Pathological Communication

On the Efficiency of Silver Nanoparticles Synthesized using *Streptomyces* spp. against Human Pathogens

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ABSTRACT

Silver nanoparticles have changed the scenario of applications in various fields like food, medical, textile, health care, bio-sensing systems, etc. This work is dedicated to find out the Invitro killing ability profiles of silver nanoparticles producedby actinomycetes (*Streptomyces* AK3) against human pathogens and contributes to the enhancing knowledge about the effectiveness of silver nanoparticles for the treatment of human diseases. The better relevant strain of actinomycetes has been examined and sequenced (GenBank MT626067). The soil sample was collected from Tapi river, Surat. The river is polluted with heavy metals and can bear the heavy metal resistant bacteria. *Streptomyces* spp. AK3 strain was isolated with the actinomycetes isolation media and then sub cultured in starch casein broth. The broth was filtered then cell free filtrate was added with $AgNO_3$ and kept in shaking incubator. Intensity of colour change from yellow to brown was measured with Shimadzu UV-Vis 1601 spectrophotometer. Size distribution and Zeta potential of silver nanoparticles were known with Malvern, and TEM analysiswas done to have a detailed account of prepared silver nanoparticles' characteristics. The diffusion method was used for analyses of efficiencies shown by the prepared silver nanoparticles and the antibiotics against various human pathogens. In result, the zones of inhibition were formed with strain synthesized silver nanoparticles as 22 mm, 24 mm, 25 mm and 24 mm for *Salmonella*, Co-agulase negative *Staphylococcus, Klebsiella*, and *Enterococcus* respectively. In conclusion, silver nanoparticles synthesized by *Streptomyces* spp. AK3 have been found a better alternative to antibiotics in terms of their efficiencies against human pathogens and side effects.

KEY WORDS: SILVER NANOPARTICLES, STREPTOMYCES SPP. AK3, TEM ANALYSIS, ZETA POTENTIAL ANALYSIS, ZONES OF INHIBITION.

INTRODUCTION

Use of silver dates back to 69 B.C.E.The use of silver has been happening for a long time and was reported from Before Christ to mentioned year (Hill et al.,1939; Alexander 2009). However chemical reduction has been the major technique, production of nanoparticles is possible with various life forms (Mohanpuria et al. 2008; Kholoud et al.,2010). NADH co-factor of nitrate reductase plays a role in the reduction process (Husseiny et al., 2007). AgNO3 concentration, pH, and reaction temperature are decisive in obtaining the silver nanoparticles of desired size (Gurunathan et al., 2009). Bio-synthesized nanoparticles should not only be effective against pathogens but also be nontoxic to the patient and this property is possessed by silver nanoparticles (Song et al., 2009). *Pseudomonas stutzeri* can produce AgNPs in its periplasmic space, whereas *Verticillium* has the ability to produce them on the surface of mycelia as its surface contains nitrate reductase, inturn, this enzyme can bind silver ions with its negatively charged groups and gives the output as reduced silver ions (Klaus et al., 1999; Senapati et al., 2004; Krishnamurthy et al., 2010). Silver nanoparticles can accumulate inside the bacterial cells and kill them, in turn; these killed cells can act as reservoirs of AgNPs and release silver cations slowly in the environment of pathogenic bacteria(Mohamed et al., 2020).

Microbes arthrospira commonly usedas dietary supplements have also been found to have the ability with some biochemical alterations during the process to produce nanoparticles (Cepoi et al., 2015). AgNPs are

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potential agents for treating viruses, cancers of breast, liver, skin and blood (Wei et al., 2015). High doses of antibiotics have side effects, if silver nanoparticles added to antibiotics cause the reduction of antibiotics' dose with the same or increased efficiency as compared to the previous high dose of antibiotics' alone, harmful effects of high doses can be minimized (Singh et al., 2015). Not only the enzymes like nitrate reductase but also the substances having carboxyl, amino, or hydroxyl groups have been noticed to reduce and cap the ions (Abdel-Raouf et al., 2017). Due totheir smaller size, Silver nanoparticles can cross the membranes of pathogens and cause the production of free radicals (Siddiqi et al., 2018). Since silver nanoparticles in high doses can cause the leakage of hemoglobin from erythrocytes hence exhaustive examination of the In Vivo effects must be followed (Hamouda et al., 2019). This work presents a comparative account of lethal effects of the strain synthesized AgNPs and antibiotics against human pathogens.

MATERIAL AND METHODS

The soil sample was collected at the bank of the heavily polluted Tapi River, Surat (Gujarat). The sample was sprinkled on actinomycetes isolation agar media, four strains naming AK1-AK4 were isolated, transferred to starch casein broth in Erlenmeyer flasks and grown at 120 rpm, and 30 ^{0}C for 72 hours. Preliminary identification of these strains was done with citrate utilization, methyl red, indole, urea hydrolysis, and nitrate reduction tests. Broths of flasks; AK1-AK4 in two sets, were filtered through bacteriological filter paper, and two types of filtrates; cell filtrate and cell-free filtrate, were obtained. One set was left as control.10ml of each filtrate; AK1-AK4, from one set was made up to 48.5 ml with phosphate buffer of 0.1M and then 1ml of 1mM $AgNO_3$ each and 0.5 ml of 1mM of methionine and cysteine each were added to these filtrates. The same was followed with a set of controlsexcept the addition of $AgNO_3$. Flasks of these filtrates were kept in a shaking incubator at 120 rpm and 30 ^{0}C for 72 hours and any colour change to brown; an indication of silver nanoparticles' synthesis, was noticed (Sukanyaet et al., 2013).

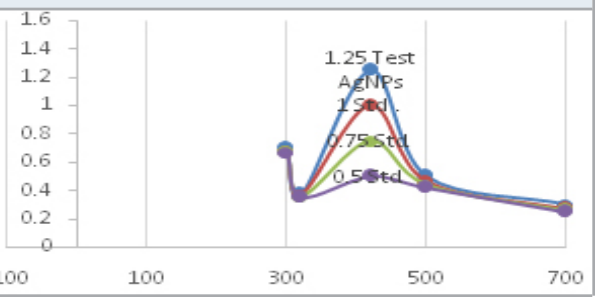
This 50 ml was evaporated to have 5 ml as a semi-final volume and a further 10 times dilution of this semi-final volume for readings with spectrophotometer was done leaving one set as undiluted semi-final volume for the highly resistant bacteria. Separation of antibiotics was done with a dialysis membrane and phosphate buffer of 6.5pH as silver nanoparticles are conjugated with bio-polymers and cannot pass through the pores of the membrane. Spectrophotometric analysis was done at 420 nm. On finding themaximum absorbance given by the product of strain AK3, the candidate was got sequenced for 16S rRNA with ABI 3130 genetic analyzer at Biokart Ltd. Bangaluru and zeta potential and size distribution of silver nanoparticles synthesized by *Streptomyces* spp. AK3(Gen Bank MT626067) were measured and checked for the degree of agglomeration at PERD, Ahmedabad. Strain AK3 was analyzed for shape and size with TEM at Sprint testing, Powai, Mumbai after some improvement in procedure with the addition of amines to prevent the agglomeration. *Enterococcus foecails*, Coagulase negative *Staphylococci* spp., *Klebsiella pneumoniae, Staphylococcus aureus* and *Salmonella* spp. were selected for testing the silver nanoparticles' activity. These microbes were grown on Petri plates with different concentrations of synthesized silver nanoparticles; given in table-1, and antibiotics in the wells.

RESULTS AND DISCUSSION

Morphological and physiological analyses: Colonies were wrinkled, raised, opaque and, white andstrainswere visualized as long positive rods. Strains could grow in the range between 26 ^{0}C to 40 ^{0}C and the best growth was observed at 32 ^{0}C. Production of AgNPs was checked at pH ranging from 4.5 to 8.5. The optimum pH for production was found as 6.5. Slightly acidic pH may impart a stronger binding ability to capping agents, in turn; smaller sized nanoparticles are formed (Gan et al., 2012). 2% NaCl was the optimum concentration at which strain can grow the best as opposed to halophytic actinomycetes which grow at 15%-25% salt concentration (Jiang et al., 2016).

Biochemical tests: The strain showed positive results for citrate utilization, methyl red, urea hydrolysis and nitrate reduction, and negative for indole.

Figure 1: Absorbance on Y axis at wavelengths in nm



UV-Visible spectroscopic analysis of synthesized nanoparticles:

Figure of absorbance versus wavelength was developed with Shimadzu UV-1601 spectrophotometer. An inference can be made for the synthesis of silver nanoparticles with the colour change to light brown. The test sample was diluted ten times and readings were noted with standards of 40 µg/ml, 30 µg/ml and 20 µg/ml as 1.25, 1, 0.75, and 0.5 absorbances respectively from the test sample to the least concentrated standard. The test sample was diluted later also as per the matches with the most effective antibiotics available in terms of efficiencies against human pathogens, whose data are described in this document. Surface plasma resonance of AgNPs synthesized by *Streptomyces* spp. ranges from 440-450 nm (Eid et al., 2020).

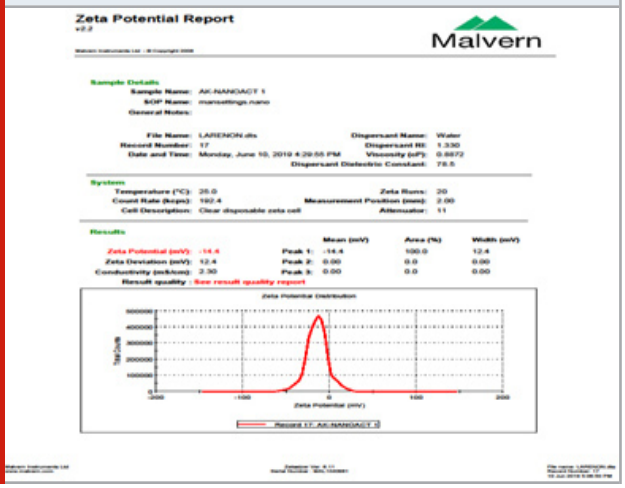
16S rRNA sequencing of strain AK3: Strain AK3 (GenBank MT626067) was found as *Streptomyces* spp. closer to *Streptomyces atacamensis*. A few species of *Streptomyces* are known to have the ability to convert silver ions to silver nanoparticles. Primers such as 27F and 1492R can be used for the amplification of *Streptomyces* spp.(Khadyat et al., 2020).

Zeta potential silver nanoparticles: Zeta potential was-14.4 which shows agglomeration-free silver nanoparticles to the most extent.AgNPs can be sterically stabilized by natural surfactants (Chartarrayawadee et al., 2020).

Features of produced silver nanoparticles known with TEM: TEM produced the images with 20 nm-sized silver nanoparticles having various morphological characteristics such as spherical, prism-shaped etc. A role against destabilization of silver nanoparticles is played by sulphur-containing amino acids, thiols amines, alcohols to some extent as different results are seen for control and stabilizers added silver nanoparticles synthesizing cell-free filtrate (Iravani et al. 2014). Properties can be manipulated by changing the concentration of capping agents, however the efficiency of silver nanoparticles against pathogens is affected with increased concentration adversely. Antimicrobial activity of silver nanoparticles depends on the shapes also. Dispersity of AgNPs can be modulated by the flow rate in a continuous flow tubular microreactor (Dawadi et al., 2021).

Comparative antibacterial activities of synthesized silver nanoparticles and antibiotics: Well diffusion method was performed and data of affected microbes by different antibiotics and SNPs with zones of inhibition have been represented here as Petri plates and the table is shown as well below them.

Figure 2: Synthesized silver nanoparticles' Zeta potential measured by Zeta sizer



24 (a)-*Enterococcus foecalis* with SNPs, LE5, and HIG-120
24 (b)-*Enterococcus Foecalis* with LZ30, AMP10, TEI30, IPM10, VA30, and CIP5
346-Co-agulase negative *Staphylococci* spp. with SNPs, LZ30, TEI30, VA30, DO30, and TE30
1019-*Klebsiella pneumoniae* with SNPs, CPT30, CFM5, C30, TOB10, TFC15
13-*Staphylococcus aureus* with SNPs, TE30, LE5, CIP5, TEI30, DO30, and MI30
341-*Salmonella* spp. with SNPs, PB300, CL10, CFS30, TE10, CFM5, AZM15, and PF5

Figure 3: Images of silver nanoparticles with TEM

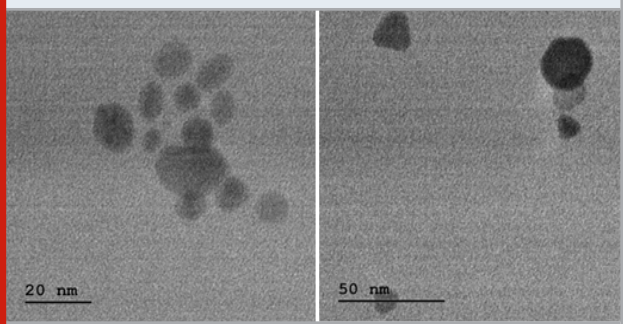
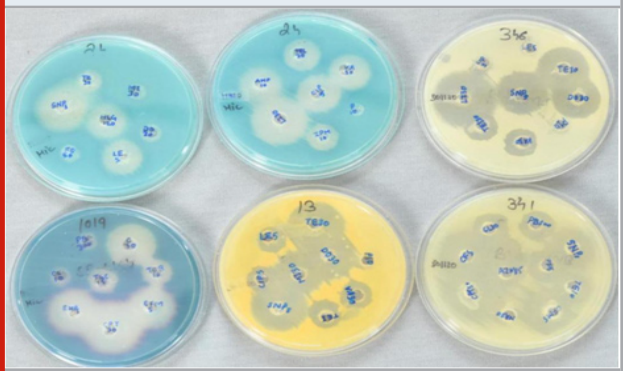


Figure 4: Inhibition zones of SNPs andantibiotics



1. *Staphylococcus aureus*, *Salmonella typhi, Pseudomonas aeruginosa, E.coli*, Co-agulase negative *Staphylococci, Enterococcus foecalis*, and *klebsiella pneumoniae* were selected as test pathogens. 2. *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa, E.coli,* and Co-agulase negative *Staphylococci* were tested on Mueller Hinton agarwhereas *Enterococcus foecalis* and *klebsiella pneumoniae* were tested on Hi-chrome media. Plates were placed in the incubator for 20 hours at 370 C. 3. *Staphylococcus aureus* was highly sensitive to tetracycline 30, almost resistant to penicillin-G 10, and sensitive to silver nanoparticles (10 µg/ml) with a 25 mm zone of inhibition. Addition of silver nanoparticles to the tetracycline, produced in the current work, could have enhanced lethal effect on *S. aureus* (Hussein et al. 2019). Effect of silver nanoparticles on the closer species *Staphylococcus epidermidis* has been reported (Swolana et al., 2020). 4. Test organism *Salmonella* was the most sensitive to cefapazone sulbactam 30, the least sensitive to polymyxin B300 and sensitive to silver nanoparticles with a 22 mm inhibition zone.

Table 1. Effect of *Streptomyces*'s strain AK3 synthesized Silver Nanoparticles (SNPs) on test organisms

Test Organism	Antibiotics-µg/ SNPs	Zone of inhibition (mm)	Plate No.
Staphylococcus aureus	Tetracycline 30 (TE30)	28	13
	SNPs	25	
	Levofloxacin 5 (LE5)	12	
	Ciprofloxacin 5 (CIP5)	9	
	Penicillin-G 10 (P10)	7	
	Vancomycin 30 (VA30)	15	
	Teicoplanin 30 (TEI 30)	14	
	Doxicycline 30 (DO 30)	13	
	Minocycline 30 (MI 30)	23	
	Clindamycin 2 (CD2)	18	
Salmonella spp.	SNPs	22	341
	Polymyxin B 300 (PB300)	15	
	Colistin 10 (CL10)	17	
	Cefopazone sulbactum30 (CFS30)	24	
	Tetracline 10 (TE10)	17	
	Cefixime 5 (CFM5)	25	
	Azithromycin 15 (AZM15)	16	
	Pefloxacin 5 (PF5)	18	
Coagulase negative *Staphylococci*	Linezolid 30 (LZ30)	26	346
	SNPs	24	
	Teicoplanin 30 (TEI30)	15	
	Vancomycin 30 (VA30)	16	
	Doxycycline 30 (DO30)	20	
	Tetracycline 30 (TE30)	23	
Enterococcus faecalis	Linezolid 30 (LZ30)	25	24 (a)
	Ampicillin10 (AMP10)	19	
	Teicoplanin 30 (TEI30)	17	
	Imipenam 10 (IPM10)	15	
	Vancomycin 30 (VA30)	19	
	Ciprofloxacin 5 (CIP)	20	
Enterococcus faecalis	SNPs	24	24 (b)
	Levofloxacin 5 (LE5)	18	
	High level gentamycin	20	
	120 (HIG120)		
Klebsiella pneumoniae	SNPs	25	1019
	Ceftaroline 30 (CPT30)	27	
	Cefixime 5 (CFM5)	22	
	Chloramphenicol 30 (C30)	22	
	Tobramycin 10 (TOB10)	15	
	Tigecycline 15 (TGC15)	13	

The sensitivity of *Salmonella* to silver nanoparticles (10 µg/ml)has been documented as the "sensitivity depends on the strain, and dose" (Losasso et al. 2014; Petrus et al. 2011).When *Salmonella braenderup* was exposed to AgNPs, membranes of the microbes were ruptured (Diego et al., 2020).

5. Co-agulase negative *Staphylococci* can cause infections when they reach the blood stream. An iinhibition zone of 25 mm was produced by prepared AgNPs. Linezolid was the only antibiotics with a bigger zone of 26 mm than that of AgNPs.

6. Treatment of *Enterococcus foecalis* is difficult when it reaches the urinary tract (Kau et al., 2005). Linezolid 30 is effective against this species. In the current study, Linezolid 30 produced a 25 mm of zone of inhibition. Prepared AgNPs (30µg/ml) had almost the same effect

as that of Linezolid with a 24 mm zone of inhibition. Traditional approaches are less effective in treatment of *Enterococcus foecalis* how ever; silver nanoparticles could be a potent solution to treat them (Sadony et al., 2019).

7. *Klebsiella pneumoniae* produces extended spectrum β-lactamase, in turn, this compound can breakdown many medicines. Carpabenam has been found effective against ESBL-producing *Klebsiella pneumonia* (Patersonet al. 2004). However, *Klebsiella pneumoniae* shows greater resistance against considerably safe AgNPs but at higher doses as 100µg/ml, AgNPs formed a 25 mm zone of inhibition. *Klebsiella pneumoniae* has been resistant to ampicillin and AgNPs were tested against this species to get effective results (Hamida et al., 2020).

CONCLUSION

Streptomyces Spp. AK3 has been found to synthesize expected quality silver nanoparticles. Synthesized silver nanoparticles with this strain are much closer to the most effective antibiotics against human pathogens in terms of the formation of zones of inhibition as represented in table-1. The strain synthesized silver nanoparticles should be preferred to antibiotics for the sake of having lesser side effects, being cost-effective, and working as a tool against multi-drug resistant bacteria.

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