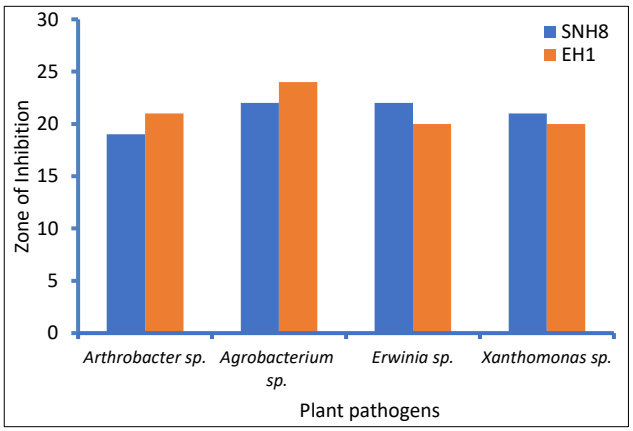

Fig 6 Antimicrobial potency of biosynthesized AgNPs

Antimicrobial activity

As these particles are to be used as tools in agriculture it becomes necessary to check their efficiency against plant pathogens. For this purpose, the antibacterial activity of synthesized AgNPs was evaluated against plant pathogenic bacteria, which included *Xanthomonas campestris*,

Agrobacterium tumifaciens, *Arthrobacter sp.*, and *Erwinia chrysanthemi burkholder*. (Fig 5-6, Table 1) shows the results of the zones of inhibition produced by AgNPs after 24 h. The highest zone of inhibition 26 mm against *Agrobacterium tumifaciens* was obtained through EH1AgNPs. SNH8AgNPs showed less efficiency of inhibition against *Arthrobacter* sp. as compared to EH1AgNPs. But in some cases, it shows a higher zone of inhibition compared to EH1AgNPs.

Table 1 Antimicrobial activity of biosynthesized silver nanoparticles against plant pathogens

Bacterial isolates / Plant pathogens	Control ($AgNO_3$)	SNH8	EH1
	Zone size in (mm)		
Arthrobacter sp.	0 ± 0.0	19 ± 0.2	22 ± 0.1
Agrobacterium *tumifaciens*	0 ± 0.0	24 ± 0.1	26 ± 0.2
Erwinia chrysanthemi burkholder	0 ± 0.0	23 ± 0.2	21 ± 0.3
Xanthomonas campestris	0 ± 0.0	21 ± 0.1	20 ± 0.2

CONCLUSION

Agrochemicals such as fertilizers, pesticides, herbicides etc., are used in agriculture for improvement of plant growth and plant protection from pests and pathogens. But the chemicals used for these are toxic and costly as well as it affects the environment and farmers also. Nowadays, green approach nanotechnology has been used in agriculture for crop improvement. In the present study, we reported the simple biogenic approach for the microbial fabrication of AgNPs. The extracellular mode of synthesis is presented in this study as it is a simple and fast technique. The main mechanisms for the fabrication of AgNPs involve enzyme nitrate reductase. The enzyme converts nitrate to nitrite and an electron shuttle is induced as a result of that reduction of silver ions to silver nanoparticles occurs. The AgNPs were successfully synthesized by using bacterial strains *Bacillus paramycoides* (SNH8) and *Bacillus aerius* (EH1). The UV- visible spectra and Scanning electron microscopy images provide strong evidence that AgNPs were synthesized successfully. Optimization of the study shows that the particles were favored at 30°C temperature, 1mM concentration and 7 pH. Also, the extracellular synthesized AgNPs showed an excellent antibacterial property against plant pathogens. Hence, the biological method appears to be an efficient alternative to the conventional method of silver nanoparticles synthesis and is easily amenable to large scale production.

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