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Biogenic synthesis of silver nanoparticles mediated by *Acinetobacter indicus* and its biomedical applications

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A B S T R A C T

Nanotechnology, an attractive branch of science deals with smaller particles with incredible efficiency. The applications of nanoparticles were widely distributed in all fields of science to enhance the reaction with ease. Despite of chemical and physical method of synthesis, biological methods has gained importance due to its less toxicity and cost efficiency. In the present study, silver nanoparticles (Ag NP's) were synthesized using the culture supernatant of *Acinetobacterindicus*VLE-1 isolated from paper mill effluent. The synthesized nanoparticles were characterized using UV- visible spectroscopy, FTIR, XRD, SEM and TEM analysis. Biogenic Ag NP's were evaluated for its antagonistic ability against selected pathogenic bacterial strains such as *Bacillus cereus, Streptococcus pyogenes, Shigella dysentriae, Escherichia coli and Proteus vulgaris*. Maximum zone of inhibition was recorded against *Escherichia coli* and *Shigella dysentriae* when compared with the control. The nanoparticles were determined for its larvicidal activity against *Culex* mosquito larvae at various concentrations. Larvicidal activities were observed to be directly proportional to the concentration of biogenic Ag NP's.

Introduction

Nanoparticle is a unique subset of the extensive scientific research area called as nanotechnology. Nanoparticles are those particles ranging in size from 10 nm to 100 nm. Currently, they are gaining importance due to its use vast array of applications such as medicine, drug delivery, information, energy and environmental technologies (Murphy, 2008). Nanoparticles can be classified into two types viz engineered nanoparticles and non-engineered nanoparticles. Engineered nanoparticles are those created or synthesized artificially such as silver nanoparticles, gold nanoparticles etc for their use in several techniques where as non-engineered nanoparticles are those that are freely available in the environment such as atmospheric nanoparticles that are produced during combustion, aerosols etc. Both engineered and non-engineered nanoparticles pose their uses in several industries (Wagner *et al.*, 2014). Several techniques have been developed to synthesize nanoparticles such as chemical mediated synthesis, gas and liquid phase process etc. Despite the above mentioned "Bio-mediated (Biogenic) synthesis" of nanoparticle has been growing from last decade to develop eco-friendly

technologies in material synthesis. Biosynthesis of nanoparticle from microorganisms, enzymes, fungus, and plants or plant extracts has been considered as animpeccable alternative for the chemical mediated synthesis due to their cheap cost and economically benign nature (Phanjom *et al.,* 2012). Further the chemical and physical synthesis of nanoparticles was found to involve hazardous materials that impart toxic impact to environment (Okuyama *et al.,* 2004).

Microbial synthesis of nanoparticles is considered to be a highly efficient process since it involves biological entity in synthesis, its cost effective and it does not impart any side effects to both biotic and abiotic factors. Several microorganisms such as *Pseudomonas stutzeri*(Klaus *et al.,* 1999), *Escherichia coli* (Du *et al.,* 2007), *Serratia marcescens* (Karthika et al 2015), *Shewanellaalga* (Konishi *et al.,* 2007), *Chromohalobacter salexigens* (Tharanya et al 2015), Yeast cells, *Actinomycetes* sp., (Thirumangai *et al.,* 2015), etc.have been explored for the synthesis of nanoparticles. Nature has opened several ways for fabricating nanoparticles in smaller size. In 1999, Klaus and his co-workers demonstrated bacterial mediated biosynthesis of nanoparticles after identifying the accumulation of silver inside the bacterial cell (Klaus *et al.,* 1999). Reduction of metal ions to form nanoparticles by bacteria extracellularly may occur using any one of the two methods. Reduction may occur by the involvement of biomolecules liberated by the bacterial cells into the culture medium or the bacterial cell itself may for the nanoparticles inside the cell wall and liberate it outside the system (Mahdieh *et al.,* 2012). Interestingly, reduction of metal ions into nanoparticles can also be mediated in the absence of cell biomass, that is the bacterial supernatant can be used for the synthesis of nanoparticles that comprises of active biomolecules secreted by the bacteria. In the present study, silver nanoparticles have been synthesized by bacterial species and their characterization has been performed. This study highly emphasizes on the antagonistic efficacy of bacterial mediated silver nanoparticle against the selected clinical pathogens and larvicidal efficacy of synthesized material against *Culex* mosquito larvae.

Materials and Methods

Sample Collection

Effluent samples were collected from paper mill contaminated sites in and around Erode, Tamilnadu. Samples were collected in sterile polythene containers and transported to laboratory aseptically to avoid contamination. The transferred samples were kept in air tight container and stored in refrigerator at 4ºC for future use.

Isolation of bacterial strains

Samples were serially diluted to varying concentration and about 0.5 ml of the diluted sample were plated on freshly prepared nutrient agar plate maintaining at pH 7. The agar plates were incubated at 37º C for 48 h in an incubator. Following incubation, morphologically distinct colonies (size, shape and color) were selected and streaked over the above-mentioned medium until pure cultures were obtained. The pure colonies were designated as VLE 1 to VLE 3and maintained as glycerol stock at 4 ºC for further use.

Synthesis of Silver Nanoparticle

To 100 ml of freshly prepared nutrient media, loopful cultures of the isolates (VLE-1 to VLE-3)were inoculated and incubated at 37 ºC for 24 h in shaking incubator at 150 rpm. Following incubation, the grown cultures were centrifuged at 10,000 for 15 minutes. The

supernatantswere stored in a sterile screw cap tube for synthesis of nanoparticles. To 10 ml of culture supernatants, 5 ml of silver nitrate ($AgNO_3$) solution (10 mM) was added and incubated at 30 ºC for 24 h. Control was maintained without the addition of culture broth. Both the test solutions were kept in dark to avoid undesired photochemical reactions during the study. After 24 h of incubation, the solutions were observed for colour change from yellow to reddish brown which confirms the formation of AgNP's. The silver nanoparticles (Ag NP's) were purified by centrifugation at 10,000 rpm for 5 min twice, and the samples were collected for further characterizations(Karthika *et al.,* 2015). The potential strain was further subjected to phenotypic, microscopic and morphological identification.

Characterization of biogenic Ag NP's

UV-Vis Spectroscopy

The efficiency of biogenic approach in reducing Ag ions was evident by the appearance of brown colour which confirms the formation of silver nanoparticles in reaction mixture. The solution was subjected to measuring the absorbance against distinct wave lengths to confirm the formation of silver nanoparticles using UV-Vis Spectroscopy. Formation of silver nanoparticles is easily detected by spectroscopy since the coloured nanoparticle solution shows a peak at ~400 nm. In this study, Jasco spectrophotometer (V- 730) was used to measure the optical density of solution (Pugazhendhi *et al.,* 2017).

Fourier Transform Infra-Red Spectroscopy (FTIR)

To remove any free biomass residue or compound that is not capping the ligand of the nanoparticles, after complete reduction, synthesized silver nanoparticles were concentrated by repeated centrifugation (3 times) at 15,000 rpm for 20 min. The supernatant was replaced by distilled water each time. Thereafter, the purified suspension was freeze dried to obtain dried powder. The dried biogenic nanoparticles were made into KBr pellet and the functional groups were analysed using ALPHA FT-IR Spectrometer (from Bruker, Germany). Presence of various functional groups were determined by viewing peaks between the region 4000 cm^{-1} to 500 cm^{-1} (Guan *et al.,* 2018).

X- Ray Diffraction (XRD) Analysis

The reduced solution was centrifuged at 8000 rpm for 40 min and resulting supernatant was discarded and pellet obtained was re-dispersed in deionized water. Non-adsorbed substances were removed from the nanoparticles by repeated centrifugation. Thus, obtained pellet was purified and dried. The dried pellets were subjected to X-ray diffraction (XRD) analysis. For XRD studies, dried Ag NP's were coated on XRD grid, and the spectra were recorded by using Phillips PW 1830 instrument operating at a voltage of 40 kV and a current of 30 mA with Cu Kα1 radiation (Sadhasivam *et al.,* 2012).

Scanning Electron Microscopy (SEM)

The particle size and morphology of the silver nanoparticles were examined using Scanning Electron Microscopic observations. SEM measurements were performed on a JEOL JSM 6390 instrument operated at an accelerating voltage at 15kV. The sample was sonicated prior to examination for uniform distribution(Tharanya *et al.,* 2015).

Transmission Electron Microscopic analysis

The synthesized nanoparticles were subjected to TEM analysis for determining its size and

morphology. The biogenic Ag NP's were measured using Transmission electron microscopy – Hitachi H-7100) using an accelerating voltage of 120 kV and methanol as solvent (Tharanya *et al.,* 2015).

Antibacterial activity:

Antibacterial activity of biosynthesized AgNP's has been studied against the selected pathogenic strains *Bacillus cereus, Streptococcus pyogenes, Shigella dysentriae, Escherichia coli and Proteus vulgaris.* To determine the antibacterial efficacy of silver nanoparticles, Muller Hinton agar plates were made freshly and wells were made using sterile well cutter. The above-mentioned pathogenic strains were swabbed over the agar media using sterile cotton swab. Test wells were inoculated with synthesized biogenic Ag NP's (100 µg/ml) and controls were maintained. The plates were incubated in incubator at 37 ºC for 24 h and left undisturbed. Following incubation, the plates were observed for zone of inhibition (mm) (Shanmuga Praba *et al.,* 2015).

Larvicidal activity of Biogenic Ag NP's against *Culex* mosquito

Antagonistic activity of Ag NP's was investigated towards the *Culex* larvae. The above-mentioned samples were treated with varying concentration of Ag NP's ranging from (10 µg/ml, 25µg/ml, 50µg/ml, 75µg/ml and 100µg/ml). 20 late third and fourth instar larvae of *Culex* mosquitoes were collected from Department of Zonal Entomology, Vellore, Tamilnadu. Collected larvae were equally distributed in 5 sterile trays containing dechlorinated water with increasing concentration of synthesized Ag NP's. Average larval mortality in the test and control samples were observed after 24 h of incubation. Triplicates were maintained and the rate of mortality in percentage was calculated using the mortality formula

Mortality percentage = No. of larvae killed / Total No. of Larvae tested X 100.

Results and Discussion

Synthesis of Silver Nanoparticle

Synthesis of silver nanoparticles were done using the bacterial cell free culture supernatant where, the supernatant was mixed with 5 ml of $AgNO_3$ (10 mM) and incubated for 24 h. Following incubation, the reaction mixture was observed for visible color change where, the mixture has been changed to reddish brown from yellow color which confirmed the reduction of silver metal into silver nanoparticles. The suspension was completely dried on hotplate and powder form of the silver nanoparticles were collected.

Identification and characterization of the isolate

Among various colonies, single colony based on its ability to synthesis silver nanoparticle was selected and sub cultured on sterile nutrient media. The pure culture was subjected to microscopic, biochemical and molecular analysis. The isolate VLE-1 was found to be Gram negative coccobacillus in nature. The biochemical analysis of the isolate was tabulated in table 1.

Characterization of biogenic Ag NP's
UV-Vis Spectroscopy

UV- Visible absorption spectra of synthesized silver nanoparticles was subjected to UV-Visible spectroscopic analysis between the range 350 to 600 nm. Reduction of silver ions to silver nanoparticles was confirmed by visible color change of the reaction mixture from yellow (broth) to reddish brown (synthesized Ag NP's). In this study, absorption behavior arises due to surface plasmon resonance (SPR), was notices as

sharp peak at 464 nm which shows the presence of silver nanoparticle in the sample (Fig 1).

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR analysis of the synthesized silver nanoparticles (AgNP's) were carried out to determine the functional groups of active molecules involved in reducing and capping the silver metal to silver ions in the synthesized material. The FTIR spectra of the silver nanoparticle was shown in Fig 2in which the spectrum of the bacteria mediated silver nanoparticles exhibited transmittance band at 3259 cm^{-1} represents the presence of O-H free bond, aldehyde C-H stretching (2953 $cm^{-1}$and 2920 cm^{-1}). Peak at 1587 cm^{-1} corresponded to amide I, arising due to carbonyl stretch in proteins. Peak at 1041 cm^{-1} corresponded to C-N vibrations of the amine. Hence, it proves that the synthesis of biogenic Ag NP's involving in the biological reduction of the $AgNO_3$ was mediated by the bacterial metabolites.

X- Ray Diffraction (XRD) Analysis

X-ray diffraction is an important method to determine the nature of the synthesized material from the X-ray diffraction pattern. It enables to understand the structure of crystalline material and used for the lattice parameters analysis of single crystals, or the phase, texture or even stress analysis of samples. The crystal structure of the AgNP's was analyzed by X-ray diffractometer. Formation of silver nanoparticles synthesized using the culture supernatant of *Acinetobacter indicus* StrainVLE-1 was supported by X-ray diffraction measurements. X-ray diffractogram of synthesized AgNP's showed distinct diffraction peaks at 25.46°, 31.90°, 37.15°, 37.90°, 38.73°, 48.23°, 54.07°, 55. 26°, 62.34°, 62.87°, 68.95°, 70.45°, 75.22° and 76.20° indexed to the planes 110, 111, 211 and 220 (Fig. 3).

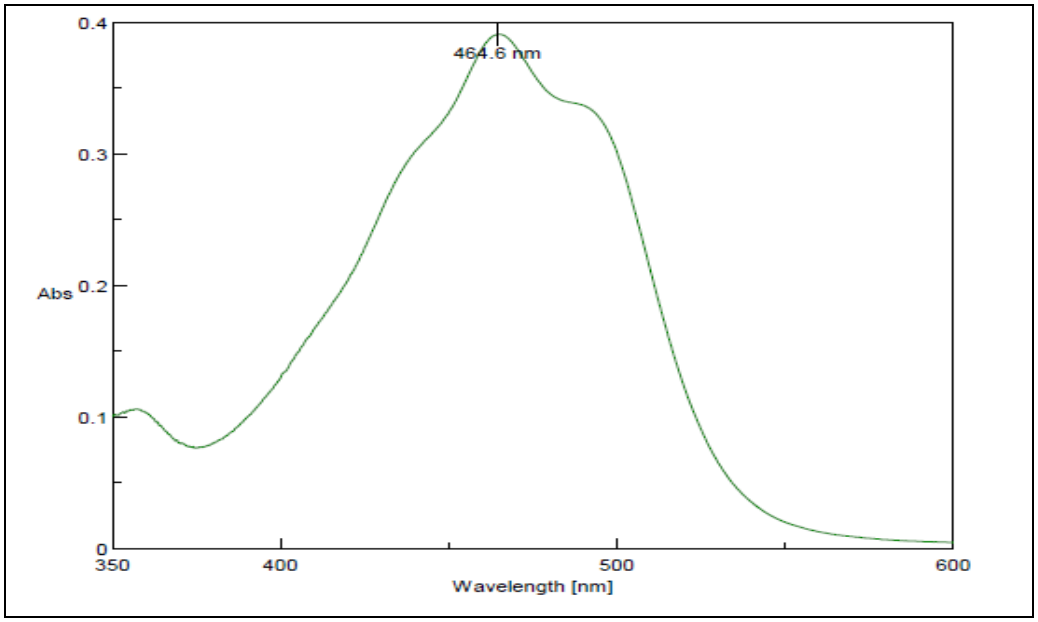


Fig.1 UV-Vis spectrum of Ag NP's biosynthesized using *Acinetobacter indicus* VLE-1

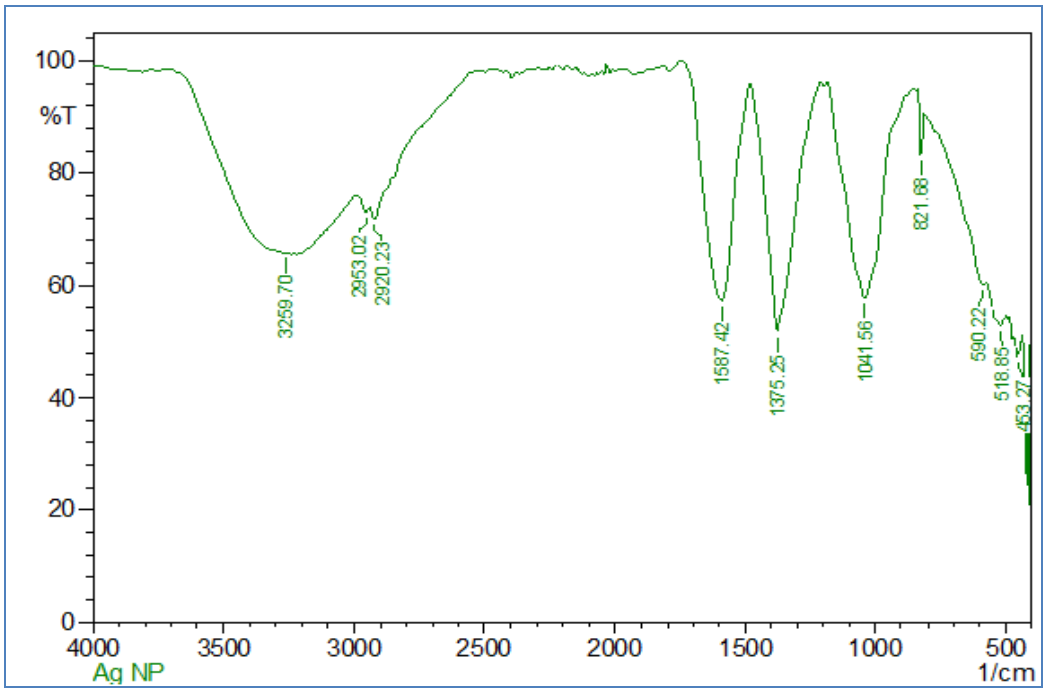


Fig.2 FTIR spectra of Ag NP's biosynthesized using *Acinetobacter indicus* VLE-1

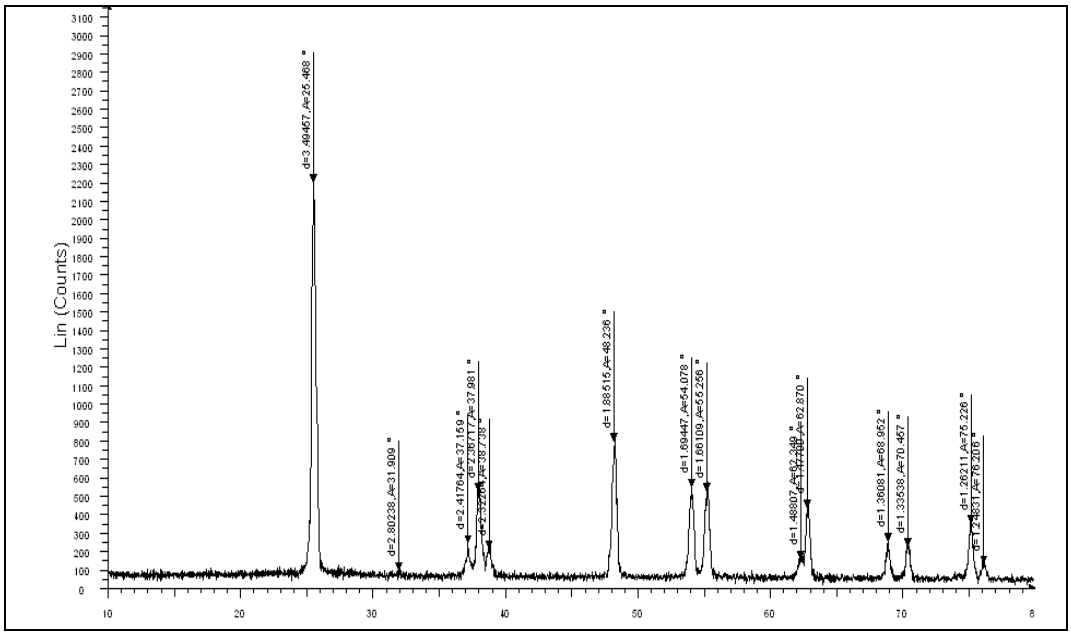
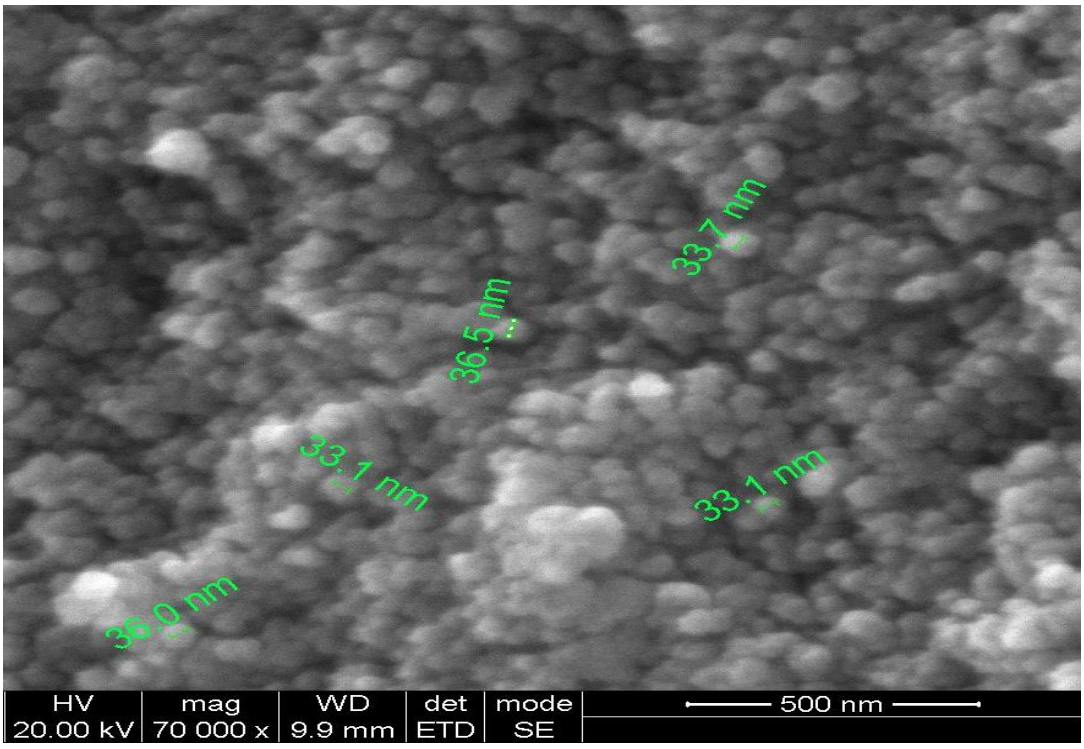


Fig.3 XRD analysis of Ag NP's biosynthesized using *Acinetobacter indicus* VLE-1



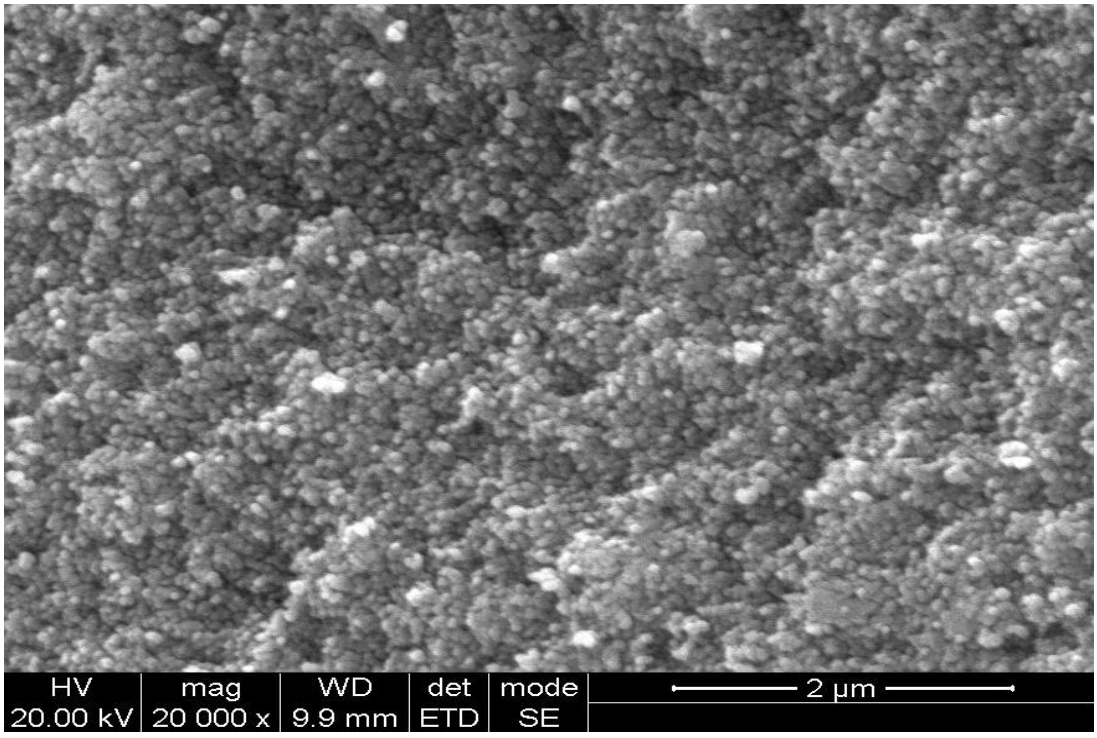


Fig.4 SEM Images of Ag NP's biosynthesized using *Acinetobacter indicus* VLE-1

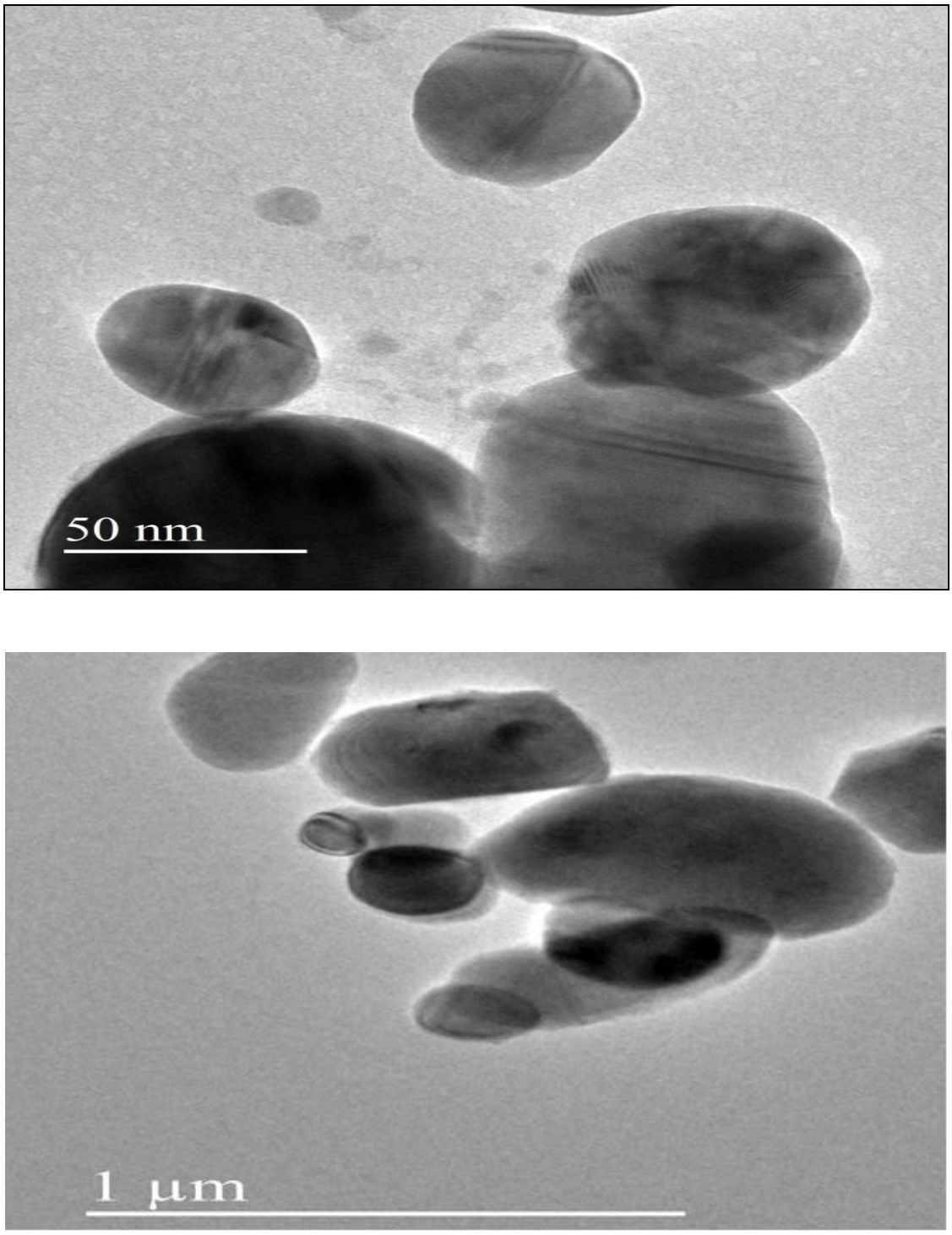


Fig.5 TEM Images of Ag NP's biosynthesized using *Acinetobacter indicus* VLE-1

Table.1 Morphological, Physiological and Biochemical Characteristics of strain VLE-1

S. No	Test	Observations
1	Morphology	
	Grams staining	Negative
	Cell shape	Cocco-bacilli
	Motility	Non-Motile
	Cell arrangement	Single, paired and short chains
2	Colony Characters on Nutrient agar	
	Colony morphology	Spherical
	Colony size	2.5 -3.0 mm
	Colony elevation	Raised
	Colony density	Dull, opaque
	Colony edge	Entire
	Pigmentation	Greyish white
3	Sugar Fermentation	
	Lactose	Negative
	Maltose	Positive -Acid
	Glucose	Positive -Acid
	Sucrose	Positive -Acid
4	IMVIC	
	Indole	Negative
	Methyl Red	Positive
	Voges Proskauer	Positive
	Citrate	Negative
5	Enzyme Reaction	
	Urease Production	Negative
	Catalase Activity	Positive
	Oxidase	Negative
	Coagulase	Negative
6	H_2S Production	Negative
7	Caseinase Activity	Negative
8	Gelatin	Negative

Table.2 Antibacterial activity of Ag NP's against selected bacterial pathogens

S.No	Bacterial pathogens	Zone of Inhibition (mm)	
		Bio Ag NP's (100 µg/ml)	Standard Antibiotics
1.	*Bacillus cereus*	09 ± 0.2	14 ± 0.2
2.	*Streptococcus pyogenes*	11 ± 0.3	13 ± 0.2
3.	*Shigella dysentriae*	14 ± 0.3	13 ± 0.1
3.	*Escherichia coli*	15 ± 0.2	14 ± 0.2
4.	*Proteus vulgaris*	10 ± 0.1	12 ± 0.3

Table.3 Larvicidal activity of AgNP's synthesized using *Acinetobacter indicus* VLE-1

Conc. of Ag NP's (µg/ml)	Mortality of larva after 24 h Replica N=3			Average	Mortality (%)
	Trial 1	Trial 2	Trial 3		
Control	0	0	0	0	0
10 µg/ml	3	4	4	3.66	18.30
25 µg/ml	7	7	8	7.33	36.65
50 µg/ml	10	11	10	10.33	51.65
75 µg/ml	14	13	15	14.0	70.00
100 µg/ml	16	17	16	16.33	81.65

Scanning electron microscope (SEM) analysis

The SEM images of AgNP's obtained with the culture supernatant of *Acinetobacter indicus* were also subjected for Scanning Electron Microscopy to determine the size and morphology of the synthesized nanoparticles.SEM analysis revealed that the average size of the nanoparticles ranged between 33.1 nm to 36.7 nm with interparticle space. The shape of the synthesized particles was observed to be spherical and ellipsoidal with uniform distribution (Fig4).

Transmission Electron Microscope (TEM) analysis

Morphological features of the synthesized silver nanoparticles were also investigated by TEM analysis and the results represents the size and distribution of the nanoparticles. The size determined using TEM shows the particles ranges from 20 nm to100 nm. This analysis also confirms that the synthesized silver nanoparticles were spherical and few with irregular shape (Fig 5).

Antibacterial activity

Antibacterial activity of the synthesized biogenic Ag nanoparticles was carried out by conventional Kirby-Bauer well diffusion method against *Bacillus cereus*, *Streptococcus pyogenes, Shigella dysentriae, Escherichia coli and Proteus vulgaris.* Silver nanoparticles exhibited maximum antagonistic activity towards *Escherichia coli* (15 mm) and *Shigella dysentriae* (14 mm) when compared with the standard antibiotics. Notable antagonistic effect was observed for *Streptococcus pyogenes*(11 mm)*,Proteus vulgaris* (10 mm) and *Bacillus cereus* (9 mm) (Table 2).

Larvicidal activity of Biogenic Ag NPs against *Culex* mosquito

Larvicidal activity of synthesized silver nanoparticles were studies against *Culex* mosquito larvae. Larvicidal activity of the nanomaterial increased with increasing concentration of biogenic Ag NP's. Mortality rate reached 50% at 50 µg/ml and highest rate of mortality was recorded at 100 µg/ml. Interestingly, notable antagonistic activity of Ag NP's against *Culex* larvae was encountered at minimal concentration (10 µg/ml) with the rate of 18% and maximum concentration (100 µg/ml) kills larvae at the rate of 81. 66% (Table 3). Thus, this shows that the synthesized Ag NP's at lower concentration also has the efficacy to kill the *Culex* mosquitoes at larval stages.

Silver nanoparticles has received tremendous significance among the several other nanoparticles due to its less toxicity, easy synthesis and higher applications in medicine and environmental applications. Biological methods of nanoparticle synthesishave gained much attention due to its compatibility to ecosystem when compared with the chemical methods. In this study, bacteria isolated from paper mill effluents samples were cultivated and the extracellular components were investigated for the synthesis of nanoparticle with antagonistic efficacy against clinical pathogens. Cell free supernatant of *Acinetobacter indicus*was used for the synthesis of silver nanoparticles.Similarly, cell free supernatant of *Thermoactinomyces*sp. was reported to synthesize spherical silver nanoparticle ranging from 20-40 nm in size (Deepa *et al.*, 2013). Culture supernatant comprising nutrient broth and other bacterial metabolites enables the reduction of Ag metal to Ag nanoparticles. Knowledge about the reduction of silver ions and formation of silver nanoparticles were still not clear, but it is believed that protein molecules and enzyme, including nitrate reductase enzyme which may act as good regulating agent in the formation of silver nanoparticles.

Synthesis, formation and stability of nanoparticles are determined using UV-Vis spectroscopy. Excitation of SPV leads to the formation of AgNP's which was confirmed by the visible color change from yellow to reddish brown. This conversion remains as the signature feature of AgNP's in the solution (Chung *et al.,* 2016). In the present study, synthesis of silver nanoparticle was confirmed by the presence of sharp peak at 464 nm. Broad spectral length denotes the poly-dispersed silver nanoparticles. The reduction of silver ion to silver nanoparticles was confirmed by the peak between the respective spectral range. Similar results were recorded in the green synthesized silver nanoparticles where, leaf extract of *A. indica* were used and the plasmon resonance band was observed between 436 – 446 nm (Ahmed *et al.*, 2015). FTIR results of the synthesized silver nanoparticle confirmed the presence of functional groups such as alcohols, aldehydes, amides and amines. Similar spectral results were reported by Sharma et al. (2018) in which silver nanoparticles were synthesized using plant extracts. Phase angle and crystalline nature of the synthesized bacteria mediated silver nanoparticles exhibited diffraction peaks at 25.46°, 31.90°, 37.15°, 37.90°, 38.73°, 48.23°, 54.07°, 55. 26°, 62.34°, 62.87°, 68.95°, 70.45°, 75.22° and 76.20° indexed to the planes 110, 111, 211 and 220. The synthesized nanoparticles were crystalline in nature. Anandan et al. 2019 also reported similar results from silver nanoparticles synthesized using aqueous leaf extract of *Dodonaeaviscosa.* Scanning electron microscopic analysis reveals that the particles were spherical and ellipsoidal with average size ranging from 33.1 nm to 36.5 nm.

TEM analysis also confirmed that the Ag NP's

were spherical and the size ranges within 100 nm. Antibacterial activity of the synthesized nanoparticles exhibited maximum resistance towards *Escherichia coli* (15 mm) and *Shigella dysentriae* (14 mm). Notable antagonistic activity was found for other strains used in the study. Similarly, silver nanoparticles synthesized using *Coffea arabica* seeds exhibited effective antibacterial ability against *E. coli* and *Staphylococcus aureus* (Dhand et al. 2016). On the other hand, Ag NP's synthesized using banana peel extract expressed efficient antagonistic ability against Gram negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) when compared with Gram's positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) (Ibrahim, 2015). Larvicidal activity of biogenic silver nanoparticle were found to have higher potential in killing the *Culex* mosquito at larvae stage. The mortality was observed at both minimum (10 µg/ml)as well as maximum concentration (100 µg/ml). Mortality rate of *Culex* larvae was higher at 100 µg/ml (81.65%).

Ever since the discovery of the first antibiotic Penicillin, bacterial community acquired resistance through various evading mechanisms. MDR against the bacterial pathogens has emerged as the most dreadful concern among the human population around the globe. This study embodies, an efficient invention which act as proficient antibacterial agent against infections caused by *Bacillus cereus, Streptococcus pyogenes, Shigella dysentriae, Escherichia coli and Proteus vulgaris*. Biogenic nanoparticles also remain as a potential antimicrobial agent that sensitize resistance against bacterial pathogens. This novel approach also aids to overcome drug resistance among pathogenic bacteria. These biogenic nanoparticles also mediate cytotoxicity via cell wall disruptions and possess larvicidal activity against*Culex* mosquitoes. Hence, it is essential to focus on the developing applications of Ag NP's in environmental and medicinal sectors.

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