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Nanosilver Fabrication Mediated by Exopolysaccharides from *Pseudomonas fluorescens* and Its Biological Activities

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ABSTRACT

Synthesis of silver nanoparticle (SNP) has become an emerging trend in the field of nanotechnology. Here a greener method was approached to develop silver nanoparticles using extracted exopolysaccharides (EPS) from *Pseudomonas fluorescens*, a soil isolate from rhizosphere of the medicinal plant, *Catharanthus roseus*. The exopolysaccharide acted as a reducing and stabilizing agent in converting silver nitrate to silver nanoparticles. The fabricated SNPs were characterized by UV-Visible Spectroscopy, Scanning Electron Microscopy (SEM) and FT-IR Spectroscopy. The FT-IR analysis revealed that proteins present in the polysaccharide served as a capping agent. This simple process was carried out for 5 days at room temperature at optimum pH 7. The dried SNP sample is found to be stable for more than 6 months. The present work also focused on SNPs' antimicrobial effect by agar well diffusion technique and antioxidant activity by DPPH method. The EPS reduced SNP was found to be more active against Gram negative than Gram positive and also worked in inhibiting *Candida* sp. DPPH radical scavenging effect of SNP was highly effective than standard at a maximum concentration of 1mg/ml. The investigation thus showed that EPS stabilized SNP could be employed in several pharmaceutical purposes.

Keywords: *Pseudomonas fluorescens*, exopolysaccharide, silver nanoparticle, SEM, EDS, Agar diffusion, DPPH scavenging activity

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INTRODUCTION

The emergence and rapid hike of multidrug resistant pathogens pose a jeopardizing effect on humans and this has led the researchers to discover new effective drugs. Nanotechnology is a recent burgeoning area serving in various areas especially in pharmaceutical field. Nanoparticles exhibit unique physiochemical features like good conductivity, magnetic, electrical, optical and catalytic properties and also biological functionalities based on salient characteristics like morphology and size when compared to bulk macro materials [1-3]. These particles are generally 10-100nm in size [4] and have high surface area to volume ratio [2]. Various noble metals are involved in the synthesis of nanoparticles like titanium, copper, platinum, silver, gold, zinc, magnesium [5, 6].

Metallic silver as an antimicrobial agent had been in use for several centuries [7]. It has been reported that silver is highly toxic to microorganisms and less harmful to mammalian cells [8]. Silver nanoparticles (SNP) are found to be efficiently applied in chemistry as a good catalyst, in electronics and in therapeutics as anticancer, immunomodulatory, antibacterial, antifungal and antioxidant [3, 9-12]. Due to their tiny structures, they allow themselves to interact easily with the cellular components thus increasing their antimicrobial efficiency. Numerous chemical methods to synthesize SNPs are available namely thermal deposition of silver, radiation mediated, sonication and chemical reduction of silver compounds [4, 13, 14] . These methods are harmful, cost intensive and highly reactive which impart malicious effect on environment and mankind [7]. Hence green synthesis is the latest and advanced approach for the generation of silver nanoparticles which could be ecofriendly, cost effective, carried out with no high temperature and energy and easily scaled up [15, 16].

Biological synthesis of SNP involves naturally occurring polysaccharides. These biogenic nanoparticles are water soluble and biocompatible [15]. Several plant and animal polysaccharides like starch, agar, alginate, chitosan had been used in biosynthesis of SNPs as reducing and stabilizing agents[2,5.] Microorganisms also synthesize a wide spectrum of multi functional polysaccharides that are intense bioactive substances employed extensively in textile, food, leather, pharmaceutical industries[17-21]. Extracellular or exo-polysaccharides (EPS), secreted outside the cell, are high molecular weight polymers of monosaccharides [17]. They are renewable in nature, non-toxic and biodegradable [22]. EPS exhibits remarkable thickening and shear thinning properties and display high intrinsic viscosity. Most of the EPS are negatively charged; it could be indulged in preparation of positively charged nanoparticles through polyelectrolyte

complexation or ionotrophic gelation [23], thus finding its new niche in nanobiotechnology. Presence of hydroxyl (OH^+) ions in polysaccharide plays the most essential role in reduction of silver ions to silver nanoparticles[2,7]. Additionally, mucoadhesion property of EPS provides greater neutral coating with low surface energy and restricts non specific protein receptor recognition[1].

The present study aimed at exopolysaccharide mediated silver nanoparticles synthesis by reduction of silver nitrate solution to SNP. This is the first report on the nano silver generation from a rhizosphere soil isolate of *Pseudomonas* sp. The fabricated SNPs were characterized by UV-Visible Spectroscopy, SEM analysis and FT-IR spectroscopy. An attempt was also made to examine in vitro antibacterial and antioxidant activities of SNP.

MATERIALS AND METHOD

Microbial Strain

The culture was isolated from the rhizosphere soil of *Catharanthus roseus* grown in the campus of Annamalai University, Tamilnadu, India (11.3908^0 N; 79.7147E). The soil sample was suspended in sterile distilled water and subjected to serial dilution (10^{-1}-10^{-7}). An aliquot of 0.1 ml of each dilution mixture was spread on nutrient agar medium containing peptone (5g/L), yeast extract (2g/L), NaCl (5g/L) and Agar (20g/L). The final pH was adjusted to 7 using 0.1N NaOH and diluted HCl. After sterilization the plates were incubated at 28^0C for 48h. After incubation, mucoid colonies were selected and purified by streaking on selective, King's B Agar medium (HiMedia Laboratories, Mumbai, India). The isolated, pure, yellow pigmenting colonies were then subjected to routine microbiological and biochemical characteristic techniques, with which the culture isolated was determined as *Pseudomonas fluorescens*. The organism was stored on nutrient agar slant and maintained at 4^0C for further studies.

Molecular Identification of the strain

The molecular identification of the characterized culture was done by analyzing the genomic DNA. PCR analysis was performed with 16SrRNA primers: 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'- TAC GGT TAC CTT GTT ACG ACT T-3'). A volume of 25µl reaction mixture for PCR was carried out using 10ng of genomic DNA, 1X reaction buffer (10mM Tris HCl pH 8.8, 1.5mM $MgCl_2$, 50mM KCl and 0.1% Triton X 100), 0.4 mM dNTPs each, 0.5U DNA polymerase and 1mM reverse and forward primers each. The reaction was performed in 35 amplification cycles at 94^0C for 45 sec, 55^0C for 60 sec, 72^0C for 60 sec and an extension step at 72^0C for 10min. The sequencing of 16S amplicon was performed according to

manufacturer instructions of Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The 16S rRNA gene sequence obtained from the organism was compared with other Pseudomonas strains for pairwise identification using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and multiple sequence alignments of the sequences were performed using Clustal Omega version of EBI (www.ebi.ac.uk/Tools/msa/clustalo). Phylogenetic tree was constructed by Clustal Omega of EBI (www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny) using neighbor joining method.

Production and Isolation of Exopolysaccharides

EPS produced was extracted by ethanol precipitation method. The culture was centrifuged at 11,000 rpm for 10 min at 4^0C. The supernatant obtained was mixed with two volumes of ice cold ethanol and kept at 4^0 C for 24 hr. The mixture was then centrifuged at 2500 rpm for 20 min at 4^0C. The obtained pellet was suspended in distilled water, which was centrifuged at 2500 rpm for 30 min at 4^0 C with two volumes of ice cold ethanol [24]. The process was repeated twice and the EPS obtained was dried, weighed and lyophilized. The total carbohydrate content of the biopolymer was studied by phenol sulfuric acid method [25] using glucose as standard.

Synthesis of SNP

The inoculum was prepared separately for the production of silver nanoparticles. A volume of 1 ml culture broth was inoculated in 100ml of Nutrient broth medium (HiMedia Laboratories, India). The flasks were incubated at 28^0C for 48h. The fermented medium was then centrifuged and biopolymer was precipitated using ethanol. A volume of 3% biopolymer was prepared for its use in SNP generation. In a 250ml Erlenmeyer flask, 50 ml of 1mM $AgNO_3$ solution was added to 100ml of 3% EPS solution. EPS solution without $AgNO_3$ solution was maintained as blank. The flasks were then left for 5days at room temperature in brightness. The color change from pale yellow to brown was visually checked, which indicated the extracellular synthesis of SNPs. The SNPs were obtained by centrifuging at 12000rpm for 10 min, washing the pellet with double distilled water and repeating the protocol thrice. The obtained SNP pellet was then lyophilized.

Characterization of SNP

Primary characterization of SNP was performed by UV-Vis spectroscopy (Model- SL 159, ELICO Ltd, India), with wavelengths ranging between 200-600nm. FTIR spectroscopy was also used to analyze the nanoparticles. A quantity of 50mg of lyophilized SNP was taken, mixed with 150mg of KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement in the frequency range

of 4000-600cm^{-1}. The exopolysaccharide was characterized using a Fourier Transfer Infrared Spectrophotometer (Bruker Optics, GmBH, Germany). Stability of nanosilver was checked for 4 months under normal room conditions.

Scanning Electron Microscopy and EDX studies

Morphology of synthesized SNP was examined using Scanning Electron Microscopy (Model-JSM 5610 - Jeol, Japan). Thin films of samples were coated onto a carbon tape and allowed to dry for 5 min. The SEM images were obtained under 30kX magnification. The SEM machine was operated at an accelerating voltage of 20kV. The low voltage was maintained so that damage to thin sections of sample could be minimized. The presence of elemental Ag in nanoparticles was analyzed using a SEM equipped with an EDS attachment.

Antimicrobial Activity of SNP

The antimicrobial activity was determined by well diffusion method. Test organisms namely *Bacillus subtilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli, Salmonella typhii* and *Candida albicans* were used for the study. A volume of 100µl culture was spread on sterile Mueller Hinton agar plates and wells were made. A volume of 20 µl EPS reduced SNP were added to each plate and incubated at 37^{0}C for 24h.

Antioxidant activity of SNP – DPPH Scavenging Effect

The antioxidant activity of EPS stabilized SNP was evaluated on the basis of the free radical scavenging effect of 1, 1- diphenyl 2- picrylhydrazyl (DPPH), by the method of Shimada [26] with slight modification. In brief, sample solutions at various concentrations of 0.2, 0.4, 0.6, 0.8 mg/ml were made upto to 1 ml with distilled water. 1 ml of DPPH solution (0.004% in methanol) was added to sample and standard solutions. After incubating the solutions for 30 min at dark, the absorbance was read at 517nm. Vitamin C and distilled water with DPPH were used as the reference and blank respectively. Percent scavenging ability was calculated using the formula:

$$\text{Percent (\%) scavenging activity} = 1 - (A/B) \times 100$$

RESULTS AND DISCUSSION

Molecular characterization of the bacterial isolate

The plant rhizosphere soil is an ecological niche for multitudinous plant associated microorganisms. An attempt was made to isolate an exopolysaccharide producing bacterium from the medicinally important plant, *Catharanthus roseus*. Ten bacterial cultures, which were mucoid were isolated from preliminary screening and cultured on *Pseudomonas fluorescens* selective agar medium to identify the polymer producing, yellow pigmenting culture. The

bacterial strain was then subjected to morphological, biochemical and 16S rRNA sequence analysis. The amplified gene sequence was compared with BLAST database, acquiring sequences displaying maximum homology. The strain of the study exhibited maximum percentage of similarity, 100%, with the sequences of other *P.fluorescens* strains with a high score. The target rRNA was aligned with all homologous sequences using Clustal W2 and a phylogenetic tree was eventually constructed (Figure 1). The phylogenetic analysis confirmed that the isolated strain was *P.fluorescens*. The nucleotide sequence of the organism, referred to as *P.fluorescens* CrN6, has been deposited in the GenBank database under the accession number KF359766.

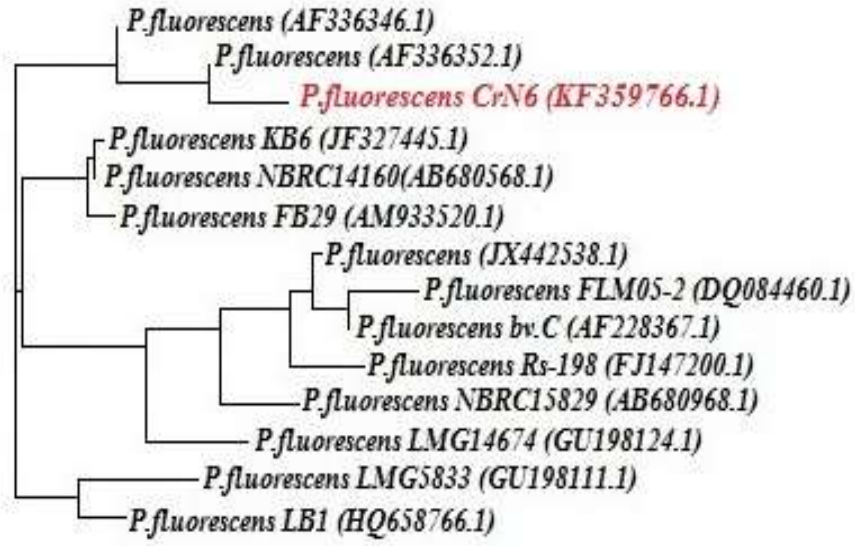


Figure 1 representing phylogenetic tree of the isolated strain with the homologous sequences

Characterization of EPS reduced SNPs

Exopolysaccharides extracted from plant and microbial sources had been used as reducing and stabilizing agents [6, 27, 28]. The silver nanoparticles were synthesized with the addition of exopolysaccharide from *P.fluorescens* which reduced Ag^+ ions to Ag^0 at room temperature under light. *Pseudomonas fluorescens* serve in generation of SNP due to the secretion of cofactor NADH and NADH dependent enzymes which involve in reduction of Ag^+ to Ag^0, thereby forming silver nanoparticles. The process gets initiated with the electron transfer from NADH to NAD^+ catalysed by NADH- dependent reductase as electron carrier, which reduces Ag^+ ions to metallic silver [29, 30, 31].

$$AgNO_3 + NADH \xrightarrow{\text{NADH dependent nitrate reductase}} Ag^0 + NAD^+$$

Various commercially available polymers and microbially produced polymers had been used for silver nanoparticle reduction and stabilization. Spherical silver nanoparticles of 6nm were synthesized using exopolysaccharides from *L.plantarum*, which reduced silver nitrate and stabilized the generated SNPs [32]. Dextran, chitosan, starch and pullulan are certain polymers that are used for greener generation of SNPs [1,33,34]. Exopolysaccharides produced by different species of *Bacillus* are being involved in stabilizing SNPs [2, 35].SDS has also been used as capping polymer in synthesis of SNPs [36].

Visual Observation

The simple method to find out the synthesis is the visual observation of color change (Figure 2). The reduction of silver nitrate resulted in the conversion of pale yellow to dark brown color indicating clearly the formation of silver nanoparticles. No color change was observed in the control sample. In case of *P.fluorescens*, the change of color initiated after 3h of addition of silver nitrate in EPS solution. The color gradually increased as the time of conversion increased. The color intensity was ascending due to the concentration of silver nanoparticles. After 120h, the color remained the same which indicated the complete reduction of silver ions.

Figure 2 illustrating the visual observation of SNP synthesis by EPS reduction and stabilization pale yellow to dark brown coloration at 120h.

The appearance of dark brown color was due to the excitation of surface plasmon resonance. The specific color is observed when the frequency of the electromagnetic field becomes resonant with the coherent electric motion thereby a strong absorption takes place.

UV-Visible spectroscopy

The visual observations were confirmed with the analysis by UV-Visible spectroscopy (Figure 3), which is a unique and simple protocol analyzing samples with their optical properties. The report showed no increase in absorbance after 120h denoting the complete formation of SNPs. The sharp absorption peak was noted at 400nm which confirmed the presence of SNPs. The

negatively charged EPS bonded with nanosilver will confine free electrons of the nanoparticles in a smaller volume. This results in a high free electron density which in turn leads to high plasmon frequency. Hence a sharp peak was observed at a lower wavelength[37].

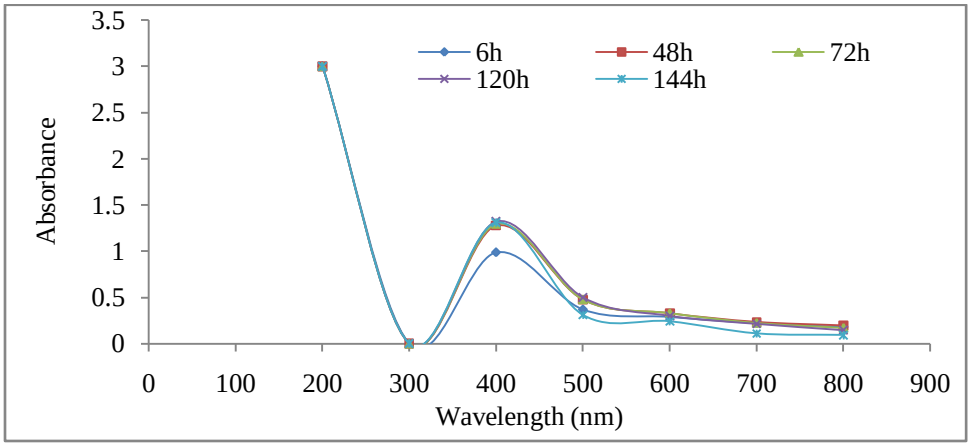


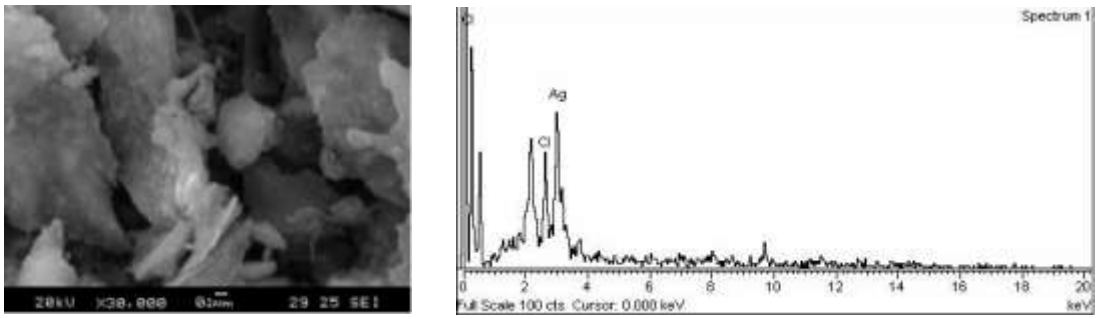
Figure 3 UV-Vis spectrogram of SNP formed at various time periods

Similar results were observed with octasaccharide stabilized SNP[13]. Various other studies also revealed that absorption band of silver nanoparticles occurred in the visible range of 400-500nm [2, 4, 5, 7, 13]. Surface Plasmon resonance band may be due to the electrons vibrating in resonance with the light waves. The absorption peak remained the same even after prolonging the time suggesting that the EPS stabilized SNPs were efficiently capped, well dispersed and did not agglomerate. The nanoparticles thus stabilized by EPS remained stable upto 4 months under normal conditions and storage after which the particles started conglomerating and increased in size. This might be because of the increased force of attraction between the particles and surface passivation by polysaccharides due to hydrogen bonds[1].

SEM and EDS examination of SNPs

SEM analysis was carried out to examine the morphology of the generated SNPs. Figure 3A illustrates the image of silver nanoparticle under SEM with 30,000X magnification. The microscopic observations confirmed the presence of spherical silver nanoparticle. The particle size relies upon the rate of nucleation and growth process and agglomeration [38, 39]. The SEM results showed that the average size of the particles were 90nm. Formation of nanosilver by microbial strains are recently becoming the interest of researchers due to their optical, chemical, photo electrochemical and electronic properties, enabling their synthesis of different chemical compositions, well defined sizes and distinct morphologies [31, 37.] Many investigations reported similarly on particles of different shapes and varying sizes. *Aspergillus fumigatus* served in forming SNPs of 7-19nm in size. Exopolysaccharide produced by *L.rhamnosus* GG stabilized

silver nanoparticles of differing shapes like hexagonal, triangular, spherical and rod with an average size of 10nm [1]. *Verticillium* sp. and *F. oxysporum* yielded SNP of diameter 2- 20 and 2-50nm respectively [28]. Triangular shaped nanosilver particles were synthesized and reduced by supernatant of *Bacillus flexus* [1]. Size of spherical nanoparticles synthesized by *P.aeruginosa* was examined to be 80nm [38]. The size and shape of the SNPs depend upon the concentration and type of reducing and stabilizing agents used [1].



(A) **(B)**

Figure 3 showing the SEM image (A) and EDS spectrum (B) of EPS reduced SNPs

Energy Dispersive Spectroscopy (EDS) was performed to check the presence of elemental silver in the nanoparticles. The vertical axis represented the X-ray count number and the horizontal axis displayed energy in keV. The EDS profile of stabilized SNPs showed strong signals at ~3keV (Fig.3B). The sharp peak at the signal was apparently due to surface plasmon resonance which is referred to as the collective oscillation of conduction electrons [40-42]. Weaker signals of C (0.2keV), O (0.5keV), P (2keV) and Cl at respective signals were also observed due to X ray emissions by protein molecules present in EPS [28,43,44].

FTIR analysis of EPS reduced SNP

FT-IR spectral analysis was carried out to scrutinize the functional groups present in the EPS reduced SNPs (Figure 4B). The spectrum depicted the varied range of absorption from 3446 - 1022 cm^{-1}. A broad absorption band was observed at 3446.32cm^{-1} which indicated the hydroxyl stretch. Intense peaks at 2961.91, 2922.64 and 2855.07 cm^{-1} denoted the symmetric CH_3 and CH_2 stretches. Absorption peaks at 1653.05 and 1637.00 cm^{-1} indicated carbonyl groups of amide I bands and 1558.57 and 1541.86 cm^{-1} marked the presence of N-H stretching of amide II bands. An intense peak at 1263.51 cm^{-1} indicated C-O stretching. Absorption peaks at 1118.32 and 1022.45 cm^{-1} were characteristic peaks for C-O-C stretching for all sugar moieties. Additional peak at 1458 cm^{-1} designated the presence of SNPs [2, 29, 45]. The protein (amide) denoting bands showed that the silver nanoparticles were capped and protected by the proteins present in the polysaccharides. Several results suggested that proteins were the major constituent

capping and stabilizing SNPs by electrostatic forces [13, 15, 29, 46]. The peaks of pure EPS (Figure 4A) and the peaks of EPS reduced SNPs totally dissented to each other which indicated that there occurred a strong and efficient interaction between Ag and hydroxyl groups in polysaccharide which aided in coordination and stability [1].

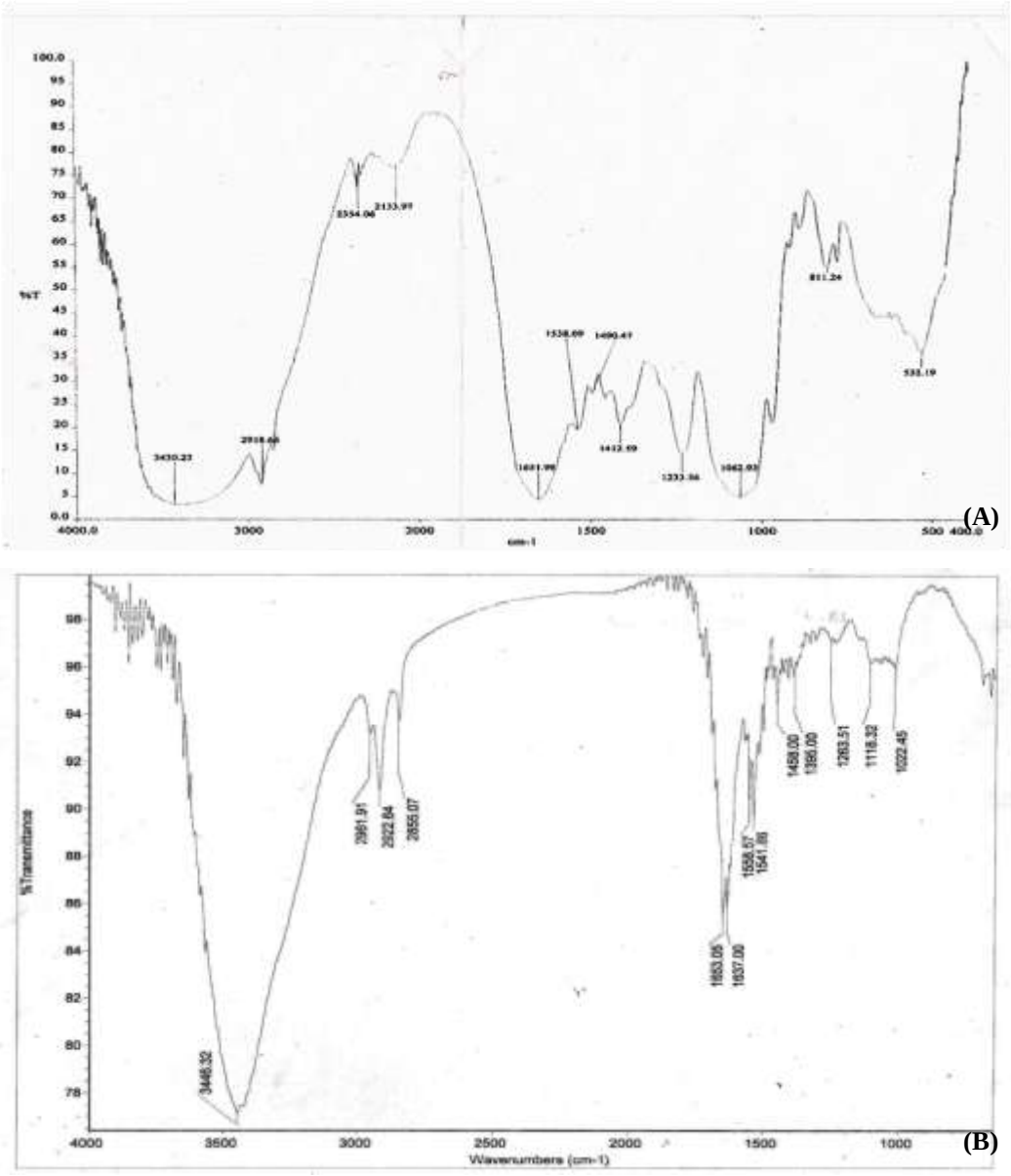


Figure 4 FTIR spectrum of pure EPS (A) and EPS reduced SNPs (B)

Antimicrobial activity of SNPs

The effect of EPS reduced SNPs against certain microorganisms were examined in this study. Silver had been of medicinal value for many centuries but silver nanoparticles are recently proving to be highly effective against diseases. The antimicrobial effect is due to its smaller size, thus possessing a larger surface area [4] .The activity was checked by the formation of zone of

inhibition and the diameter of each zone was measured and tabulated (Table 1).The results showed that the effect was pronounced more against Gram negative bacteria than that of Gram positive. Maximum zone was observed in plate inoculated with *Escherichia coli* (27±0.32). In E.coli cells, it was stated that the SNP form pits on the cell wall and enter the cell. The cell loses the structural integrity and DNA, the replication ability, thus the organism ultimately lyses [47,48.] The activity was lesser against Gram positive bacteria due to the nature of the cell wall. Presence of thick peptidoglycan layer, composed of cross linked polysaccharides, inhibited the penetration of silver nanoparticles [40], thus the effect was deteriorated. The mechanism of inhibition of microorganism growth remains unclear. Many reports suggest that the silver ions, since tiny in structure, might enter the cell and bind to the cellular proteins thereby inactivating them by denaturation [8,9.] SNPs bind to the phosphorus and sulfur compounds on the cell membrane, which in turn alter the structure of cell wall and increase permeability [1]. Cell division mechanism also changes finally resulting in cell death. The study revealed that the synthesized SNP could be utilized as a broad spectrum antimicrobial agent as it worked well against Gram positive and negative bacteria.

Table 1: Antibacterial and anticandidal effects of EPS stabilized SNP

Organism	EPS reduced SNP	Positive control
Bacillus subtilis	10±0.23	25±0.02
Pseudomonas aeruginosa	17±0.46	23±0.02
Klebsiella pneumoniae	12±0.12	21±0.12
Escherichia coli	27±0.32	22±0.07
Salmonella typhii	26±0.16	28±0.13
Candida albicans	19±0.08	24±0.10

Antioxidant effect of SNPs

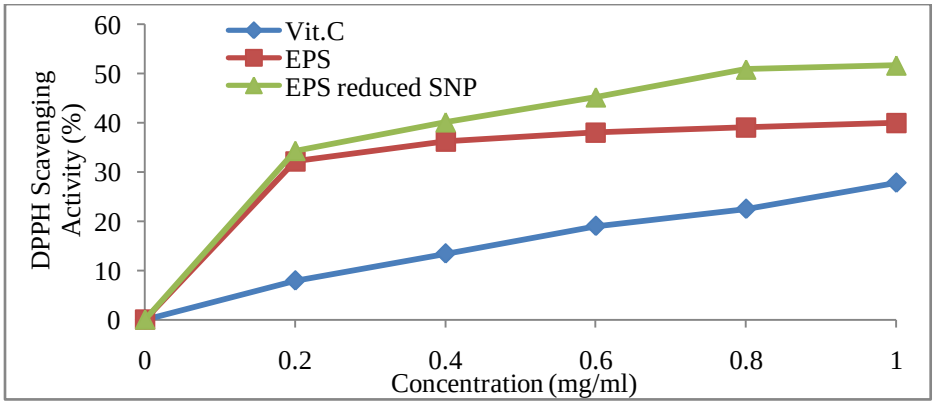


Figure 5 DPPH scavenging efficiency of EPS reduced SNP

DPPH assay was performed to check the free radical scavenging activity of EPS reduced SNPs. Antioxidants are those macromolecules that could scavenge the free radicals of reactive oxygen

species, i.e, superoxide anions like hydrogen peroxide, singlet oxygen and free hydroxyl anions, which are generated by transfer of one electron [49]. DPPH could be scavenged on accepting one hydrogen or an electron [50.] Thus this activity results in the reduction of stable DPPH radical (purple) to non-radical DPPH-H (yellow) form .The antioxidant activity of EPS stabilized SNP happens on a dose dependent manner [49]. The SNPs was found to be a stronger antioxidant than the standard Vitamin C (Vc). The maximum scavenging activity of 51.69% was revealed the concentration of 1mg/ml (Figure 5). The activity found to increase with increase in dosage and also was found to be higher than that of the crude EPS. The study revealed that the silver nanoparticle stabilized by EPS was found to be a potent antioxidant.

CONCLUSION

An attempt was made to synthesize silver nanoparticles using exopolysaccharides from *Pseudomonas fluorescens*, a greener approach of nanosilver formation. SNP synthesis was primarily observed by the color change and UV-Vis Spectroscopy indicated the formation of SNPs. The morphological analysis by SEM revealed that the average diameter of the particles was 90nm and strong signals in the silver region confirmed the presence of metallic silver reduced by EPS. EPS reduced SNP was stable for 4 months after which nanosilver started clustering. The generated SNPs showed antimicrobial effect and exhibited high antioxidant activity than the standard Vitamin C. The present investigation was eventually a potential study on synthesis of an essential and ecofriendly component that was multifunctional in biotechnological field.

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