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Green Approach for Synthesis of Silver Nanoparticles from Marine Streptomyces- MS 26 and Their Antibiotic Efficacy

A. Zarina[1] and Anima Nanda[2]
[1,2]*Department of Biomedical Engineering, Sathyabama University, Rajiv Gandhi Salai, Chennai - 600119, India.*

Abstract -Antibiotic resistant pathogens pose an enormous threat to the treatment of various serious infections. To overcome this condition, a periodic replacement of the new and existing antibiotic is necessary. Antibiotics in combination with biosynthesized silver nanoparticles minimize the antibiotic doses to cure the dreaded diseases. In this study, Silver nanoparticles were bio-synthesized by extracellular method using Marine *Streptomyces sp.,* isolated from Muttukadu estuary. It was observed that the aqueous silver ions, when exposed to a filtrate of *Streptomyces sp.,* were reduced in solution, thereby leading to formation of extremely stable silver nanoparticles. These silver nanoparticles were characterized by means of several techniques. The biosynthesized silver nanoparticles were also evaluated for their synergistic effect with twelve different antibiotics against the MTCC pathogens. The UV Spectrophotometer revealed the conformation of silver nanoparticles by yielding silver plasmon absorption maxima at 420 nm and Field emission scanning electron microscopy [SEM] micrograph and Particle size analysis indicates the uniform spherical particles at the size range of 50 to 76 nm. The Fourier Transform Infrared [FTIR] Spectroscopy confirmed the presence of proteins as the stabilizing agent surrounding the silver nanoparticle. Atomic force microscopy [AFM] showed the particle height, average roughness of the particles. In this study the combined formulation of silver nanoparticles from Marine Streptomyces species MS-26 with twelve standard antibiotic discs selected from seven groups having a different mode of action was found to be enhanced greatly against the MTCC pathogens *Micrococcus luteus [1538]*, *Pseudomonas sp.,[129] Staphylococcus aureus[96]* and multi drug resistant pathogen.

Keywords: *Streptomyces sp.,* Synthesis of Silver Nanoparticles, UV-Vis Spectrophotometer, FTIR, FESEM, AFM, Particle size analysis, Antimicrobial activity.

INTRODUCTION

Resistance to antibiotics is a ubiquitous and relentless clinical problem that is compounded by a dearth of new therapeutic agents that block resistance mechanisms [1]. Novel antibacterial formulation is being searched by the researchers and pharmaceutical companies. The most effective vicinity of nanotechnology is the competence of modulating metals into their nanosize [2] which offers unique approaches to probe and control a wide variety of biological and medical processes that occur at nanometer length and is believed to have a successful collision on biology and medicine [3,4]. Owing to their high antibacterial properties, nanoparticles of silver, oxides of Zinc, titanium, copper, and iron are the most commonly used nanoparticles in antimicrobial studies. Furthermore, these nanoparticles have been used to deliver other antimicrobial drugs to the site of pathological process [5].Metallic nanoparticles provides an attractive alternative to antibiotics to the pharmaceutical field by developing novel applications The synthesis of nanomaterials of specific composition and size is a burgeoning area of materials science research. The properties of these materials in applications as diverse as catalysis, sensors and medicine depend critically on the size and composition of the nanomaterial [6].Thus, researchers have used biological synthesis, since this technique provides particles with good control over the size distribution. The main reason for this may be that the processes devised by nature for the synthesis of inorganic materials on nano- and micro- scales have contributed to the development of arelatively new and largely unexplored area of research based on the use of microbes in the biosynthesis of nanomaterials [7, 8]. Biological synthesis of nanoparticles is a green chemistry approach. Green synthesis of nanoparticles is capable to meet the requirements of diverse industrial application [9, 10, 11]. It has been known that silver [Ag] and its compound processes broad spectrum of antibacterial activities since ancient times [12, 13]. Eco-friendly materials like plant leaf extract [14], bacteria [15], fung[(16], actinomycetes and yeasts have been used for the green synthesis of silver nanoparticles as it offers numerous benefits. Marine microbial biotechnology has opened up unexpected new horizons for finding novel organism for trapping their potential resources. Oceans account for more than 70% of the earth's surface and the microorganisms growing in marine environments are metabolically and physiologically diverse from the terrestrial organisms [17]. Actinomycetes are Gram-positive filamentous spore formers with high G+C (>55%) content of DNA. They are free living saprophytic bacteria forming a major group of soil population. *Actinobacteria* are widely distributed in terrestrial and aquatic ecosystems, especially in soil, where they decompose complex mixtures of polymers in dead plant. They also play main role in recycling of organic matter [18].Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and actinomycetes alone produce 10,000 of these compounds[19]. Many of these secondary metabolites are potent antibiotics, which has made *Streptomycetes* the primary antibiotic-producing organisms exploited by the pharmaceutical industry. Marine source provides a promising source of actinomycetes that can ruin the

resistant pathogens. The present study deals with the synthesis of silver nanoparticles biologically from marine *Streptomyces species* and their antibiotic efficacy against the MTCC pathogens.

MATERIALS AND METHOD:

Sample collection

The Soil samples were collected from Muttukadu estuary, the coastal region of Tamil Nadu, India (Lat. 13°.59' N, Long. 80°.15' E) from its top layers at a depth of 20 cm depth after removing approximately 3 cm of upper soil surface with help of sterile spatula, in sterile polythene bags. The samples were closed tightly transported to the laboratory aseptically and stored in the refrigerator at 4°C until further use.

Isolation and screening of actinomycetes

Collected soil samples were treated with 2% calcium carbonate and air dried for 3 to 4 days under *in vitro* lab condition. The soil samples were serially diluted from 10^{-1} to 10^{-7} and 100µl of diluted samples were inoculated in Starch Caesin agar by spread plate technique. The media was supplemented with flucanazole to avoid fungal contamination. The plates were incubated at 30°C for 7-10 days. The isolated colonies were purified by quadrant streaking technique and maintained for further studies [20]. Preliminary screening was done using cross-streak method [21] by streaking Actinomycetes isolates in the centre of starch casein agar plates. After incubation at 28°C for 6 days, 24 hrs cultures of MTCC pathogens *Staphylococcus aureus, Micrococcus luteus, Bacillus ceresus, Escherichia coli, Pseudomonassp., Klebsiella pneumonia, Vibrio cholarae and Proteus vulgaris* were streaked perpendicular to the central strip of actinomycetes culture. All plates were again incubated at 30°C for 24 hrs and inhibition was measured.

Identification of potential actinomycetes

The spore chain morphology such as sporangia spore motility and spore surface ornamentation of the isolate was evaluated by phase contrast microscope magnifications 100x.This is done by using cover slip method [22].The generic level identification of potential actinomycetes was carried out. The morphological, cultural, physiological, biochemical, colour and carbon utilization were carried out using standard procedure and were identified based on the keys of Bergey's manual of determinative bacteriology [23]..

Synthesis of silver nanoparticles

For the synthesis of silver nanoparticles ,the spores of potential *Streptomyces sp*., was inoculated in MYGP media (Malt extract-3.0 g, Yeast extract-3.0 g, Glucose-10.0 g, Peptone-5.0 g, Distilled water-1000 ml, pH-7.2) in Erlenmeyer flasks incubated at 25°C on a shaker (150 rpm) for 72 hours. The biomass was filtered using Whatman filter paper (No.1) and washed extensively with distilled water to remove any medium components. Fresh and clean biomass was taken in the Erlenmeyer flasks containing 100cm^3 of milli-Q deionized water. The flasks were agitated at the same conditions as described above and again the biomass was filtered through Whatman filter paper. For the synthesis of silver nanoparticles extracellulary 1mM final concentration of silver nitrate AgNO3, was mixed with 50 ml of cell filtrate in a 250 ml Erlenmeyer flask and agitated at 25°C in dark for 72 h under static and at 200 rpm and the silver nanoparticle production monitored. Control (without the silver ion, only culture) was also run along with the experimental flask [24].

Characterization of silver nanoparticles

The bioreduction of the Ag+ ions in the solution was monitored by changes in color. Periodically, aliquots of the reaction solution were removed and the absorptions were measured in a UV-Vis spectrophotometer between 350-750nm range of the reaction medium The dried silver nanoparticles were subjected to FTIR analysis. The samples were scanned using infrared in range of 4000- 500 cm-1 using FTIR. The spectrum obtained was compared with reference chart to identify functional groups present in sample. Scanning electron microscope (SEM) was used to obtain the surface image and the size of the microbially synthesized silver nanoparticle silver nanoparticles. The silver nanoparticles solutions were sonicated, centrifuged and the obtained dried powdered silver nanoparticles were subjected to SEM analysis. An atomic force microscopy (AFM) was performed to identify the topological appearance, and the size of the biosynthesized silver nanoparticles. For this the sonicated silver nanoparticles were formed as a thin film in slide.

Antibacterial activity and synergitic effect of silver nanoparticles

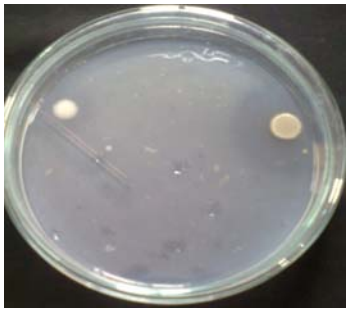
The antimicrobial activities of the microbiologically synthesized silver nanoparticles were tested against pathogenic organisms obtained from MTCC, Chandigarh, India. The organisms used were *Micrococcus luteus* ,*Staphylococcus aureus*, *Pseudomonas sp.* and multi drug resistant pathogen. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C. Overnight culture in the nutrient broth was used for the present experimental study. The combined formulation of silver nanoparticles with standard antibiotic discs selected from seven groups having a different mode of action includes Amoxicillin (β-lactams), Azithromycin and Erythromycin (Macrolides), Cefazolin (Cephalosporins1stgeneration), Cefataxime and cefixime(Cephalosporins3rdgeneration),Chloramphenicolan dTetracycline(sulphoamides),Streptomycin (aminoglycoside), Sparfloxcin and Oflaxcin(quinolones/fluoroquinolones), Piperacillin/Tazobactam (Penicillin combination) were used to find out the synergistic effect against the above pathogens. The zone of inhibition was measured after overnight incubation at 37°C.

Assessment of increase in fold area

Increase in fold area was assessed by calculating the mean surface area of the inhibition zone generated by an antibiotic alone and in combination with silver nanoparticles [25]. The fold increase area was calculated by the equation, Fold increase (%) = (b-a)/a*100 where a and b refer to the zones of inhibition for antibiotic alone and antibiotic with silver nanoparticles.

RESULTS AND DISCUSSION:

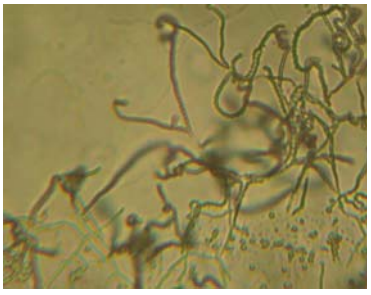
A total of eleven isolates [MS 25-36] of Actinomycetes [Fig 1-a] from marine sediments of Muttukadu estuary, were isolated. By primary screening method, the potential isolate which showed more inhibition against the MTCC pathogens was selected shown in [Fig1-b]. Among eleven isolates MS-26 [Fig1-b] showed inhibition against all the MTCC pathogens and also reduced silver ions extracellularly, within 24 hours. The selected potential actinomycete was viewed under microscope [Fig-2] and was identified as *Streptomyces species* based on the keys of Bergey's manual of determinative bacteriology.



a] Potential actinomycetes MS- 26 on

b] Primary screening of the isolate MS- 26starch casein agar plate

Fig.1-



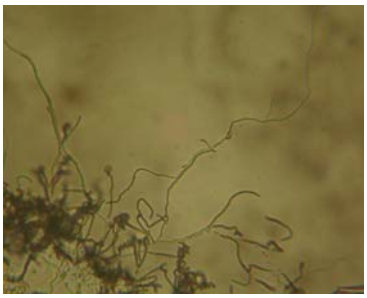


Fig. 2-Microscopic view Of Streptomyces MS-26

Silver nanoparticle synthesis

Silver nanoparticles were biologically synthesized from the culture supernatant of Marine Streptomyces *sp.*, MS-26. The Erlenmeyer flasks with the cell filtrate of *Streptomyces* were a pale yellow color [Fig 3-a] before the addition of Ag+ ions and this change to a brownish color [Fig 3-b] on completion of the reaction with Ag+ ions for 28h.The appearance of a yellowish-brown color in the silver nitrate treated flask was a clear indication of the formation of silver nanoparticles in the reaction mixture [26] due to the reduction of metal ions and formation of surface plasmon resonance, whereas no color change was observed in the control flasks.

a] Before treatment

b] After treatment of silver nitrate

Fig.3-Synthesis of silver nanoparticles from marine *Streptomyces sp.*,MS-26

UV–Visible spectroscopic analysis

In this study, silver nanoparticles were successfully synthesized in the culture supernatant *Streptomyces* sp., MS-26. The formation and stability of the reduced silver nanoparticles in the colloidal solution was monitored by a UV–vis spectrophotometer [27]. In the UV–visible spectrum [Fig-4], a strong peak was observed at 420 nm, and the surface plasmon resonance [SPR] confirmed successful formation of silver nanoparticles.

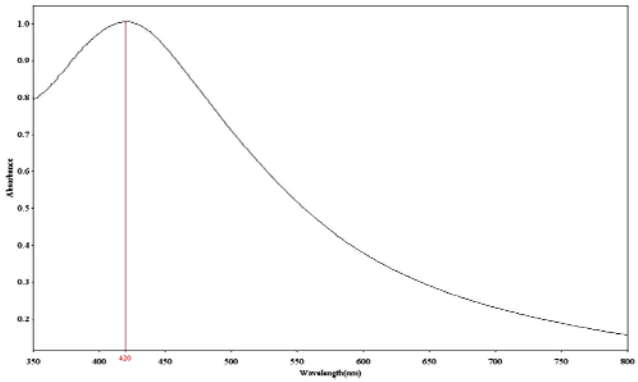


Fig.4-UV–Vis spectrum of silver nanoparticles synthesized from Marine*Streptomyces* MS-26

Fourier transform infrared spectroscopy

FTIR spectral analysis showed array of absorbance bands in 500 cm-1to 4000 cm-1. The silver nanoparticles synthesized using *Streptomyces sp*., MS-26 showed strong bands [Fig 5]. The FTIR spectrum analysis of AgNPs showed intense absorption bands at 3438.96, 2923.78, 2855.64, 1743.07, 1639.85, 1460.95, 1105.43, 1029.70 and 422.86 cm−1 respectively. The intense broad absorbance peak at 3438.96 [N-H stretch] is the characteristic of amines. The intense medium absorbance peak at 2923.78 [O-H stretch] is the characteristic of H bonded functional group in carboxylic acid. The band at 2855.64 [C–H stretch] can be assigned to the alkanes group. The strong band at 1743.07 [C=O stretch] can be assigned to aldehydes, ketones, carboxylic acid and ester group. The intense medium absorbance at 1639.85 cm−1 [N-H bend] is the characteristic of the amine group. The intense medium absorbance at 1460.95 cm−1 [C-H scissoring and bending] is the characteristic of the alkanes group. The intense medium absorbance at 1105.43 and 1029.70cm−1 [C–N stretch] is the characteristic of the amine group.

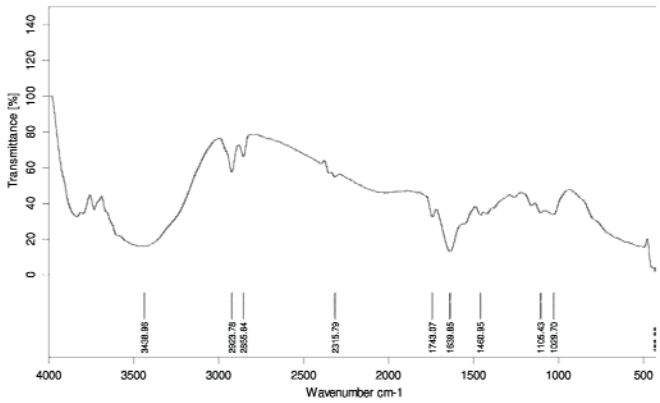


Fig.5-FTIR analysis of Silver Nanoparticles synthesized from Marine *Streptomyces* MS-26

Scanning electron microscope

The dried silver nanoparticles were obtained by centrifugation at 10000 rpm for 20 mts. The size and shape of the silver nanoparticles biosynthesized was studied by SEM. The nanoparticles size was spherical and poly dispersed whose range was in between 50 to 76 nm [Fig 6].

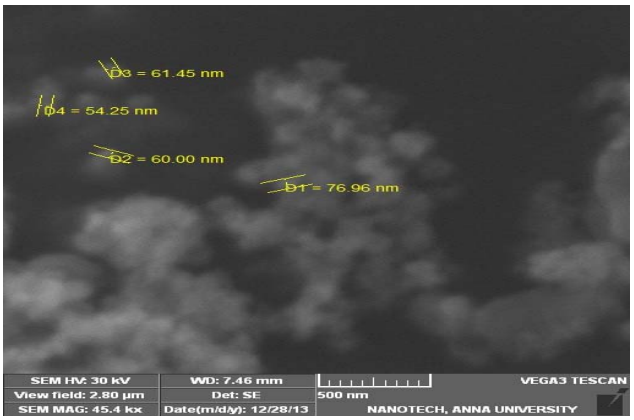


Fig. 6- SEM image of the biosynthesized silver nanoparticles MS -26

Particle size analysis

The particle size determination of the formulated nanoparticles was shown based on intensity**.**

Particle size analysis shows that, when scanning from 1 nm, the particles count is very low and its gradually reached the higher value at 76 nm and again it gradually decreased. So this indicates that the maximum nanoparticles in the range of 50 to 76 nm and only very few particles are present below and above this range [Fig 7].

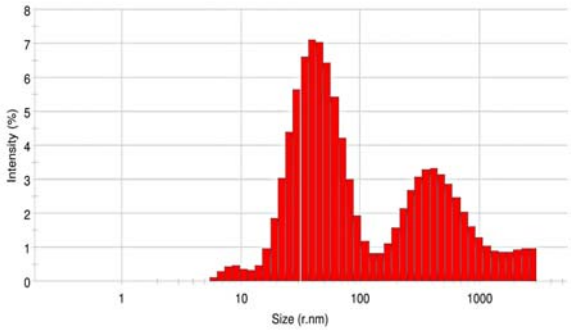


Fig.7 -Particle size distribution for silver nanoparticles using Streptomyces sp. MS -26

Atomic force microscopy

The particle size and average roughness were further characterized by AFM .Three dimensional image showed the particle height, average roughness [Fig 8.a]. Two dimensional image [Fig 8.b] showed the agglomeration of the particle. Nanoparticles size was spherical and poly dispersed whose range was in between 50 to 76 nm.

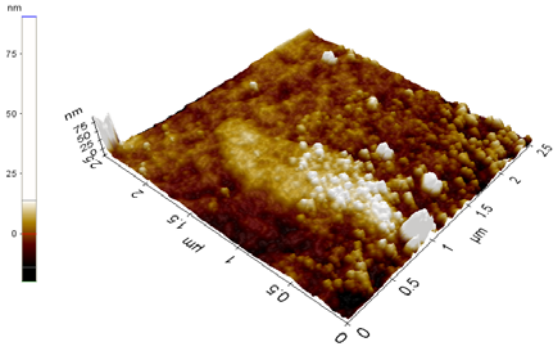


Fig.8-a:3D picture of Atomic Force microscopy of silver nanoparticles synthesized from Marine Streptomyces MS-26

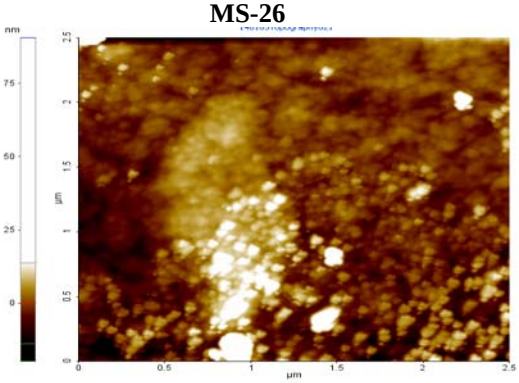
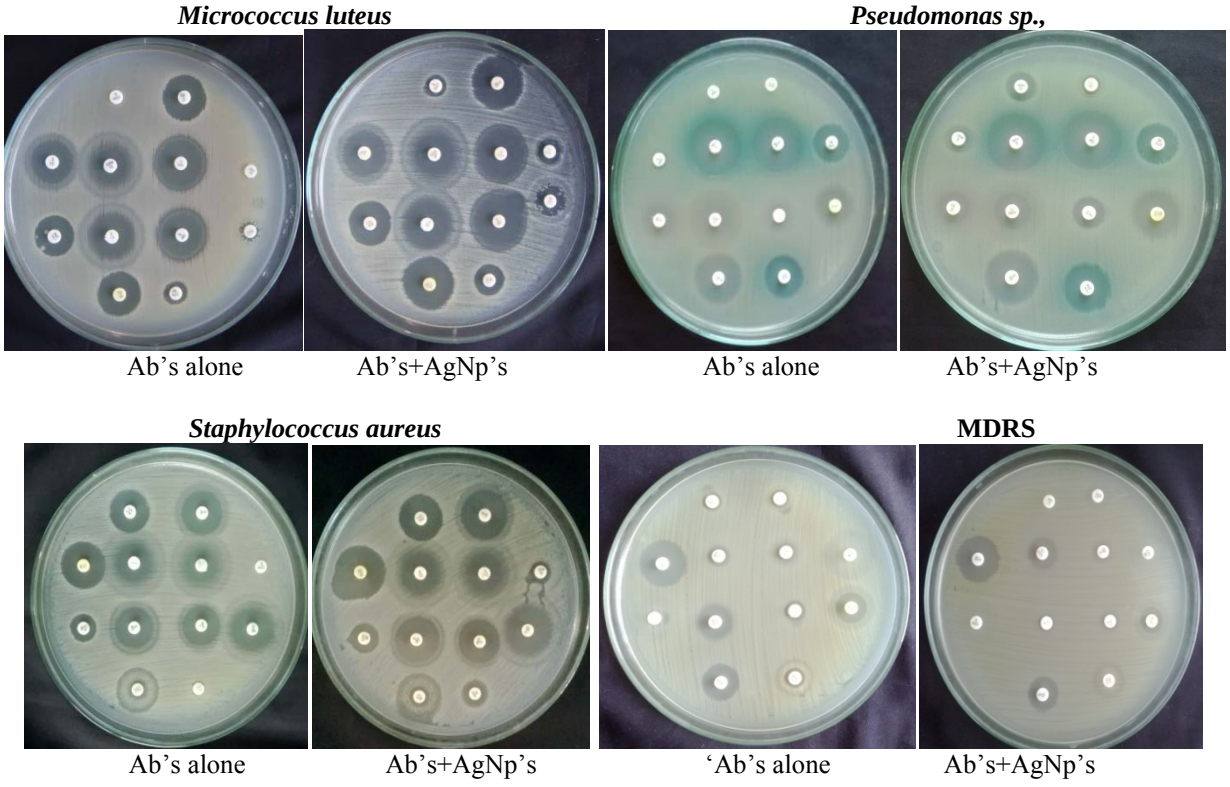


Fig. 8-b:2D picture of Atomic Force microscopy of silver nanoparticles synthesized from Marine Streptomyces MS-26



Ab's-Antibiotics AgNp's-Silver Nanoparticles MDRS-multi drug resistant pathogen

Fig.9 - Synergistic effect of silver nanoparticles of Marine Streptomyces MS- 26 with antibiotics

Synergitic effect of silver nanoparticles with antibiotics

Due to overuse of antibiotics and a growing problem of antibiotic resistance, nanoparticles are being researched as an alternative antibacterial agent. The inhibitory activity of the silver nanoparticles was evaluated against mtcc pathogens and their potency was assessed qualitatively by the presence of inhibition zones. Different classes of bacteria exhibit different susceptibilities to nanoparticles. The combined effect of silver nanoparticles with twelve standard antibiotic discs was done against the MTCC pathogens and multi drug resistant pathogen [Fig 9]. The diameter of inhibition zones for antibiotics alone and in combination with silver nanoparticles showed significant increase in fold area in all the cases [Tables 1-4]. The highest fold increase in area was observed for Amoxicillin [7 mm], Cefixime [8 mm], Amoxicillin [6 mm], and Erythromycin [5 mm] against *Micrococcus luteus, Pseudomonas sp., Staphylococcus aureus* and multi drug resistant pathogen respectively. Among the tested strains, silver nanoparticles showed highest fold increase [40.5%] on *Pseudomonas sp.,*

Types of antibiotics	Name of the antibiotics	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold increase (%)
		Ab	Ab+AGNP'S		
β-lactams	Amoxicillin	-	13	7	116
Macrolides	Azithromycin	13	17	4	31
	Erythromycin	13	16	3	23
Cephalosporins1st generation	Cefazolin	9	14	5	56
Cephalosporins 3rdgeneration	Cefataxime	10	15	5	50
	Cefixime	-	10	4	67
Sulphonamides	Chloramphenicol	20	25	5	25
	Tetracycline	20	26	6	30
Aminoglycosides	Streptomycin	19	22	3	16
Quinolones/Fluoroquinolones	Sparfloxcin	26	28	2	8
	Oflaxcin	24	29	5	21
Penicillin combination	Piperacillin/Tazobactam	19	21	2	11
	Overall synergistic bacterial effect (%)			**37.83%**	

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

Table 1: Synergistic effect of different antibiotics with and without silvernanoparticles against *Micrococcus luteus*

Types of antibiotics	Name of the antibiotics	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold increase (%)
		Ab	Ab+AGNP'S		
β-lactams	Amoxicillin	-	12	6	100
Macrolides	Azithromycin	12	17	5	42
	Erythromycin	8	14	6	75
Cephalosporins1st generation	Cefazolin	-	10	4	67
Cephalosporins 3rdgeneration	Cefataxime	19	19	-	0
	Cefixime	-	14	8	133
Sulphonamides	Chloramphenicol	12	15	3	25
	Tetracycline	16	19	3	19
Aminoglycosides	Streptomycin	19	21	2	11
Quinolones/Fluoroquinolones	Sparfloxcin	23	23	-	0
	Oflaxcin	22	24	2	9
Penicillin combination	Piperacillin/Tazobactam	20	21	1	5
	Overall synergistic bacterial effect (%)			**40.5%**	

Table 2: Synergistic effect of different antibiotics with and without silver nanoparticles against *Pseudomonas sp.,*

Types of antibiotics	Name of the antibiotics	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold increase (%)
		Ab	Ab+AGNP'S		
β-lactams	Amoxicillin	-	12	6	100
Macrolides	Azithromycin	15	17	2	13
	Erythromycin	14	17	3	21
Cephalosporins1st generation	Cefazolin	8	11	3	38
Cephalosporins 3rdgeneration	Cefataxime	13	15	2	15
	Cefixime	-	10	4	67
Sulphonamides	Chloramphenicol	17	20	3	18
	Tetracycline	24	26	2	8
Aminoglycosides	Streptomycin	22	24	2	9
Quinolones/Fluoroquinolones	Sparfloxcin	20	23	3	15
	Oflaxcin	21	24	3	14
Penicillin combination	Piperacillin/Tazobactam	21	23	2	10
	Overall synergistic bacterial effect (%)			**27.3%**	

Table 3: Synergistic effect of different antibiotics with and without silvernanoparticles against *Staphylococcus aureus*

Types of antibiotics	Name of the antibiotics	Zone of inhibition (mm)		Increased Zone Size (mm)	Fold increase (%)
		Ab	Ab+AGNP'S		
β-lactams	Amoxicillin	-	9	3	50
Macrolides	Azithromycin	15	17	2	13
	Erythromycin	-	11	5	83
Cephalosporins1st generation	Cefazolin	-	9	3	50
Cephalosporins 3rdgeneration	Cefataxime	-	-	-	0
	Cefixime	-	-	-	0
Sulphonamides	Chloramphenicol	21	23	2	10
	Tetracycline	12	15	3	25
Aminoglycosides	Streptomycin	17	19	2	12
Quinolones/Fluoroquinolones	Sparfloxcin	-	10	4	67
	Oflaxcin	-	10	4	67
Penicillin combination	Piperacillin/Tazobactam	-	10	4	67
	Overall synergistic bacterial effect (%)			**37%**	

Table 4: Synergistic effect of different antibiotics with and without silvernanoparticles against *MDRS*

CONCLUSION

In the present work, marine *Streptomyces species* is capable of producing the silver nanoparticles extracellularly and these nanoparticles are stable inside the solution and revealed antimicrobial property against gram-negative as well as gram-positive MTCC pathogens and multi drug resistantpathogen.The conjugation of antibiotics[betalactams,Macrolides,Cephalosporins1st&3rdgeneration,Sulphonamides,Aminoglycosides,Quinolones/Fluoroquinolones and Penicillin combination] with silver nanoparticles would prevent development of resistance by microbes and enhance the antimicrobial property of the antibiotic and minimize the dosage of antibiotics against multi drug resistance pathogens. The biosynthesis of silver nanoparticles derived from microbial origin from marine source has lower toxicity and excellent biomedical application. The cytotoxicity activity of the silver nanoparticles will be analyzed in future study to find its biocompatibility with animal and human beings for drug designing .

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