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BIOSYNTHESIS OF SILVER NANOPARTICLES USING THE CELL-FREE FILTRATE OF *Streptomyces atacamensis*: CHARACTERIZATION AND ANTIBACTERIAL ACTIVITY

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ABSTRACT

A bacterial strain was isolated from a soil near the bank Tapi river, Surat (India) and identified as *Streptomyces atacamensis* AK3 on the basis of 16s rRNA gene sequencing. The strain possessed reducing agents which enabled it to produce silver nano-particles (AgNPs). The peak observed at 420 nm and turning the broth culture colour to brown indicated the synthesis of AgNPs. Zeta potential of -14 mV revealed a low degree of aggregation of AgNPs. Mostly, AgNPs were spherical, ovoid, and rhomboid. TEM analysis revealed that AgNPs of size ~20 nm were most abundant in the sample. The zones of inhibition (ZOI) against *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* were 23 ± 2, 17 ± 1, 24 ± 2, and 22 ± 2 mm, respectively. The antibiotics or AgNPs: GM30 and MET30 only produced bigger ZOI against *S. aureus* than 35 µg mL^{-1} AgNPs. It was only CFS30 that made bigger ZOI against *E. coli* than 10 µg mL^{-1} AgNPs. The use of 35 µg mL^{-1} AgNPs against *B. cereus* did not yield the similar output as they barely made 17 ± 1 mm ZOI while the use of 35 AgNPs µg mL^{-1} yielded fruitful results against *P. aeruginosa* in comparison to those of antibiotics.

Keywords: Antibacterial activity, phylogenetic tree, silver nano-particles, TEM, Zeta potential

INTRODUCTION

Silver nanoparticles (AgNPs) are of great interest to the researchers due to their wide application in areas like electronics, agriculture, energy, sensors, biomedicine, etc. (Rudrappa *et al.*, 2022). AgNPs have emerged as antibacterial, antiviral, antifungal, anticancer, and antiangiogenic agents (Zhang *et al.*, 2016). The bacterial species are frequently exposed to drugs at different times; however, none of those drugs have worked effectively due to the natural selection of resistant Ruddaraju *et al*., 2020; Mozafari *et al*., 2021). Due to the specific physical properties the nano-particles have been reported as potential agents against harmful bacteria (Khan *et al.*, 2019; de Melo *et al*., 2020). AgNPs show a greater outcome in comparison to gold, zinc, iron, etc. (Manikprabhu and Lingappa, 2014). AgNPs of < 10 nm size have shown adverse cytotoxic effects on human cells (Liao *et al*., 2019; Ferdous and Nemmar, 2020). Since AgNPs are thermally stable and their nucleation rate constant increases at a higher temperature (80-90°C), the manipulation in size is possible by changing the temperature of the process (Liu *et al*., 2020). The concentration of AgNPs need to be optimized to reduce their electrical conductivity and keep them from becoming large crystals (Chen *et al*., 2009). Biosynthesized AgNPs have more effective therapeutic applications than chemically synthesized ones besides, toxic chemicals can be absorbed on AgNPs

in chemical synthesis (Firdhouse and Lalitha, 2015; Kotcherlakota *et al*., 2019). Synthesis of AgNPs with the actinomycetes is human cells friendly and cost-effective (Al-Dhabi *et al.*, 2018). Statistical models should be applied for providing optimum conditions for the process of AgNPs biosynthesis (Naser *et al*., 2016).

Actinomycetes are dual-natured bacteria having the characteristics of both bacteria and fungi and are inhabitants of moist soil (Pepper and Gentry, 2015; Shivabai and Gutte, 2019). Actinomycetes have been explored for the production of secondary metabolites meant for curing human diseases (Abdel-Aziz *et a*l., 2019; Selim *et al*., 2021). The synthesis of AgNPs by *Streptomyces* inhabiting heavy metal polluted sites and their therapeutic use have rarely been reported (Sukanya *et al*., 2013; Abdeen *et al*., 2019). This paper is based on the biosynthesis of AgNPs by *Streptomyces,* inhabiting the heavy metal polluted site on the bank of Tapi river, Surat, Gujarat (India), and their medicinal applications for humans.

MATERIALS AND METHODS

Soil collection and isolation of actinomycetes

The soil samples were collected from a heavy metal polluted site on the bank of Tapi river, Surat, Gujarat (India). The soil was sieved to remove the large particles and kept at 26°C for 7-8 h (Sengupta *et al*., 2015). Soil sample (1 g) was dissolved in 10 mL 1% NaCl solution, diluted to 10^{-7}, and then 100 μL^{-1} of each dilution was spread on Petri-dishes having "actinomycetes isolation medium" (supplemented with 25 $\mu g\ mL^{-1}$ cycloheximide or 50 $\mu g\ mL^{-1}$ nystatin or 25 $\mu g\ mL^{-1}$ nalidixic acid, separately). All the Petr-dishes were incubated at 30°C of 1-2 weeks. Based on the morphological characteristics, the actinomycetes were isolated (Chakraborty *et al*., 2022) and preserved in 50% (v/v) glycerol at -40°C for future use and sub-cultured till further use.

Silver nanoparticles synthesizing ability-based selection of isolate

The isolates from the above step were transferred to the flasks containing 100 mL starch casein broth (SCB) for mass multiplication and incubated in a shaking incubator (120 rpm) at 30°C for 72 h. Then the mycelia produced along with SCB was filtered through a bacteriological filter to get cell-free filtrate (CFF). To CFF, 1 mM $AgNO_3$ solution was added and kept in an incubator (120 rpm) at 30°C for 48 h. The incubated solution was centrifuged for pelleting the $AgNO_3$ at 17000 rpm (Fig. 1). The colour of added CFF turned pale brown on 3rd day of incubation [8.5 pH] (Nayak *et al*., 2020c; Sreenivasa *et al*., 2021). The biosynthesized AgNPs were compared with antibiotics for their anti-bacterial activity.

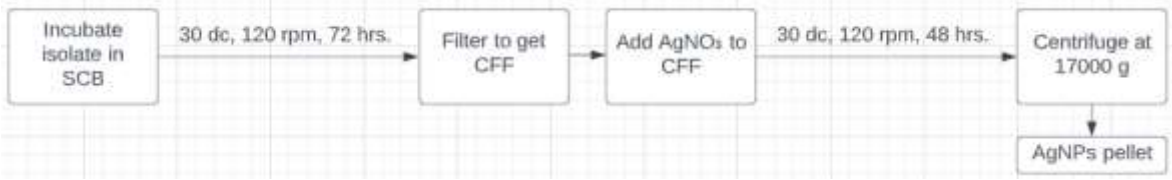

Fig. 1: Flowchart of the biosynthesis of AgNP's

Quantification and structural properties of synthesized silver nanoparticles

The quantification of $AgNO_3$ was done spectrophotometerically [Shimadzu UV-VIS 1700, range 190 -1100 nm]. The size distribution and Zeta potential reports were obtained using Malvern. The characterization of $AgNO_3$ was accomplished by using a ttransmission electron microscope (accelerating voltage, 80 to 200 kV, Joel.).

16s rRNA gene sequencing of actinomycetes

The 16s rRNA gene was amplified in 100 µL PCR mixture [54.3 ng DNA, 0.5 mM of each dNTP, 400 ng of each primer (5`GGATGAGCCCGCGGCCTA3` and 5`CGGTGTGTACAAGGCCCGG

GAAC3`), 3.2 mM $MgCl_2$, and 1 µL 3U Taq DNA polymerase]. The amplification was accelerated for 30 cycles and 2 h in a PCR machine. The timings of each cycle were 1 min for denaturation, 1 min for annealing, and 2 min for extension. The amplified DNA was sequenced with an ABI3130 Genetic Analyzer, homology in sequences was checked with BLAST, and a phylogenetic tree was created with the Weighbor's (Bruno, Socci and Halpern, 2000).

Treatment of human pathogens with various concentrations of silver nanoparticles

Gram-positive and Gram-negative bacteria were categorized on the basis of their sensitivity to AgNPs. Since peptidoglycan layers in Gram-positive bacteria are very thick and densely packed, the AgNPs do not exhibit similar effects on Gram-positive bacteria (Dakal *et al.,* 2016). Gram-positive bacteria namely *Staphylococcus aureus* (ATCC 33862) and *Bacillus cereus* (ATCC 13061) were treated with 10 and 35 µg mL^{-1}; whereas Gram-negative bacteria namely *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 10536) with 7 and 10 mL^{-1} $AgNO_3$ as lower and higher concentrations, respectively, along with both types of pathogenic bacteria treated with the antibiotics (Table 1). All the experiments were performed in triplicate and the mean values of the zones of inhibition were calculated.

RESULTS AND DISCUSSION

Selection of actinomycetes for the synthesis of AgNPs

The *Streptomyces atacamensis* strain AK3 caused a change in colour from white to brown after 48 h of the addition of $AgNO_3$ to the cell free-filtrate. The properties of AgNPs were studied with UV-VIS spectrophotometer, Zeta sizer, and TEM (Fig, 2-4). The maximum absorption was observed at 420 nm as a confirmation of AgNPs.

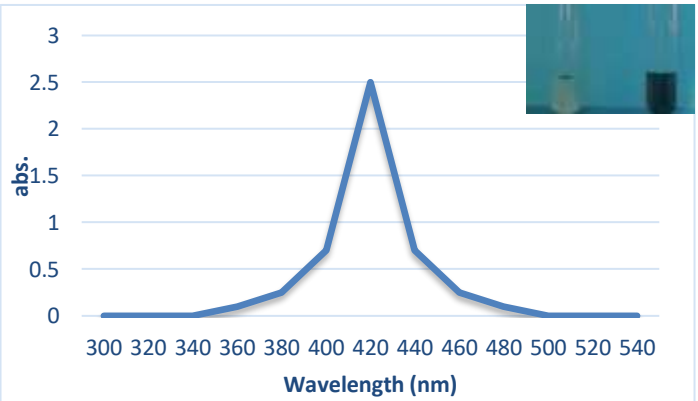

Fig. 2: UV visible spectra of AgNPs

The polydispersity index (PDI), which goes lower than 0.03 to reveal a low level of agglomeration, was reported as 0.3. The Zeta potential of AgNPs was -14 mV, confirming the agglomeration of AgNPs to a lesser extent.

The TEM micrograph revealed the shape of AgNPs as ellipsoidal to spherical with size as ~20 nm (Fig. 5).

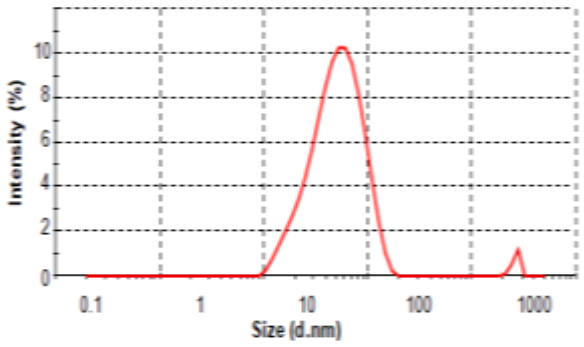

Fig. 3: Intensity graph of biosynthesized AgNPs produced with Malvera

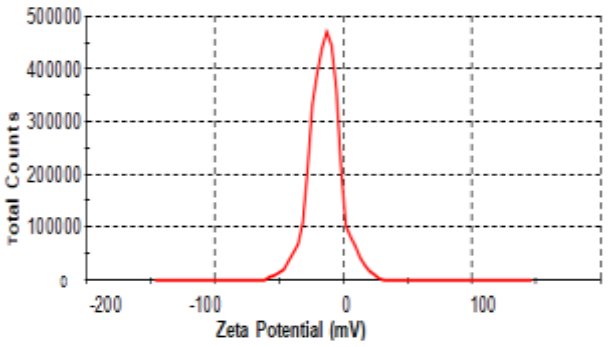

Fig. 4: Zeta potential of biosynthesized AgNPs produced with Malvera

Morphological and molecular identification of the actinomycetes isolate

The colonies were wrinkled, raised, and like chalk in appearance, and the strain grew well under aerobic conditions at 32°C. The 16s rRNA gene was used to identify strain at molecular level. The ssequencing produced an image of a 1040 bp long gene for analysis. The sequence alignment with NJ method established the strain 99.42% similar to *S. atacamensis* C60 (Fig. 7). The 16s rRNA gene sequencing data of the strain is available at the NCBI gene bank (Accession No. MT626067).

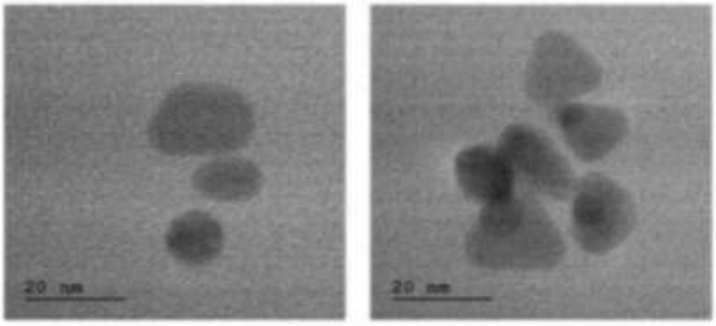

Fig. 5: TEM micrograph of AgNPs showing their shapes

Fig. 6: *Streptomyces* strain AK1, AK2, AK3 and AK4 on the bacterial medium

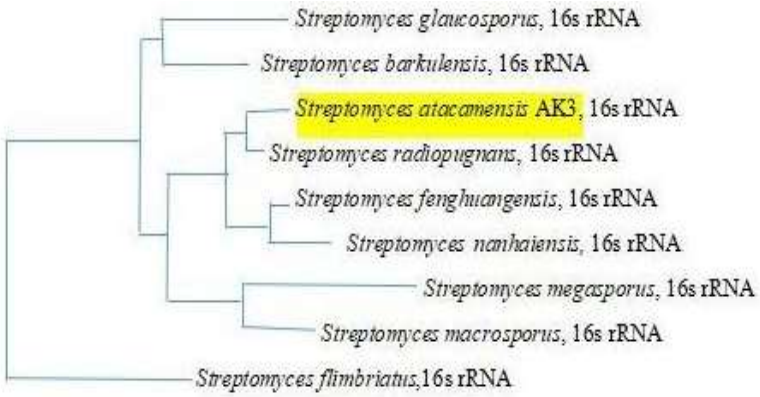

Fig. 7: Phylogenetic tree of *Streptomyces atacamensis* AK3

Comparison between the strain synthesized AgNPs and antibiotics

The strengths of synthesized AgNPs and antibiotics against some elected human pathogens were compared. The synthesized AgNPs produced the desired results. The physical and chemical methods of AgNP synthesis have become expensive and harmful from the environmental point of view. The microorganisms have been used exhaustively for AgNP synthesis in the last decade (Li *et al.*, 2011). The production media for AgNP biosynthesis were optimized and temperature, pH and $AgNO_3$ concentration are considered the three key parameters for optimization (Rose *et al.*, 2019). As compared to the common biomolecules, AgNPs have greater resistance ability against severe production conditions. The actinomycetes are fast emerging source for AgNP synthesis (Abd-Elnaby *et al.*, 2016; Manimaran and Kannabiran, 2017). This is the first report of AgNP synthesis using *S. atacamensis*, isolated from industrially polluted site near Tapti river, for the treatment of some human pathogens. The surface area, shape, and size of AgNPs are optimizable with sulphur amino acids containing media and play a crucial role in the degree of efficiency rendered by AgNPs (Zhang *et al.*, 2016). The efficiency of AgNPs reduces as the thickness of bacterial peptidoglycan increases (Dakal *et al.*, 2016). In current study, *E. coli* and *P. aeruginosa* were found more sensitive to the synthesized silver nanoparticles than *S. aureus* and *B. cereus*. The increasing trend of inhibition zones existed up to an optimum concentration of AgNPs only, then came down at even higher concentrations with an implication that increased level of AgNPs caused agglomeration. On entry into the bacterial cell, AgNPs destroy the chromatin through production of ROs (Butler *et al.*, 2015). In contrast to the previous work, the cell-free filtrate on addition of $AgNO_3$ synthesized AgNPs. Zeta potential is the measurement of repulsion between charged particles and stability of particles with time, and a higher negative value of -14 mV indicates the stable and well dispersed AgNPs. The phylogenetic relationship of the expected results-producing strain was established on

Fig. 8: *In vitro* performance of AgNPs (SNP7, SNP10 and SNP35) against some human pathogens in comparison to various antibiotics A) *S. aureus* as against DO30, TEI30, GM30, SNP10 GM10, E15, MET30 and SNP35; B) *P. aeruginosa* against CX30, C30, GM10, PB300, MI30, SNP7, AMP10 and SNP10; C) *E. coli* against SNP7, SNP10, CX30, CFS30, CL10 and CTX30; D) *B. cereus* against TE10, CB100, PB300, CTX10, S10, NOR5, SNP10 and SNP35. For details of abbreviated antibiotics see Table 1.

Table 1: Selected human pathogen's resistance (R) and sensitivity (S) to various concentrations of synthesized silver nanoparticles and the antibiotics

S. No.	Antibiotics/AgNP & their concentration tested	Zone of inhibition (mm)			
		S. aureus	*P. aeruginosa*	*E. coli*	*B. cereus*
1	Doxycycline 30 µg (DO30)	13	Untreated (UT)	UT	UT
2	Teicoplanin 30 µg (TEI30)	14	UT	UT	UT
3	Gentamicin 30 µg (GM30)	28	UT	UT	UT
4	AgNP 10 µg mL^{-1} (SNP10)	6	22	24	12
5	Gentamicin 10 µg (GM10)	18	6	UT	UT
6	Erythromycin 15 µg (E15)	16	UT	UT	UT
7	Methicillin 30 µg (MET30)	27	UT	UT	UT
8	AgNP 35 µg mL^{-1} (SNP35)	23	UT	UT	17
9	Ampicillin 10 µg (AMP10)	UT	6	UT	UT
10	Cefoxitin 30 µg (CX30)	UT	6	6	UT
11	Chloramphenicol 30 µg (C30)	UT	6	UT	UT
12	Polymyxin 300 µg (PB300)	UT	15	UT	17
13	Minocycline 30 µg (MI30)	UT	12	UT	UT
14	AgNP 7 µg mL^{-1} (SNP7)	UT	6	16	UT
15	Cefoperazone sulbactam 30 µg (CFS30)	UT	UT	26	UT
16	Colistin 10 µg (CL10)	UT	UT	20	UT
17	Cefotaxime 30 µg (CTX30)	UT	UT	14	UT
18	Cefotaxime 10 µg (CTX10)	UT	UT	UT	23
19	Streptomycin 10 µg (S10)	UT	UT	UT	27
20	Norfloxacin 5 µg (NOR5)	UT	UT	UT	27
21	Tetracycline 10 µg (TE10)	UT	UT	UT	30
22	Carbenicillin 100 µg (CB100)	UT	UT	UT	28

the basis of 16s rRNA gene sequencing; the product of this gene, 16s rRNA, is a component of 30S subunit of prokaryotic ribosome and has the conserved and hypervariable sequences for the identification of species (Nayaka *et al*., 2020a). The 16s rRNA gene of strain, 1040 bp long, was discovered as a close relative to *Streptomyces atacamensis* C60, and the strain was named as *Streptomyces atacamensis* AK3. The size and shape analysis of AgNPs were accomplished with TEM, and in turn their effects on human pathogens could be deduced. As AgNPs enter the microbial cell, they initiate a chain of reactions leading to cell death (Nayaka *et al*., 2020b). TEM micrograph elucidated the size of biosynthesized AgNPs as ~20 nm and the shape from spherical to ellipsoid. When pathogens interacted AgNPs, *S. aureus* and *B. cereus* exhibited different behavior from *E. coli* and *P. aeruginosa* and were more efficient in lessening the inhibition zone on the same

concentration of AgNPs. Acceleration of AgNP activity was greater in *S. aureus* than in *B. cereus* as AgNPs at 10 µg mL^{-1} formed a 6 mm zone of inhibition (ZOI) against *S. aureus* and 12 mm ZOI against *B. cereus* but AgNPs at 35 µg mL^{-1} formed 23 mm ZOI against *S. aureus* and only 17 mm ZOI against *B. cereus*; acceleration was greater in *P. aeruginosa* than *E. coli;* however, *E. coli* had greater ZOI than *P. aeruginosa* at 35 µg mL^{-1} AgNPs. The antibacterial activities of antibiotics against particular species were compared with AgNPs' activities experimentally (Table 1). GM30 and MET30 only could make bigger ZOI than 35 µg mL^{-1} AgNPs against *S. aureus*; CFS30 only could produce bigger ZOI than 10 µg mL^{-1} AgNP against *E. coli*; application of 35 µg mL^{-1} AgNPs on *B. cereus* did not give the similar output as they could hardly make 17 ±1 mm ZOI, and the application of 35 AgNPs µg mL^{-1} showed most fruitful results against *P. aeruginosa* on comparison with used antibiotics.

Conclusion: Due to the side effects and decreasing potential of antibiotics, the use of AgNPs in moderate quantities are safer. The toxic effects of AgNPs were greater in Gram-negative bacteria than in Gram-positive bacteria. The use of AgNPs showed increased inhibition activity against *P. aeruginosa*, to appreciable extent against *E. coli* and *S. aureus*. The synergistic activity of AgNPs with other molecules against *B. cereus* needs to be explored. The AgNPs synthesized by using actinomycetes strain AK3 is an eco-unfriendly alternative against multi-drug resistant human pathogens.

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Conflict of interest: The authors declare that there is no conflict of interest.

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