

Extracellular Synthesis, Characterization and Antibacterial Activity of Silver Nanoparticles by a Potent Isolate *Streptomyces* sp. DW102

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ABSTRACT

The global problem of antibiotic resistance and the lack of novel antibiotics in the markets is soon to be a health care crisis. Silver nanoparticles are recently considered as a tool of the medical field, especially in therapeutic applications. *Streptomyces* sp. is a natural antibiotic producer and could be utilized for the production of nanoparticles enhanced antibiotics. In the present investigation, 17 actinomycetes were isolated from soil samples collected from various regions in Dawadmi governorate, Riyadh province. Isolate DW102 was identified as potent isolate based on the ability to synthesize silver nanoparticles along with broad spectrum antibacterial activity when compared with other isolates. The potent isolate DW102 was identified as *Streptomyces* sp. Biosynthesized AgNPs were characterized by UV-Vis Spectroscopy, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX). Biothesized Ag-NPs showed antimicrobial activity against Multi Drug Resistant (MDR) strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumonia*. The combination of antibiotics with AgNPs showed an overall percentage increase in average fold-area of zone of inhibition (ZOI) in this study.

Key words: *Streptomyces* sp., Silver nanoparticles, Antibacterial activity, Multi Drug Resistant, Zone of inhibition.

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INTRODUCTION

Nanotechnology is a revolutionary area of research which includes creation, exploitation and synthesis of different nanomaterials smaller than 1 mm. "Nano" is a Greek word which means dwarf or very little indicating one billionth of a meter or 10^{-9} .^[1] Today, nanobiotechnology is preferred for the synthesis of metal nanoparticles due to the availability of many production methods and it has simple, cost-effective and non-toxic procedures. An understanding of the biochemical

and molecular mechanisms of the reaction is required during biological synthesis for obtaining better results.^[2] Synthesis of silver, gold and platinum nanoparticles can be achieved with the help of microorganisms and plant extract.^[3] Recently, silver nanoparticles (AgNPs) have been synthesized using several bacteria like *Bacillus methylotrophicus*, *Vibrio alginolyticus*, *Enterobacter aerogenes*,^[4,5] Actinomycetes like *Streptomyces rochei*, *Gordonia amicalis*,^[6,7] fungi like *Penicillium nalgiovense*, *Macrophomina phaseolina*, *Trichoderma reesei*,^[8,9] and plant extracts from *Sida cordifolia*, *Clerodendrum phlomidis*, *Ficus talboti*.^[10,11] Today, the increased resistance of many pathogenic microorganisms to several commonly used antibiotics is a serious threat to the healthcare system. Scientists are looking for an alternative to current chemotherapeutic options. Silver has been used as antibacterial agent for the treatment of pathogenic bacteria, incorporated in wound dressing and as a coating agent for medical devices and

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implants.^[12] Outside of the clinical setting, the antibacterial properties of silver is being utilized in many products, for example textile and packaging industries, water purifiers, deodorants, paint, home appliances and children's toys.^[13,14]

Synthesis of AgNPs biologically is inexpensive, reproducible and environmental friendly compared to chemical synthesis routes.^[6,13,15] The Silver nitrate solution is frequently utilized for Ag-NPs biological synthesis from microorganisms and produce AgNPs that exhibit useful properties such as being hydrophilic, stable, with large surface area.^[16,17] Actinomycetes are Gram-positive bacteria known for their abundance in soil and for their substantial metabolic capability.^[18] They are considered as biotechnologically important since they have the capability to produce many useful secondary metabolites.^[19] The *Streptomyces* genus is the largest within *Actinobacteria*, with over 900 species described. It is estimated that *Streptomyces* as a genus produces >100,000 secondary metabolites and they are widely believed to be the top natural antibiotics producers.^[20,21] Several research groups has shown that *Streptomyces* sp. can synthesise AgNPs that have antimicrobial properties.^[6,7,22,23] Studies have reported that when AgNPs used in combination with antibiotics this could increase their activity against multi drug resistant bacteria.^[24,25]

In our study we aimed to (i) isolate actinomycetes from different soil samples of Dawadmi region, Saudi Arabia and to screen for potential isolates of actinomycetes that can synthesize silver nanoparticles for antibacterial activity, (ii) Characterization of biosynthesized AgNPs by UV-visible absorption spectrophotometer, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX) and (iii) Evaluation of synergistic effect of silver nanoparticles with different antibiotics.

MATERIALS AND METHODS

Chemicals and media

All chemicals and media were obtained from Oxoid Ltd (Basingstoke, UK) and were prepared according to the manufacturer's guidelines.

Collection of samples and bacterial strains

A total of ($n=30$) samples were collected from various regions in Dawadmi governorate, Riyadh province (latitude is 24.75387 and longitude is 46.773686) during May 2018. Soil samples were collected from 5 to 25 cm depth in sterile polythene covers with sterile spatula, labeled appropriately and transported to the Microbiology Laboratory of College of Applied Medical Sciences, Dawadmi, Saudi Arabia and stored

in the refrigerator at 4°C until further use. Strains of multi drug resistant bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumonia* were obtained from the bacterial collection of the faculty of Clinical Laboratory Sciences at the College of Applied Medical Sciences(Male), Dawadmi, Shaqra University, K.S.A. For MDR used in this study, unless otherwise mentioned, all isolates were cultured in Muller Hinton Agar at 37°C. All strains were kept in Nutrient broth with 20% glycerol at -80°C.

Isolation of actinomycetes from soil samples

The soil samples were air dried to reduce the bacterial contamination. Isolation and enumeration of actinomycetes were performed by serial dilution and spread plate technique.^[15] From each soil sample 1 g is mixed with 9 ml of sterile double-distilled water and serially diluted up to 10^{-7} dilutions and 0.1 ml of the sample were spread on the Starch Casein Agar (SCA) plates in triplicates. The antibiotics flucanazole 20 mg/L and nalidixic acid 100 mg/L were added to SCA to reduce fungal and bacterial growth respectively and the Petri dishes were incubated at 30°C for 7-10 days. After incubation the actinomycetes colonies were selected based on the colony morphology and purified by quadrant streaking technique on ISP2 -Yeast Malt Extract Agar and maintained for further studies.^[23]

Screening and Extracellular Synthesis of Nanoparticles

Selected isolates were inoculated in Starch Casein Broth (SCB) at pH 7 and incubated at 30°C in shaking condition for 5 days. After incubation the biomass were filtered, followed by extensive washing with sterile double distilled water. Then the biomass was mixed with 100ml of sterile double distilled water in an Erlenmeyer flask, incubated for 24 hr and filtered.^[26] Then the cell free filtrate is used for the extracellular synthesis of AgNPs by mixing 50ml of cell-free filtrate in 50ml of 1mM AgNO₃ solution and incubating inside a rotary shaker at 200rpm away from light at 37°C for 5 days for synthesis. Flasks were screened for visible colour changes due to Ag-NPs production. A flask containing 50ml of 1mM AgNO₃ solution and uninoculated media is also kept for incubation as a control. After the development of the yellowish-brown color in inoculated flasks, characterization of AgNPs were performed by using UV-Vis Spectrophotometer, Scanning electron microscopy (SEM) and Energy Dispersive X-ray analysis (EDX).

Characterization of silver nanoparticles

UV-vis Spectroscopy is used for confirmation of reduction of silver ions. The absorbance was measured

using UV-visible spectrophotometer (UV-2550), at wavelengths ranging from 200 to 500 nm. SEM study was done to observe the surface morphology of biosynthesized AgNPs. SEM was done by casting a drop of sample on a carbon coated copper grid, which was carried out at King Abdullah Institute for Nanotechnology, King Saud University (KAIN) using SEM (JOEL JSM-7600F). EDX analysis was done to identify the elements present in the nanoparticles and was carried out at KAIN.

Primary screening for antimicrobial activity of biosynthesized Ag-NPs

The antibacterial activity of biosynthesized AgNPs was measured by well diffusion method.^[6] Agar wells of 6mm diameter were made on Muller Hinton Agar (MHA) plates that were swabbed with a 24hr broth cultures of MDR strains. Amounts of 30 μ l of 30 μ g/ml biosynthesized AgNPs were added to the wells in each plate containing MDR strains and incubated at 37°C for 24 hr. The tests were repeated in triplicate and the mean and standard deviation of the diameter of the inhibitory zone was measured in millimetre and recorded.

Synergistic activity

To determine the synergistic effects by disc diffusion method,^[27] MHA plates were inoculated with 24 hr both culture of MDR strains by spread plate method. Standard antibiotic discs such as Ampicillin, Streptomycin and Chloramphenicol were added with 30 μ l of AgNPs. Plates were allowed to set and then standard antibiotic discs (6mm in diameter) such as Ampicillin, Streptomycin and Chloramphenicol with and without biosynthesized AgNPs were placed on the MHA plates using sterile forceps and incubated at 37°C for 24 hr. The tests were repeated in triplicate and the mean and standard deviation of the diameter of inhibitory zone (ZOI) was measured in millimeter and recorded. The increase in fold area was determined by the formula, Fold increase (%) = $(b^2 - a^2) / a^2$, (where a – ZOI for antibiotic alone; b – ZOI for antibiotic along with AgNPs (Birla et al. 2009).

RESULTS

In our pilot scale screening, out of 30 samples collected from various sites in Dawadmi governorate, a total of 17(57%) actinomycetes were isolated on starch casein agar (Figure 1) and designated as DW101 to DW117 (Table 1). The actinomycetes isolates were identified up to the genera level based on colony morphology, color of mycelium and light microscopy as a prerequisite (Figure 2). Actinomycetes isolates were purified by quadrant streaking technique on ISP2-Yeast Malt Extract Agar (Figure 3). The results of the present investigation

show that all the actinomycetes showed gram-positive, branched, filamentous, fragmented, separated, or long mycelium arrangement. Further, the actinomycetes isolates were tested for ability to synthesize silver nanoparticles and antibacterial activity. Among 17 actinomycetes isolate DW 102 was selected for further experiments based on the ability to synthesize silver nanoparticles as well as its antibacterial activity. The morphological characteristics of DW102 strongly suggested that the isolate belong to *Streptomyces* species. The biosynthesis of silver nanoparticles was observed by colour change from yellow to brown in cell-free extract after 96 h when it was incubated with 1mM AgNO₃ (Figure 4). In this study, the control which consists of un-inoculated media which was incubated with 1mM AgNO₃ under the same conditions did not show any colour change.

Biosynthesis of AgNPs was further confirmed by light absorption patterns of the supernatant solution after monitoring at regular time intervals at a range of 200nm-500nm by using UV-visible spectrophotometer. UV-visible absorption spectra of AgNPs are shown in (Figure 5).

Biosynthesized AgNPs from cell-free filtrate of actinomycetes showed an intense peak at 430 nm at OD 0.445 for the sample DW102 which indicated the presence of AgNPs. SEM analysis showed that the AgNPs are polydispersed having a roughly spherical shape. The sizes of the nanoparticles are in the range of 75-85 nm and some of them are agglomerated. (Figure 6). The EDX analysis obtained peak band in the range of 3-4 KeV which indicates the presence of AgNPs silver as the major constituent element (Figure 7).

In our study, the AgNPs produced by the filtrate of the isolate DW102 displayed antimicrobial activity against various MDR bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *E. coli* and *Klebsiella pneumonia* (Table 2 and Figures 8a-8d). The mean and standard deviation values of the diameter of inhibition zone for *Staphylococcus aureus* was the maximum (22.1 ± 0.3 mm) followed by, *Klebsiella pneumonia* (19.1 ± 0.4 mm), *E. coli* (17.0 ± 0.1 mm) and *Enterococcus faecalis* (15.2 ± 0.2 mm). In our study, we found that the combination of standard antibiotics with biosynthesized AgNPs has shown more effective antibacterial activity against different MDR strains used. The combination of antibiotics with biosynthesized AgNPs showed an overall percentage increase in average fold-area of zone of inhibition (ZOI) in the range of 0.10-12.40 against four MDR strains used (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Klebsiella pneumonia*). The percentage increase in the average fold area of ZOI for ampicillin + AgNPs was in the range of 0.10% -12.40%, for

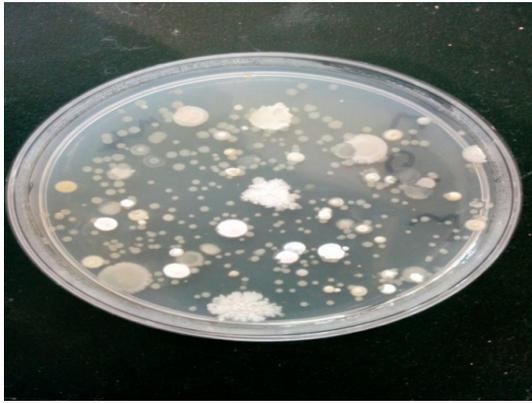


Figure 1: Actinomycetes colonies on SCA.

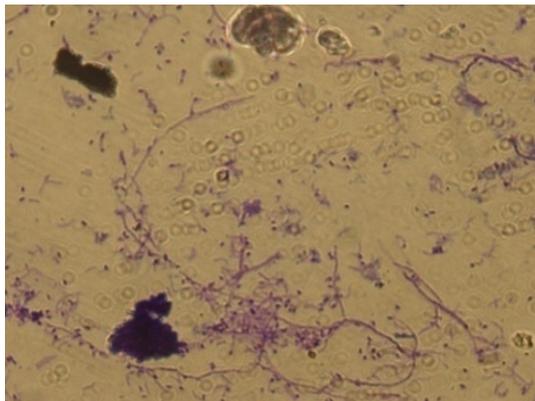


Figure 2: Light microscopic view of actinomycetes.



Figure 3: ISP2-White color aerial mycelium respectively.



Figure 4: Biosynthesis of AgNPs.

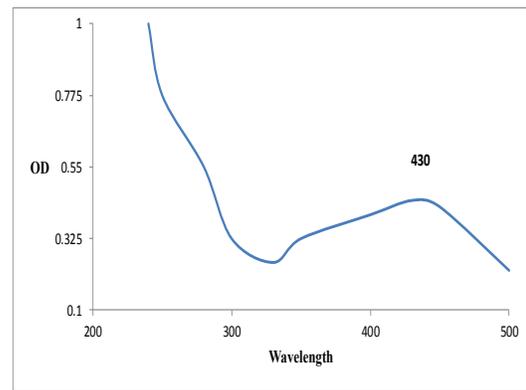


Figure 5: UV spectroscopy of biosynthesized AgNPs showing peak at 430 nm.

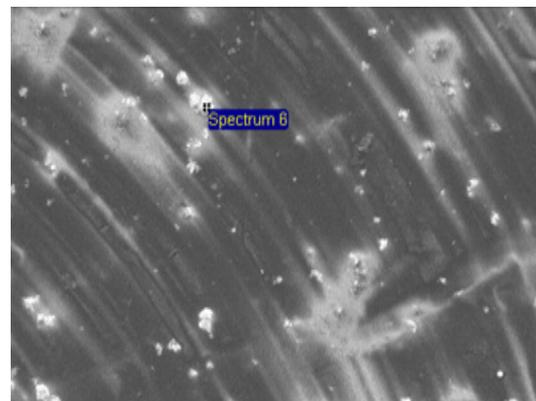


Figure 6: Scanning electron microscopy showing roughly spherical shaped AgNPs.

streptomycin + AgNPs it was 0.23 % -0.63% and for chloramphenicol + AgNPs it was 1.25% -7.03% against the MDR strains used. Typically, highest ZOIs were observed for *Staphylococcus aureus* and *E. coli* with chloramphenicol + AgNPs (24mm each) and minimum ZOI was observed for *Enterococcus faecalis* with chloramphenicol + AgNPs (14mm). The MDR strains were found to be susceptible in the range of 15-22mm for ampicillin + AgNPs combination. Streptomycin + AgNPs combination found to be have bactericidal activity in the range

of 19-23mm. Chloramphenicol + AgNPs combination found to be have bactericidal activity in the range of 14-24mm. When used without AgNPs the microbes, *Staphylococcus aureus*, *E. coli* and *Klebsiella pneumonia* were resistant against ampicillin. Similarly, *Enterococcus faecalis* and *Klebsiella pneumonia* were resistant against Chloramphenicol when used without AgNPs. Overall, antibacterial efficacy of all the three antibiotics impregnated with AgNPs was in the range of 14-24mm against all the four

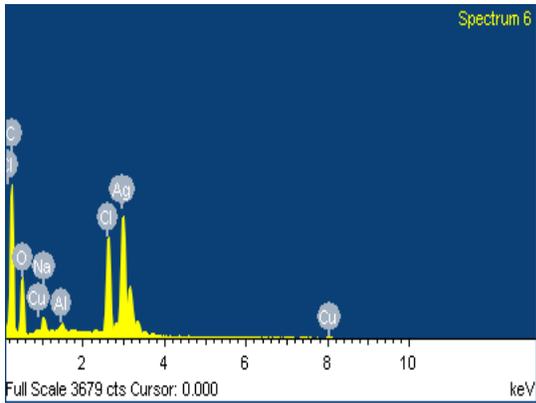


Figure 7: EDX Spectroscopy showing peak in the range of 3-4KeV.

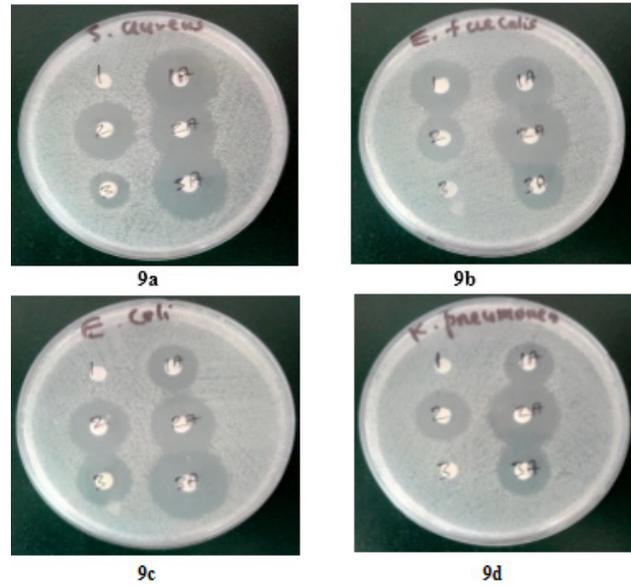


Figure 9: Synergistic effect of different antibiotics with and without AgNPs against MDR pathogens.

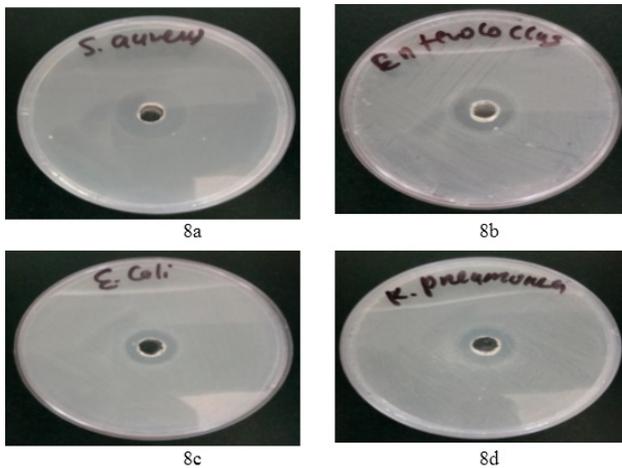


Figure 8a-8d: Showing Zone of Inhibitions produced by AgNPs against different MDR pathogen.

microbes tested. The representatives ZOI are shown in Table 3 and Figures 9a-9d.

DISCUSSION

Actinomycetes are filamentous free living bacteria which are responsible for the production of various natural bioactive metabolites especially antibiotics.^[24] In our study, a total of 17(57%) actinomycetes were isolated

on starch casein agar from 30 different soil samples collected from various sites in Dawadmi governorate. In a similar study Al-Garni *et al.*^[28] isolated 81 actinomycetes from soil samples of Jeddah and Madinah, KSA. In another study 69 actinomycetes isolates were obtained from Al-Khurmah governorate, KSA by Atta and Yassen.^[29] Also Ara *et al.*^[30] isolated 105 strains of actinomycetes from soil samples of Riyadh, KSA. Muharram *et al.*^[31] isolated 33 isolates of actinomycetes from soil samples of Al-Kharj, KSA. In our study the morphological properties showed that all the actinomycetes isolates were gram-positive, branched, filamentous, fragmented, separated, or long mycelium arrangement which falls in the findings of Sheik *et al.*^[32] Sukanya *et al.*^[33] and Sowani *et al.*^[7] Pridham and Tresner^[34] also considered colony morphology, color of mycelium and light microscopic study as important tool for identification of actinomycetes in their study.

Nanotechnology have promising applications in diagnostics since it can modify and convert metals such as

Table 1: Actinomycetes isolates from soil samples of Ad-Dawadmi region.

Collection sites	No. of samples	Type of soil	No. of isolates	Isolate Number
Site 1	7	Rhizosphere soil	6	DW101,DW104,DW105, DW106,DW116, DW117
Site 2	5	Rhizosphere soil	3	DW102,DW108, DW109
Site 3	6	House waste disposal area	6	DW103,DW107,DW110, DW113,DW114,W115
Site 4	5	Sand	0	0
Site 5	7	Cattle breeding area	2	DW111, DW112

Table 2: Antimicrobial activity of biosynthesized silver nanoparticles.

S.no	Microorganism	Zone of inhibition(mm)
1	<i>Staphylococcus aureus</i>	22.1 ± 0.3
2	<i>Enterococcus faecalis</i>	15.2 ± 0.2
3	<i>E. coli</i>	17.0 ± 0.1
4	<i>Klebsiella pneumonia</i>	19.1 ± 0.4

Note: The values are Mean ± SD of triplicates

Table 3: Synergistic effect of different antibiotics with and without biosynthesized AgNPs against pathogens.

S. no.	Micro-organisms	Antibiotics (Zone of inhibition in mm)									
		Ampicillin			F.I.%	Streptomycin		F.I.%	Chloramphenicol		F.I.%
		Ab	Ab+ AgNPs	Ab		Ab+ AgNPs	Ab		Ab+ AgNPs		
1	<i>Staphylococcus aureus</i>	0	22	12.4	18	20	0.23	10	24	4.76	
2	<i>Enterococcus faecalis</i>	20	21	0.10	19	22	0.34	0	14	4.44	
3	<i>E.coli</i>	0	15	5.25	17	19	0.25	16	24	1.25	
4	<i>Klebsiella pneumonia</i>	0	16	6.10	18	23	0.63	0	17	7.03	

Note: In the absence of inhibition zones, the disc diameter (6 mm) were used to calculate the fold increase

silver into nanomaterials which can act as carriers for antibiotics for the treatment of various diseases. In our study silver nanoparticles were biosynthesized using *Streptomyces* DW 102 isolate and colour change from yellow to brown was observed in cell-free extract. The change in colour observed while incubation may be due to the Surface Plasmon Resonance (SPR) effect and due to reduction of Ag ions especially by the enzyme reductase and electron shuttle quinines Kannan *et al.*^[35] In our study an intense peak was observed at 430 nm for the sample DW102 which indicated the presence of AgNPs. In a similar study Durán *et al.*^[36] obtained peak at the range of 420 to 450 nm which indicated the presence of AgNPs. The sizes of the nanoparticles observed in our study which were in the range of 75-85 nm was in agreement with the results of Kumar *et al.*^[26] In our study, the EDX analysis showed peak band in the range of 3-4 KeV which indicates the presence of AgNPs silver as the major constituent element. The reason for other peaks for elements such as Cl, Al, Cu, Na, O and C may be due to emissions from proteins or enzymes present in culture free supernatant. Similar additional peaks were also seen by study conducted by Singh *et al.*^[37] The antimicrobial activity shown by the AgNPs produced by isolate DW102 may be due to the penetration

of AgNPs into the bacteria, damaging the cell membrane and release of cell contents, hence the difference of zones of inhibition between Gram-positives and Gram-negatives. The ability of AgNPs to inhibit MDR strains were also studied by previous work of Sowani *et al.*^[7] and Rajeshkumar *et al.*^[5] This shows that silver nanoparticles synthesized from *Streptomyces* species have effective antibacterial properties against MDR pathogens. The combination of biosynthesized AgNPs with the standard antibiotics is an emerging practice for improvement of antimicrobial potential. In our study the combination of antibiotics with biosynthesized AgNPs showed an overall percentage increase in average fold-area of Zone of Inhibition (ZOI) against four MDR strains used. In a study conducted by Jyoti *et al.*^[27] found that organisms such as *S. epidermidis*, *E. coli*, *Klebsiella pneumonia* and *B. Subtilis* were found to be inhibited when tested with AgNPs in combination with antibiotics such as Vancomycin, Cefetaxime, Ampicillin, Kanamycin, which otherwise showed a resistant pattern in the absence of AgNPs. In a similar study conducted by Naqvi *et al.*^[25] found that the antibiotics such as ciprofloxacin, imipenem and gentamycin, when used also with nanoparticles can be efficiently inhibit microorganisms such as *E. coli*, *K. pneumonia*, *S. aureus* and *Bacillus* sp.

CONCLUSION

In this study, we have shown that environmentally isolated *Streptomyces* sp. can be selected for the AgNPs biosynthesis in an eco-friendly process with minimal effort. Biosynthesized AgNPs were confirmed by spectroscopic characterization of UV-visible, FT-IR and SEM. This study has shown that *Streptomyces* sp. DW102 produced can synthesize AgNPs which have antibacterial activity. The produced nanoparticles were stable and have medium size. The combination of AgNPs produced by *Streptomyces* sp. DW102 with clinically used antibiotics showed enhanced antibacterial activity against pathogenic MDR strains. Further analysis is needed to identify the mode of action of the biosynthesized AgNPs, the resistance frequency and cytotoxicity.

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CONFLICT OF INTEREST

No conflict of interest was declared.

ABBREVIATIONS

SEM: Scanning Electron Microscopy; **EDX:** Energy Dispersive X-ray analysis; **MDR:** Multi Drug Resistance strains; **ZOI:** Zone of Inhibition; **KSA:** Kingdom of Saudi Arabia; **SCA:** Starch Casein Agar; **AgNPs:** Silver nitrate nanoparticles; **KAIN:** King Abdullah Institute for Nanotechnology; **MHA:** Muller Hinton Agar.

SUMMARY

This study reports on isolation and identification of potent isolate based on the ability to synthesize silver nanoparticles along with broad spectrum antibacterial activity. Silver nanoparticles were biosynthesized from the potent isolate DW102 and characterized by UV-Vis Spectroscopy, Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDX). The combination of antibiotics with AgNPs showed an overall percentage increase in average fold-area of Zone of Inhibition (ZOI) in this study.

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