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Characterization and Antimicrobial Activity of AgNPs Synthesized by *Streptomyces* sp. RHS16 Against Fish Pathogens

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Abstract: *Silver nanoparticles (AgNPs) attracted great attention due to their unusual and fascinating properties, and applications in many fields. This work reports the fabrication of AgNPs by a newly isolated Streptomyces sp. RHS16. The synthesis of silver nanoparticles was mediated by nitrate reductase enzyme secreted in the culture filtrate of the bacterium and detected using UV-visible spectra that showed a strong and broad peak between 420 nm and 450 nm. The effect of crude enzyme concentration and silver nitrate molarity was evaluated. As the volume of crude enzyme increased from 1ml to 4ml, the color changed to dark reddish brown and the absorbance values increased while the color intensity increased with the increase in molarity of silver nitrate. FTIR spectral analysis of St.sp.R16 AgNPs showed an array of absorbance bands at 3422.36, 2922.88, 2393.31, 1756.94, 1631.62, 1384.06, 1035.10, 823.03, 754.58, 695.08 and 540.54 cm^{-1}. EDX spectrum revealed a strong signal in the silver region and confirmed the formation of metallic silver nanocrystals with typical optical absorption peaks approximately at 3 KeV. The characteristic XRD peaks were centered at ∼ 38.02◦, ∼44.44◦ and ∼64.51◦, 77.68◦, and 81.35◦ which could be induced by the following crystalline planes of silver; (111), (200), (220), (311), and (222), respectively. TEM showed that size of the silver particles were in the range of 1–15 nm. The Ag nano flowers (AgNFs) of average size in between 1 to 133 nm were observed with SEM. The nanoparticles possessed antimicrobial activity against some fish bacterial pathogens with strong activity against Candida albicans (inhibition zone of 18 mm).*

Keywords: *Silver nanoparticles, Streptomyces sp. RHS16, Biosynthesis, Antimicrobial activity, Fish pathogens, XRD, TEM, SEM, EDX, and FTIR*

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I. Introduction

Nanotechnology, a multidimensional science devoted to study fundamental properties of nanomaterials, has attracted interest in recent years. Nanoparticles (NPs) between 1 and 100 nm in size have attracted considerable scientific interest (**Polte, 2015**). Depending on size and shape, nanoparticles have been used for applications in medicine, healthcare, electronics, and agriculture (**Borase et al., 2014**). Silver nanoparticles (AgNPs) have been exploited for their unique properties and their vast applications in biomedicine **(Kalishwaralal et al., 2010).**

Biological approach of nanoparticle synthesis is advantageous than physical and chemical methods because of simplicity of synthesis method without requirement of high temperature, pressure, and energy besides eco-friendly, cost-effective, scalable processes (**Borase et al., 2014**). Microorganisms are efficient bio-system for the synthesis of metal nanoparticles **(Salunke1 et al., 2016).** A great interest has been given to Actinobacteria such as *Thermomonospora* sp**. (Sastry et al., 2003),** *Streptomyces hygroscopicus* **(Sadhasivam et al., 2010),** *Streptomyces parvulus* **(Prakasham et al., 2011)**, *St. albogriseolus* **(Samundeeswari et al., 2012),** *Rhodococcus* sp. **(Otari et al., 2012),** *Streptomyces* sp JAR1 **(Chauhan et al., 2013**) , the marine *Streptomyces*-MS26 **(Zarina & Nanda, 2014)**, *Streptomyces* sp. (**Saminathan, 2015)**, *Streptomyces* sp. VSMGT1014 **(Shanmugaiah et al., 2015)** and *Nocardiopsis valliformis* strain OT1**(Rathod et al., 2016). Manivasagan et al., (2016)** recently reviewed actinobacteria in synthesis of nanoparticles.

The present study aims to (a) utilize *Streptomyces* sp. RHS16 isolated from compost for the biogenic synthesis of silver nanoparticles (AgNPs) and evaluate some factors affecting their formation, (b) characterize the nanoparticles formed and (c) assess their antimicrobial activity.

II. Methodology

Streptomyces sp. RHS16 isolated from compost was used for silver nanoparticles biosynthesis. For cultivation of bacteria, GYM broth composed of (g/L): glucose, 4; yeast extracts, 4; malt extract, 10; calcium carbonate, 2 was used. For solid media 20g/L (w/v) agar was added. Cells were grown for 7 days at 30°C until sporulation and kept in the form of spore stock. For long time preservation, bacterial spores were scratched and preserved in Tween 80 (100 µl Tween 80/ 100 ml dist. H_2O **(Ben-David & Davidson, 2014).** One ml of spore suspension contained 2×10^9cells.

The antimicrobial activity of silver nanoparticles was examined against some fish pathogens; *Escherichia coli* ATCC 9739, *Salmonella typhimurium, Staphylococcus aureus* ATCC 6538P and *Candida albicans* ATCC 1023, kindly provided from the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Alexandria University. They were grown on Müller-Hinton **(Mueller & Hinton, 1941)** medium**.**

Biosynthesis of silver nanoparticles (AgNPs)

One hundred ml GYM broth were inoculated with 4ml spore stock of *Streptomyces* sp. RHS16 and incubated shacked at 200 rpm & 30 °C for 7 days (**Fayaz et al., 2010),** then cells were separated by centrifugation. The activity of cell free supernatant as a reducing agent for silver nanoparticles formation was checked by adding 1 ml of the filtrate to 100 ml of freshly prepared1mM $AgNO_3$ and incubated in the dark shacked at 200 rpm & 30 °C for 8 days. The sample was analyzed with UV-visible absorption spectroscopy to confirm the synthesis of AgNPs **(Nayak et al., 2011).**

Measurement of nitrate reductase activity

Nitrate reductase as a reducing agent for silver nanoparticles formation was examined in culture filtrate of *Streptomyces* sp. RHS 16 according to **Thamilselvi & Radha (2013)**. The enzyme activity was calculated based on the increase in nitrite concentration in aliquots over time of incubation and expressed as U/ml/min.

Effect of enzyme concentration on Ag NPs formation

Different volumes (1, 2, 3 and 4 ml) of culture filtrate were added to 24 ml of 1 mM $AgNO_3$ and incubated in the dark in an orbital shaker at 200 rpm & 30°C until the color intensity became stable (confirmed by UV-visible spectroscopy).

Effect of $AgNO_3$ concentration

4ml of culture filtrate were added to 24 ml $AgNO_3$ solution of different concentrations (1-8 mM). Flasks were incubated in the dark in an orbital shaker at 200 rpm & 30 $^{\circ}$C until the color intensity become stable.

Characterization of silver nanoparticles

The primary detection of AgNPs formation was the color transformation from clear to reddish brown and later confirmed using UV-Vis spectrum of the reacting solution scanned in the range of 300-550 nm using a double beam UV-visible spectrophotometer. This solution was centrifuged at 5000 rpm for 15 min, washed and dried, then ground with KBr into pellets (**Samundeeswari et al., 2012)**. The spectrum was recorded in the range of 3500-500 cm^{-1} using a Bruker Tensor 37FTIR spectrophotometer. Transmission electron microscope (TEM) and scanning electron microscope (SEM) were employed to comprehend the morphology, size and the distribution of nanoparticles. X Ray Diffraction (XRD) analysis was performed to determine the crystalline nature of the sample before and after washing (**Sadhasivam et al., 2010)** using PHILIPS PW, Holland. The elemental composition of the sample was determined by Energy Dispersive X-ray Spectroscopy (EDX) as described by **Jaiswal et al., (2018).**

Antimicrobial activity of silver nanoparticles

The antimicrobial activity of the synthesized AgNPs was determined by the well diffusion method **(Samundeeswari et al., 2012)**. Two controls were used; an aqueous solution of $AgNO_3$ and the cell-free supernatant. Plates were incubated at 37 °C for 24 h and the diameter of inhibition zone was measured.

III. Results& Discussion

UV–visible spectra of Streptomyces sp. RHS16 AgNPs

According to **Elgorban et al., (2016)**, the absorption spectrum of AgNPs occurs in the visible range of 380-450 nm according to their size, shape and particle interaction with the medium like agglomeration. The culture filtrate of *St*.sp.RHS16 incubated with silver nitrate solution mediated the biosynthesis of AgNPs within 24 h of incubation as color changed from clear to reddish brown as visually observed **(Fig.1).** Meanwhile, no color change was observed in either the culture supernatant without silver nitrate or the silver nitrate control experiments. The UV- visible absorption spectra showed a strong and broad peak between 420 and 450 nm **(Fig.1)** attributed to the Surface Plasmon Resonance band (SPR) of the formed silver nanoparticles. SPR peak is an important and special characteristic feature of metal nanoparticles (**Yadav et al., 2015**). The color intensity increased in relation to time and became stable after 7 days. Similar observation was reported with *St. hygroscopicus* (**Sadhasivam et al., 2010)** and *St. somaliensis* **(Nejadet al., 2015).**

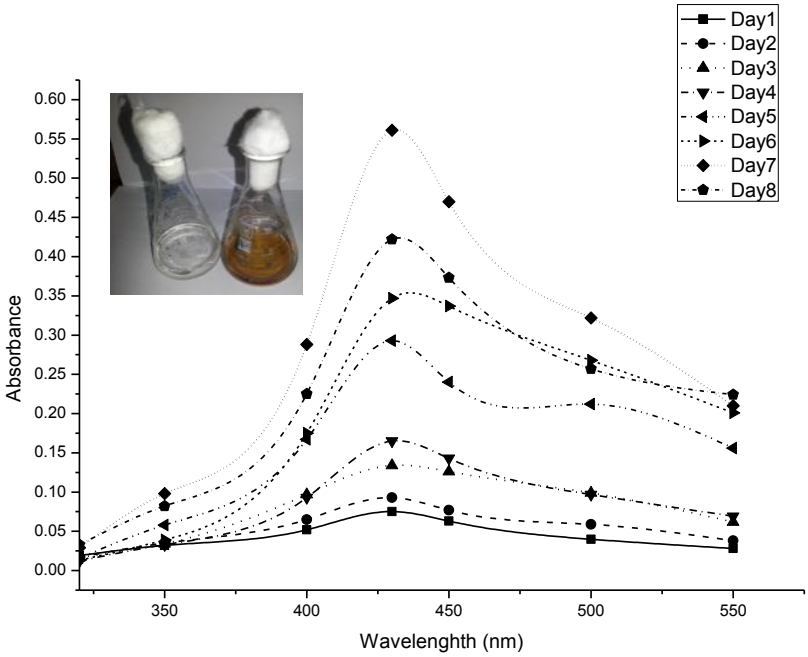

Fig.1. UV–visible absorption spectra of silver nanoparticles formed by incubating 100 ml of 1mM of silver nitrate solution and 1 ml of *St*. sp. RHS16 at 30 ˚C shacked at 200 rpm in the dark.

Mechanism of AgNPs fabrication

A possible mechanism for the formation of AgNPs is the reduction of metal ions by nitrate reductase enzyme in fungi and bacteria (**Shahrokh et al., 2014).** The extracellular synthesis of nitrate reductase was examined in the crude bacterial filtrate of the bacterium and its presence was confirmed by the change in color from pale yellow to reddish brown which was measured as 4.75 U/ml/min.

Effect of crude enzyme concentration on AgNPsformation

Conversion of Ag^+ to Ag^0 depends on the presence of equivalent amount of enzymes to the amount of challenged Ag ions (**Iravani et al., 2014**). By increasing the volume of crude enzyme from 1ml to 4ml, the color changed from light brown to dark reddish brown and the absorbance values increased **(Fig.2).** This could be due to the formation of different amounts and/ or sizes of silver nanoparticles. The color intensity became stable after 7 days.

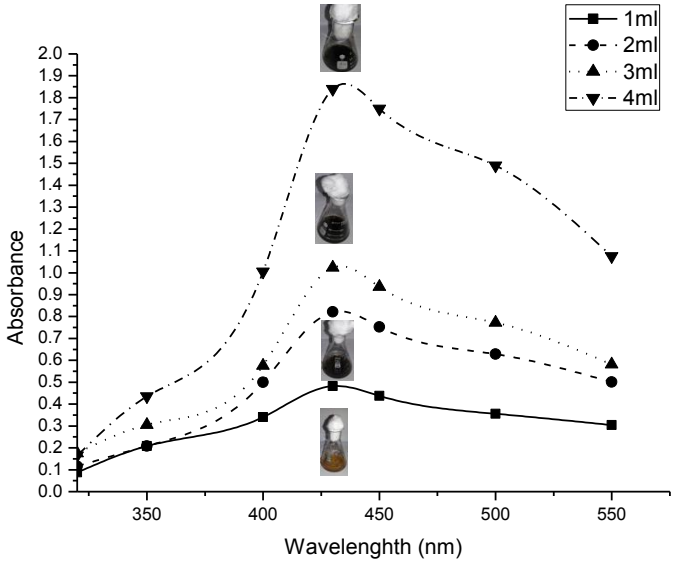

Fig.2. UV-spectra of AgNPs formed by the addition of different concentrations of *St*. sp. RHS16 crude enzyme to 1mM silver nitrate solution and incubation at 200 rpm shacked in the dark for 7 days.

Effect of $AgNO_3$ concentration

Silver nitrate concentration is a key parameter that affects the process of nanoparticle synthesis **(Nayak et al., 2011)**. Data in **Fig.3** provide evidence that the increase in $AgNO_3$ concentration up to 4 mM led to the increase in color intensity as observed by UV spectra readings. This is due to the increased number of formed nanoparticles as a result of reduction of silver ions present in the aqueous solution **(Nayak et al., 2011).** This can be explained on the basis of enzyme substrate kinetics where active site of enzyme catalyzing reduction is 66 saturated with the Ag ions and no more site is available for ions to be reduced (**Sanghi & Verma, 2009**). **Balakumaran et al., (2016)** reported that raising the concentration of $AgNO_3$ from 2 to 4 mM increased AgNPs concentration by *Aspergillus terreus*, while increasing $AgNO_3$ concentration to 3 mM enhanced AgNPs synthesis by *Aspergillus fumigates* (**Sarsar et al., 2015)**.

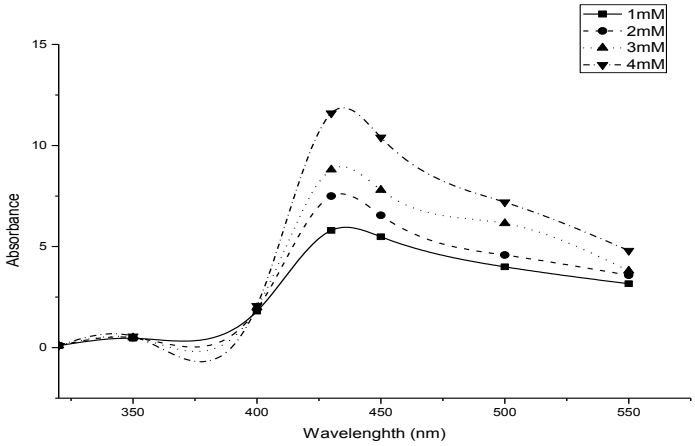

Fig.3. UV-spectra of AgNPs using different molarities of $AgNO_3$ solutions after addition of 4 ml of *St*. sp. RHS16 crude enzyme and incubation at 200 rpm shacked in the dark for 7 days.

Characterization of silver nanoparticles

Characterization of nanoparticles is important to understand and control nanoparticles synthesis and applications. Different techniques are used for determination of different parameters such as particle size, shape, crystallinity and surface area **(Abou El-Nour et al., 2010).**

Fourier transform infrared (FTIR) spectral analysis

FT-IR analysis was performed to identify the biological molecules responsible for synthesis and capping of AgNPs**(Rani et al, 2017)**. It provides evidence for the presence of proteins as possible biomolecules that helps in increasing the stability of the synthesized silver nanoparticles **(Karthik et al., 2014).** FTIR spectral analysis of *Streptomyces* sp. RHS16 AgNPs shows an array of absorbance bands **(Fig.4)**.

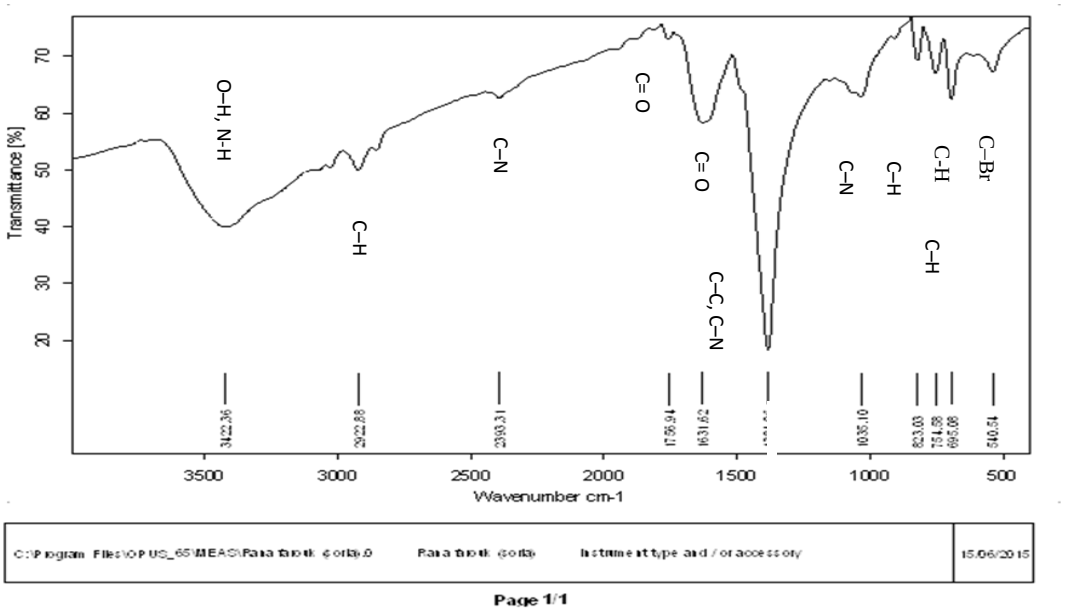

Fig.4. FTIR spectrum of AgNPs synthesized by *Streptomyces* sp. RHS16.

Intensive bands at 3422.36, 2922.88, 2393.31, 1756.94, 1631.62, 1384.06, 1035.10, 823.03, 754.58, 695.08 and 540.54 cm^{-1} were observed. The band O-H stretch of the carboxylic acid groups and the N-H stretch vibrations of the peptide linkages around 3422 cm^{-1} which may be responsible for reducing metal ions into their respective nanoparticles**,** similarly observed with *Streptomyces* sp. LK3 (**Karthik et al., 2014)**. Peaks at 3421.1, 2924.41, 1633.96, 1384.56, 1073.63, and 617.36 cm^{-1} are corresponding to N-H stretching of primary amine of the protein, alkane C-H stretching, the stretching of conjugated alkane C=C, methylene tails of the protein (CH_3-R), C-N of aliphatic amines of polyphenols and O-H stretching, respectively. The band observed at 2393.3 cm^{-1} can be assigned to the fingerprint of phenyl ring substitution overtones and the band observed at 695.5 cm^{-1} representing the bending vibrations of Alkynes. It is well known that proteins are able to bind with metals and metallic nanoparticles either through free amine groups or cysteine residues in the proteins **(Arunachalam et al., 2013)**. One or more of these proteins may be enzymes (Nitrate reductase) that reduce silver nitrate ions and form the silver nanoparticle by reduction technique (**Lakshmi et al., 2015)**.

The overall observation confirmed the presence of protein in the samples of silver nanoparticles. Proteins or peptides might have formed a coating to stabilize the nanoparticles **(Thamilselvi & Radha, 2013)**. As reported earlier, proteins can bind to nanoparticles either through free amine groups or cysteine residues in the proteins **(Arunachalam et al., 2013).**

X-ray diffraction (XRD)

XRD analysis showed that the characteristic XRD peaks were centered at ~38.02◦, ~44.44◦ and ~64.51◦, 77.68◦, and 81.35◦ which could be induced by the following crystalline planes of silver; (111), (200), (220), (311), and (222), respectively **(Fig.5).** Similar data were obtained by **Khatami et al., (2015)** and **Nejad et al., (2015)** for AgNPs from *Streptomyces somaliensis*. A comparison of our XRD spectrum with the standard sample confirmed that the silver nanoparticles had been formed in the form of nanocrystals, as was evidenced by the peaks at 2⊖values of 38.25°, 46.37°, 64.60° and 77.62° corresponding to 111, 200, 220 and 311 planes for silver, respectively **(Zonooz & Salouti 2011).**

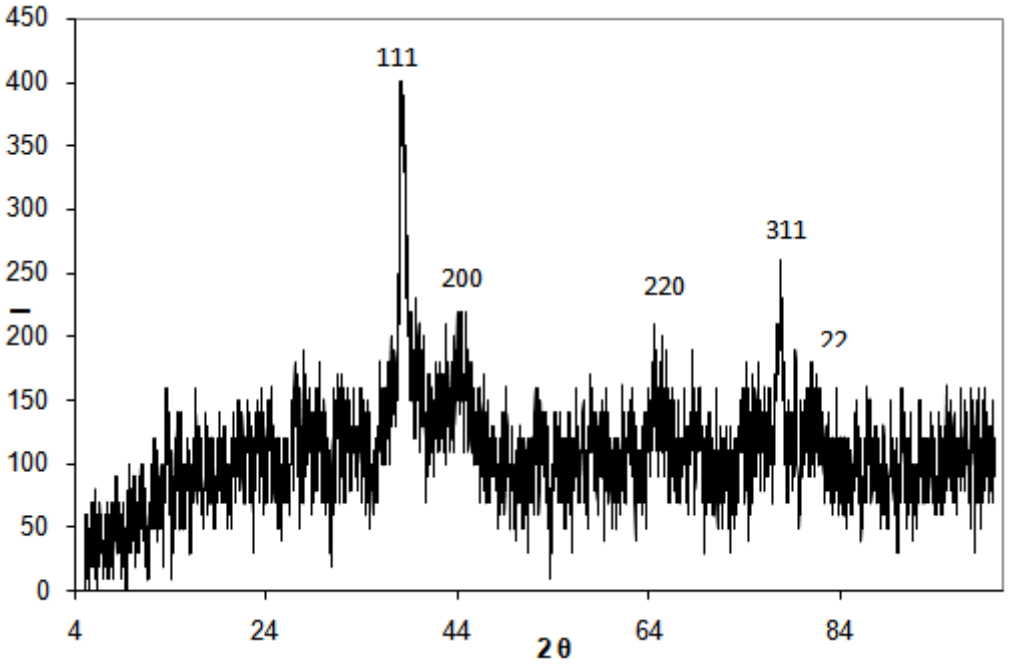

Fig.5. X-ray diffraction pattern of *Streptomyces* sp. RHS16 AgNPs

A few unassigned peaks were also noticed. These peaks might be due to the presence of capping agent stabilizing the nanoparticle. The mean particle diameter of AgNPs was calculated from the XRD pattern using the Scherrer equation: $D_p = K\lambda/\beta_{½}\cos\Theta$ where K is the shape constant, λ is the wavelength of the X-ray, $\beta_{½}$ and Θ are the half width of the peak and half of the Bragg's angle, respectively. The calculated average crystallite size of the AgNPs was found to be **32.7** nm.

Energy Dispersive X-ray Spectroscopy (EDX)

The purity of the biosynthesized AgNPs was examined by EDX combined with SEM. As shown in **Fig.6** EDX spectrum of AgNPs revealed a strong signal in the silver region and confirmed the formation of metallic silver nanocrystals with typical optical absorption peaks approximately at 3 KeV due to surface plasmon resonance (**Kaviya et al., 2011; Jyoti et al., 2016**). Silver was the only constituent element in the sample which represented 100% of the total element. This confirmed the purity of the biosynthesized AgNPs.

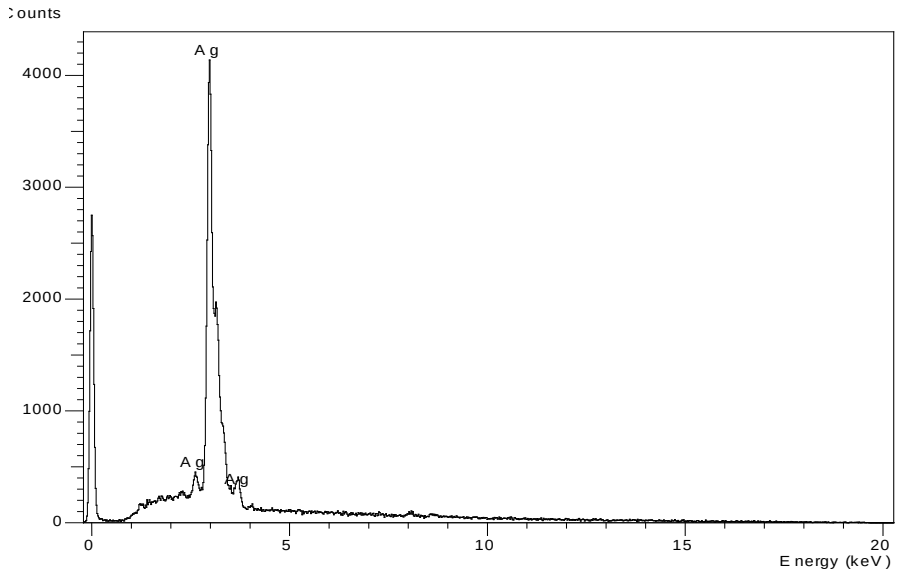

Fig.6. EDX analysis of AgNPs synthesized by *Streptomyces* sp. RHS16

Scanning electron microscope

Interestingly SEM images showed flower-like shapes of AgNPs aggregates **(Fig.7)**. The Ag nano flowers (AgNFs) have an average size in between 1 to 133 nm. **Zonooz & Salouti (2011)** studied the extracellular biosynthesis of silver nanoparticles using cell filtrate of *Streptomyces* sp. ERI-3 and reported the formation of flower-like structures. It should be noted that the directed-self-assembly of nanostructures into larger structures through nanoscale interaction is an important phenomenon in the synthesis of novel nano or microstructure materials **(Swatek & Kaczorowski, 2013**).

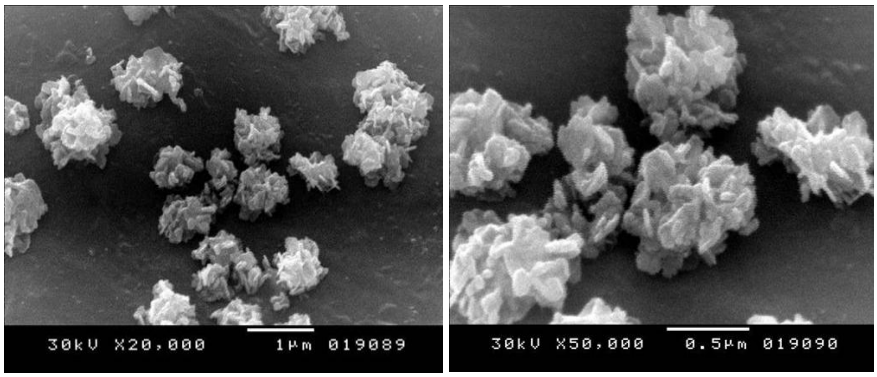

Fig.7. SEM of *St.* sp. RHS16 AgNPs showing the morphology of nanoparticles on 1 µm scale (Left) and 500 nm scale (Right).

Transmission electron microscope (TEM)

TEM image of silver nanoparticles synthesized using *Streptomyces* sp. RHS16 filtrate is shown in **Fig.8**. The AgNPs produced were spherical in shape, separate and well distributed without agglomerations. The average diameter ranged from 1–15 nm demonstrating the smallest size compared to those produced by the other microbes. **Zarina & Nanda (2014)** reported that the silver nanoparticles biosynthesized by the marine *Streptomyces*- MS 26 were spherical and poly dispersed whose range was in between 50 to 76 nm.

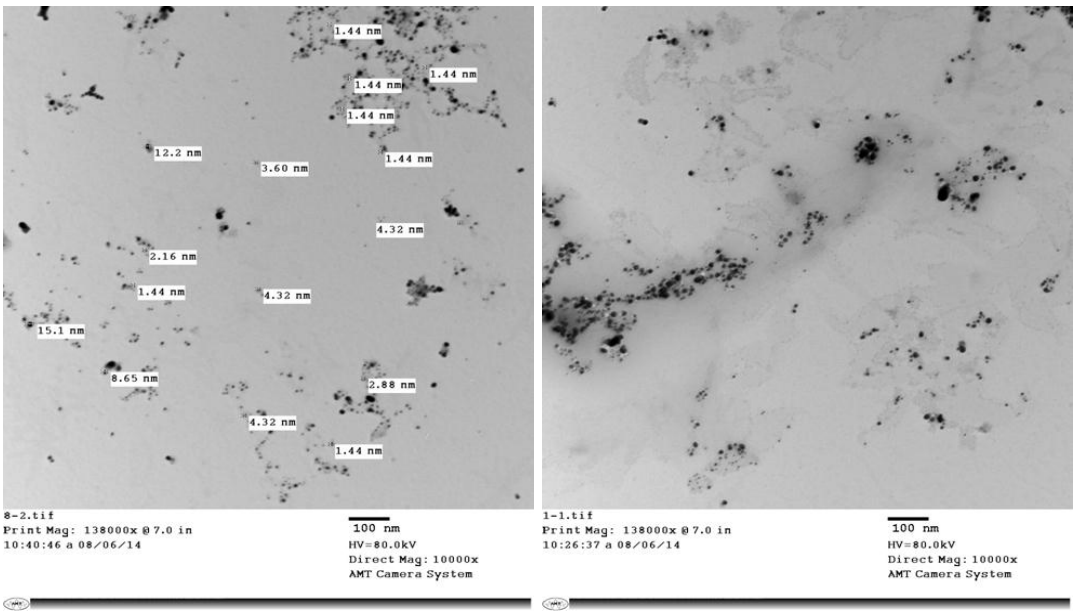

Fig.8. TEM of synthesized silver nanoparticles by *Streptomyces* sp. RHS16 shows size and morphology of nanoparticles

Previous studies showed that AgNPs are capable of performing effective antibacterial property against *Staphylococcus aureus*, *Escherichia coli*, *Vibrio cholera*, *Pseudomonas aeruginosa* and *Salmonella typhi* **(Morones et al., 2005; Li et al., 2010)**. Antimicrobial assay of *St*. sp. RHS16 NPs was performed against four fish pathogens; the gram +ve bacterium *Staphylococcus aureus*, the gram – ve *Salmonella typhimurium* and *E. coli* and the yeast *Candida albicans*.

Data presented in **Fig.9** depict that the examined AgNPs possessed antimicrobial activity against all tested pathogens. The most pronounced effect was against *Candida albicans* recording an inhibition zone of 18 mm. Silver nanoparticles showed a more inhibitory effect against the Gram –positive *S. aureus* compared to the two Gram- ve *E. coli* and *Salmonella typhimurium* as detected by the zones of inhibition formed (15, 13 and 13 mm, respectively). The antibacterial activity of AgNPs was previously reported for *Streptomyces coelicolor* klmp33 **(Manikprabhu & Lingappa, 2013**), *St. rochei* (**Selvakumr et al., 2012)** and *St. albogriseolus* **(Samundeeswari et al., 2012).**

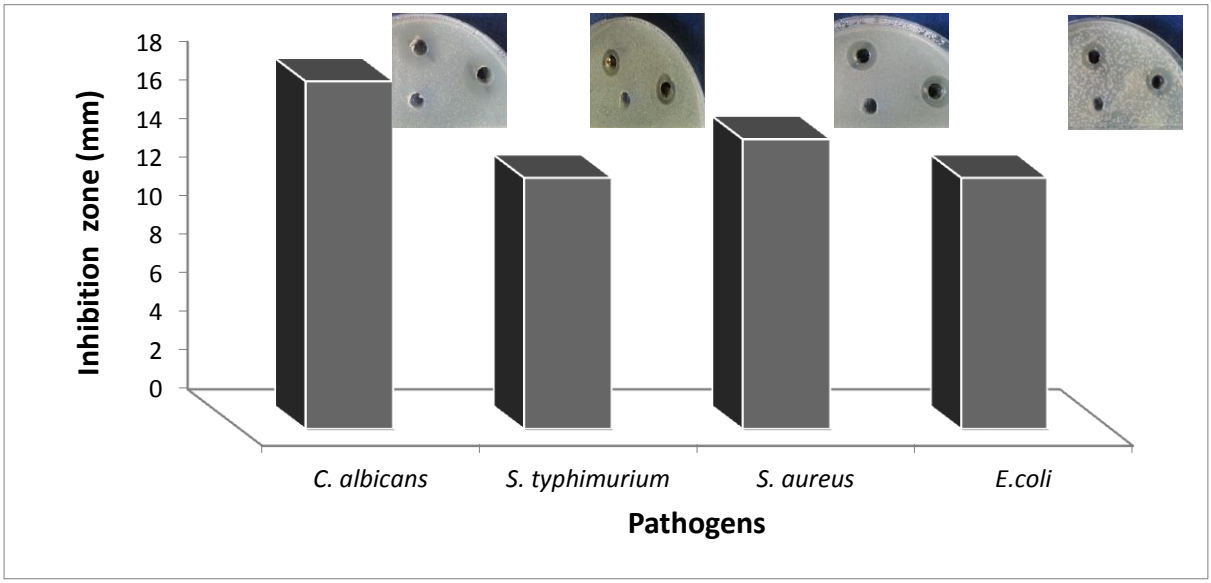

Fig.9. Antimicrobial activity of AgNPs synthesized by *Streptomyces* sp. RHS16.

Several studies proved that silver nanoparticles bind to the surface of that cell membrane, disrupting cellular permeability and the respiratory functions of the cell. The small nanoparticles have a large surface area available for interaction and greater bactericidal effect than larger silver nanoparticles **(Kvitek et al., 2008)**. Silver nanoparticles may also interact with the surface of the membrane, or penetrate inside the bacteria and inactivate their DNA replicating ability **(Morones et al., 2005)** causing cell death.

IV. Conclusion

This research provides evidence for the green synthesis of silver nanoparticles by the nitrate reductase enzyme of *Streptomyces* sp. RHS16 and $AgNO_3$. The color change was observed which proved the formation of nanoparticles. The nanoparticles were characterized by UV-vis Spectrophotometer, FTIR, XRD, EDX, TEM, SEM and measurment nitrate redutase activity. These nanoparticles have application as antibacterial and antifungal activity against some fish pathogens.

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