SYNTHESIS, CHARACTERIZATION AND ANTIMICROBIAL EFFICIENCY OF *PSEUDOMONAS STUTZERI* MEDIATED SILVER NANOPARTICLES

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Abstract:

Biogenic mediated silver nanoparticles (Ag NPs) were synthesized by biological method using marine bacteria Pseudomonas stutzeri. Synthesized nanoparticles band gap energy, functional groups, morphology and size were studied by UV-vis spectroscopy, FTIR, SEM and XRD. From the UV-visible spectroscopy, the absorption peak was found at 420 nm. In SEM images revealed the spherical shaped silver nanoparticles and most of the particles were below 100 nm in size. In XRD analysis, it was confirmed that the silver nanoparticles are crystalline in nature with 45nm grain size, which was confirmed by the FTIR peak at 518cm^{-1} corresponding to the Ag vibration present in crystalline structure. Biogenic mediated Ag NPs growth inhibition efficiency against six bacterial and four fungal strains were studied. Synthesized NPs (15 and 30µL) showed an effective bactericidal and fungicidal activity against Aeromonas liquefaciens MTCC2645 (B1), Enterococcus faecalis MTCC439 (B2), Vibrio cholerae MTCC3906 (B6) and Candida albicans MTCC1637 (F1), Cryptococcus sp. MTCC7076 (F2), Microsporum canis MTCC3270 (F3), Trichophyton rubrum MTCC3272 (F4) respectively.

Keywords*: Silver nanoparticles, Marine bacteria, Antimicrobial activity, Scanning Electron Microscopy*

1. INTRODUCTION

Nanoparticles (NPs) serve as the fundamental building blocks for various nanotechnology applications. Nanotechnology and nanomaterials are played as a promising material and an ever increasing role in science, research and development (Pallavi *et al.*, 2022) as well as also in every day's life, as more and more products based on nanostructured materials are introduced to the market. Silver nanoparticles (Ag NPs) are one of the promising products in the nanotechnology industry. Silver nanoparticles can be synthesized by several physical, chemical and biological methods, in which, one of such promising process is green synthesis (Culp *et al.*, 2019). Chemically synthesized nanoparticles methods have been replaced by either phyto or microbial mediated *i.e.,* green synthesis which reduced the synthesized compound toxicity with enhanced product quality (Nadagouda *et al.*, 2012). Ag NPs has numerous applications such as antimicrobial coatings in lab and operation theatre clothes, biomedical devices and wound dressing which are continuously release a low level of silver ions to provide protection against bacteria (Vignesh *et al.*, 2015a; Tian *et al.*, 2020).

Various microbes from our environment are utilized for the well-being of the humans (Kolar *et al.*, 2001). From the past two decades, nanoscience explored various bactericidal and fungicidal process based on the attribution of metal nanoparticles high volume to surface ratio, shape and size (Blanc *et al.*, 2005; Morones *et al.*, 2005). Bacteria are well known to produce inorganic materials either intracellularly or extracellularly. Marine bacteria are concluded as a potential biofactory for the synthesis of metallic nanoparticles like gold, silver and cadmium sulphide (Jain *et al.*, 2011). The newly emerging nanotechnology based on the metallic oxide nanoparticles with unique shape,

size and interacting abilities paved the way for the emergence of new biomedical treatments against the infectious pathogens (Hawkey 2008).

Silver nanoparticles (Ag NPs) has strong bactericidal activity against gram positive as well as gram negative bacteria including multi-resistant strains (Ahmad *et al.*, 2003). Many textile industries coat Ag NPs into their products which allowed the nanoparticles attached to the filaments. When the nano-Ag fabricated cloth exposed to sweat which releases a low concentration of Ag ions and acts as an antimicrobial agent (Duran *et al.*, 2006). The main aim of our study was to synthesize the marine bacteria mediated silver nanoparticles through biological process. The morphology, size and band gap energies were calculated by using various characterization techniques such as UV, FTIR, XRD, EDS and SEM. The antimicrobial activity of the marine bacterial mediated Ag NPs also evaluated against various pathogens.

2. Materials and Methods

2.1. Microbial mediated Ag NPs synthesis

For the marine bacterial isolation, sea water sample are collected from Nagore shore during April 2016. *Pseudomonas stutzeri* bacterial species were isolated and inoculated in nutrient broth media prepared with 25mM silver nitrate ($AgNO_3$) purchased from Sigma-Aldrich. The broth was incubated for 12hrs on a shaker at room temperature. After incubation, the broth color changed and becomes turbid, that indicated the presence of Ag NPs in the culture. Finally, the biogenic Ag NPs was prepared through marine bacterial isolate.

2.2. Characterization of nanoparticles

2.2.1. Nanoparticles Spectroscopic analysis

Marine bacterial mediated Ag NPs are anlaysed by UV-VIS and fourier transform-infra red (FT-IR) spectroscopy. Biosynthesized Ag NPs kinetic behavior and the band gap energies are calculated by using Lambda 19, UV-visible spectrophotometer (Perkin Elmer) with scanning ranges of 200 to 800nm at 480mm/min scan speed. Bio-reducing properties of the biogenic synthesized Ag NPs are measured at 400-4000cm^{-1} transmittance in FTIR spectroscopy (Kim *et al.,* 2009).

2.2.2. Scanning electron microscope (SEM) and energy dispersive spectroscopy (EDS)

The synthesized nanoparticles size, morphology and compositional analysis are analyzed by SEM and EDS technique. Scanning Electron Microscopy (Joel JSM-6480 LV) is used to characterize the nanoparticle mean size and morphology. Energy dispersive X-ray spectroscopy (EDS) is used for the compositional analysis (phases) on the biogenic Ag NPs with the help of JEOLJSM 5800 instruments (Olaitan Ogunyemi *et al.* 2019).

2.2.3. X-ray diffraction studies

By using copper grid with calcined powder along with the biogenic Ag NPs is subjected to the Philips PAN analytical X ray diffraction (Netherland) machine at 40KV with 30mA with the scan ranges of 10.00 to 90.00 at 0.6/sec respectively.

2.3. Antimicrobial analysis

For the antimicrobial efficiency of the biogenic mediated Ag NPs, various gram positive, gram negative bacterial and fungal strains such as *Aeromonas liquefaciens* MTCC2645 (B1), *Enterococcus faecalis* MTCC439 (B2), *Klebsiella pneumonia* NCIM2883 (B3), *Micrococcus luteus* NCIM2871 (B4), *Salmonella typhimurium* NCIM2501 (B5), *Vibrio cholerae* MTCC3906 (B6) and *Candida albicans* MTCC1637 (F1), *Cryptococcus* sp. MTCC7076 (F2), *Microsporum canis* MTCC3270 (F3), *Trichophyton rubrum* MTCC3272 (F4) respectively. The cultures are procured from MTCC (Chandigarh) and NCIM (Pune, India). By using agar plate diffusion method, antimicrobial sensitivity of the

microbial strains is analyzed (Pandiyarajan *et al.*, 2013; Muthukumar *et al.*, 2015; Vignesh *et al.*, 2013).

For antibacterial and antifungal activities, muller hinton agar (MHA) and potato dextrose agar (PDA) respectively are prepared based on the standard procedures (Vignesh *et al.*, 2015a). Microbial samples are inoculated into the petridishes separately. 15 and 30µL of biogenic mediated Ag NPs are poured in each disc, separately. One separate disc without NPs used for negative control. Methicillin (10mcg) and Itraconazole (10mcg) antibiotics are used as positive control for bacteria and fungi respectively. Petriplates are incubated for 24 to 48hrs at 37±1°C and for 48 to 72hrs at 25±1°C for bacterial and fungal studies. After incubation, the zone of inhibition is measured (Beevi *et al.*, 2012; Lakshmi praba *et al.*, 2013).

3. Result and Discussion

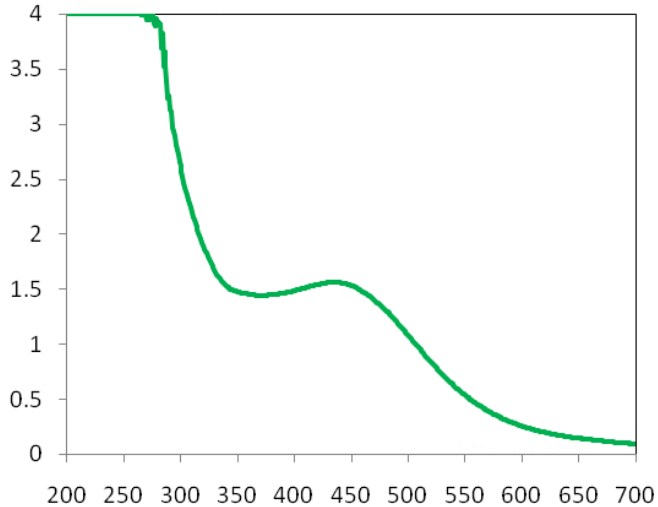
3.1. Biogenic mediated Ag NPs synthesis

The precursor silver nitrate ($AgNO_3$) solution has mixed with the *Pseudomonas stutzeri* marine bacterial extracts, the mixture are reduced into silver (Ag) nanoparticles. The mixture changed from white turbid to brown colour precipitate as silver nanoparticles. Marine bacteria are widely used as a potent source for the drug synthesis and development (Vignesh *et al.*, 2011). Bactericidal mechanism of nanoparticles are received a great attention due to their electrostatic effects on the cell membrane mainly due to their nano sizes. The effects are considered to be contributing towards enhancement of reactivity of silver nanoparticles surface.

3.2. UV-Vis spectroscopy analysis

The plasmon resonance of the silver nanoparticles was recorded. The UV-VIS spectroscopic studies revealed the presence of beard peaks at 420nm (Figure 1) whereas their band gap energy was calculated as 2.95eV. The absorption spectra of Ag nanoparticles is observed at 421nm (2.94eV). A remarkable broadening of peak at around 350nm - 480nm indicated the polydispersion of Ag particles with the broad band gap energies as 3.54eV – 5.17eV. Similar results reported by Huang *et al.* (2007) reported that the formation of silver nanoparticles when constant aqueous $AgNO_3$ at 50 ml, 1 mM with 0.1 g bio-mass produced silver nanoparticles as indicated by sharp absorbance at around 440 nm in *Cinnamomum camphora*.

Figure 1. UV-Vis spectra of biogenic mediated Ag NPs



3.3. FTIR and SEM analysis

Marine bacteria *Pseudomonas stutzeri* mediated Ag NPs FTIR spectra (Figure 2) revealed the strong bands at 3383, 2352, 1601 and 1404, 1113, 675, 518cm^{-1}. The band present at 2352cm^{-1} for O-H stretching corresponds to carboxylic acid, 1601cm^{-1} for stretching C=C corresponds to aromatic amino groups. The band at 675cm^{-1} corresponds to C-H

stretching of phenyl ring of substitution band, whereas the stretch for Ag-NPs was found around 518cm^{-1}. The morphological features of synthesized silver nanoparticles were studied by SEM analysis (Figure 3). SEM analysis revealed the presence of spherical structure.

Figure 2. FTIR Spectra of biogenic mediated Ag NPs

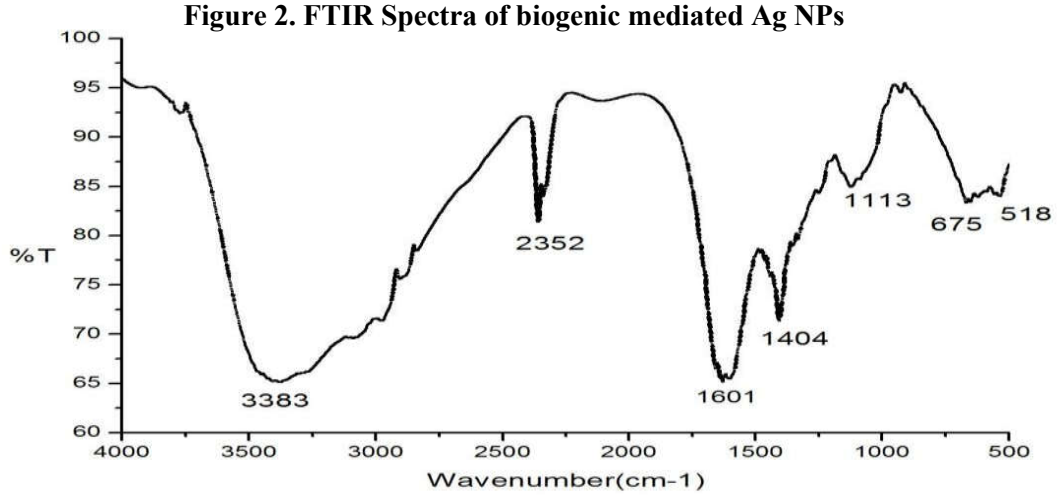
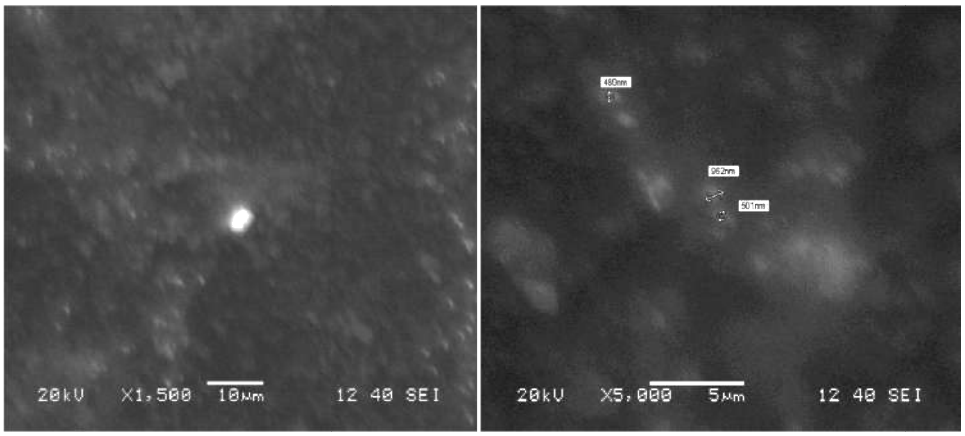


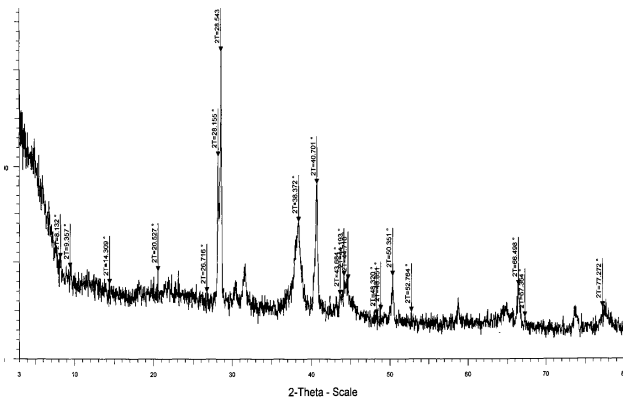
Figure 3. Scanning Electron Microscopic images of biogenic mediated Ag NPs



3.4. XRD analysis

X-ray diffraction (XRD) pattern of biogenic mediated Ag NPs were analyzed (Figure 4) and the peaks were observed at 2θ of 38°, 44°, 65° and 77° are corresponding to the Bragg's reflections such as (111), (200), (220) and (311) and their d value are calculated as 170, 266.95, 204.73 and 249.79 respectively. Other peaks were also observed along with the main peaks, which is due to the microbial isolates metabolites and ions. By using Scherrer constant value (0.94) in equation, the crystalline and grain size of the biogenic mediated Ag NPs. Marine bacteria *P. stutzeri* Ag NPs were found as 45nm. Similar XRD results were reported by green synthesized Ag NPs (Kero *et al.*, 2017; Vanaja and Annadurai 2012; Phillip 2011; Shankar *et al.*, 2003).

Figure 4. XRD spectra of biogenic mediated Ag NPs



3.5. Antimicrobial activity

Marine bacteria *Pseudomonas stutzeri* mediated Ag NPs (15 and 30µl) growth inhibition effect on six bacterial and four fungal species were reported. Among the six bacterial species, *Aeromonas liquefaciens* (B1), *Klebsiella pneumonia* (B3), *Salmonella typhimurium* (B5), *Vibrio cholera* (B6) were belongs to gram negative whereas *Enterococcus fecalis* (B2), *Micrococcus luteus* (B4) were belongs to gram positive bacteria. In bactericidal activity, high concentration (30µl) of biogenic mediated Ag NPs showed significant growth inhibition on *Aeromonas liquefaciens* (B1), *Enterococcus fecalis* (B2) and *Salmonella typhimurium* (B5) as 15.5±0.70, 15.1±0.59 and 18.3±0.62mm respectively than compared to the positive control (Methicillin) group as 14.7±0.82, 8.9±0.66 and 16.6±0.79mm.

Similarly, in fungicidal activity, all the treated fungal strains showed significant growth inhibition than positive control (Itraconazole) as dose-dependent nature. *Candida albicans* (F1) growth inhibition was observed as 11.7±0.88 and 13.7±0.68mm for 15 and 30µl concentration of biogenic mediated Ag NPs. Higher concentration (30µl) of biogenic mediated Ag NPs showed high fungicidal activity (growth inhibition) as 16.8±0.65, 14.8±0.79 and 14.8±0.69mm for *Cryptococcus* sp. (F2), *Microsporum canis* (F3) and *Trichophyton rubrum* (F4) strains respectively.

Figure 5. Antimicrobial efficiency of biogenic mediated Ag NPs

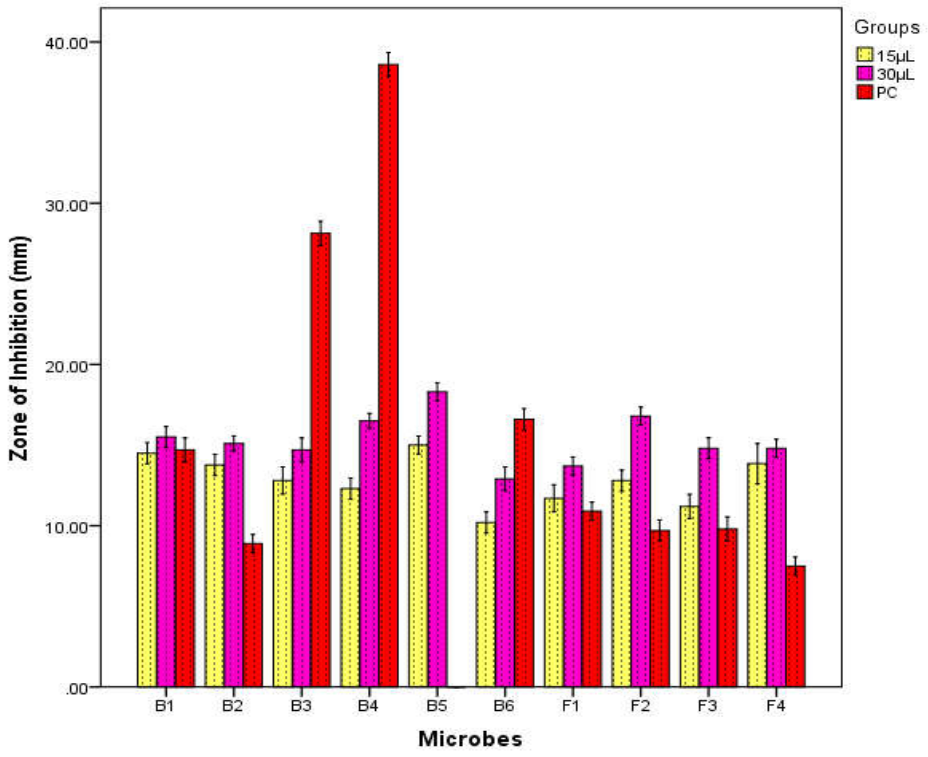


Table 1. Antimicrobial activity of *P. stutzeri* mediated Ag NPs

S.No	Microorganisms	Zone of inhibition (mm)		
		Biogenic mediated Ag NPs		PC
		15µL	30µL	
1	*Aeromonas liquefaciens* B1	14.5±0.78	15.5±0.70	14.7±0.82
2.	*Enterococcus fecalis* B2	13.7±0.82	15.1±0.59	8.9±0.66
3.	*Klebsiella pneumoniae* B3	12.8±0.90	14.7±0.81	28.2±0.73
4.	*Micrococcus luteus* B4	12.3±0.79	16.5±0.55	38.6±0.83
5.	*Salmonella typhimurium* B5	15.6±0.69	18.3±0.62	0
6.	*Vibrio cholera* B6	10.2±0.76	12.9±0.84	16.6±0.79
Fungi				
7.	*Candida albicans* F1	11.7±0.88	13.7±0.68	10.9±0.69
8.	*Cryptococcus* sp. F2	12.8±0.76	16.8±0.65	9.7±0.75
9.	*Microsporum canis* F3	11.2±0.89	14.8±0.79	9.8±0.80
10.	*Trichophyton rubrum* F4	12.9±0.79	14.8±0.69	7.5±0.63

Positive control (PC): Bacteria-Methicillin (10mcg/disc); Fungi-Itraconazole (10mcg/disc)

Silver nanoparticles have a great bactericidal effect against several bacteria including multi resistant strains and also considered as potential antifungal agent (Zeng 2007). Anitha et al (2011) reported that Ag nanoparticles have exhibited considerable activity against some human pathogens. The antimicrobial property of silver is found to be the best among different metals in the following order Ag>Hg>Cu>Cd>Cr>Pb>Co>Au>Zn>Fe>Mn>Mo>Sn (Petica, 2008). Ag ions and Ag-based compounds have strong antimicrobial effects (Furno, 2004), and also various researchers reported the utilization of inorganic compounds for nanoparticles synthesis resulted as an effective antimicrobial agent (Hamouda, 2000). Current study results enlighten that the marine bacteria *P. stutzeri* act as a metal reducing agent which was used for cost effective biogenic synthesis of metal nanoparticles with significant bactericidal and fungicidal properties.

Conclusion

Silver nanoparticle were synthesized by using of *Pseudomonas stutzeri* marine bacteria through biological method. The Surface Plasmon Resonance (SPR) property of biogenic mediated Ag nanoparticle was studied by UV-Vis spectroscopy (421nm with 2.94eV band gap energy). Nanoparticle morphology were observed as spherical structure. FTIR, XRD results concluded the marine bacteria *P. stutzeri* mediated Ag NPs nanoparticle observed

as crystalline structure with grain size as 45nm. The antimicrobial assay revealed the antibacterial and antifungal activities against B1, B2, B6 and F1, F2, F3, F4 strains respectively.

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