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Production and antimicrobial activity of silver nanoparticles synthesized from indigenously isolated *Pseudomonas aeruginosa* from Rhizosphere

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Abstract: Nanoparticles hold profound biological, pharmaceutical and industrial applications. Green synthesis of nanoparticles is considered amongst the environmentally safe and cost effective method of nanoparticle synthesis. Briefly, *Pseudomonas* strains were isolated from rhizosphere soil samples. Cell free supernatants of 36% of the isolates showed production of silver nanoparticles. Of these, three isolates, SMS13, SMS100 and SMS124 were selected as the potentially best nanoparticle producers. Amplification and DNA sequencing of 16srRNA gene identified all three strains as *Pseudomonas aeruginosa*. Scanning electron microscopy showed existence of particles between the ranges of 60nm to 70nm in the diameter with elemental composition of silver varies from 0.48% to 1.61%. Consistently, surface plasmon resonance showed maximum absorbance of nanoparticles between 352nm to 406nm. Finally, antibacterial activity of the synthesized nanoparticles was assessed by spot assay whereas Cell Free Supernatants (CFS) of respective isolates were taken as control against clinical isolates of *Salmonella typhi*, *Shigella dysentriae*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Proteus mirabilis* and *Streptococcus epidermidis.* Nanoparticles suspension from all isolates exhibited antibacterial activity against all the screened isolates, whereas no biological activity was observed in the CFS of corresponding strains.

Keywords: Nanoparticles, Rhizosphere, *Pseudomonas*, silver, antimicrobial activity.

INTRODUCTION

Nanoparticles are the ultra fine particles ranging from 1 to 100 nm in size and behave as a unit in relation to its transport and properties (Kummara *et al*., 2016). Exploitation of nanoparticles gains considerable interest due to their wide range of applications in medical, industrial pharmaceutical and environmental sciences (Khan *et al*., 2019). Among various metal nanoparticles, silver nanoparticles are proved to be the most effective due to their unique physical, chemical, biological and antimicrobial properties and are amongst the most widely used nanoparticles in textile industry, biotechnology, food industry, cosmetics, medicine and water treatment (Jyoti *et al*., 2016; Zhou *et al*., 2016*;* Khan *et al*., 2019; Simbine *et al*., 2019).

Several biological, chemical and physical methods of nanoparticles synthesis have been proposed and exploited, with biological synthesis and/or green synthesis of the nanoparticles has been found most cost effective and eco-friendly (Pantidos and Horsfall, 2014; Ijaz *et al*., 2020). Several bacterial species like *Bacillus subtilis*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas putida* have been used to synthesize nanoparticles of different metals including silver, gold, selenium and cadmium (Kumar and Mamidyala, 2011; El-Shanshoury *et al*., 2011; Zaki *et al*., 2011; Shoeibi and Mashreghi, 2017; Ijaz *et al*., 2020).

Species of *Pseudomonas* genera are well known for their primary and secondary metabolic potential and consistently observed as one of the best bacterial resource for the nanoparticle production. The mechanistic details of the nanoparticle synthesis by *P. aeruginosa* is largely unknown, however, it has been proposed that NADH dependent nitrate reductase carry out the reduction of metal ion from the environment (Oza *et al*., 2012; Quinteros *et al*., 2016; Hamedi *et al*., 2017). This in turn leads to both intracellular and extra cellular aggregation of the ions in the form of nanoparticles. Several strains of *Pseudomonas* are known for the production of nanoparticles of variety of metals. For instance *P. aeruginosa* strain BS-161R are known for the production of spherical and 13nm size silver nanoparticles (Kumar and Mamidyala, 2011). Similarly, *Pseudomonas* spp. THG-LS1.4 strain isolated from soil (Singh *et al*., 2018) and JQ989348 strains of *P. aeruginosa* isolated from deep sea have shown to produce silver nanoparticles (Ramalingam *et al*., 2014). In addition, *P. aeruginosa*

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ATCC 27853 has been demonstrated to produce silver nanoparticles of 24-45nm sizes (Quinteros *et al*., 2016).

The study presented here in deals with screening of environmental isolates of *Pseudomonas* for the nanoparticle synthesis. Subsequently, selected isolates were further examined for their potential of nanoparticle production in a time series course. The produced nanoparticles were then assessed for their physical and chemical characteristics and further examined for their antibacterial potential.

MATERIALS AND METHODS

Isolation and Microbiological Characterization of Pseudomonas Spp.

For the isolation of *Pseudomonas* strains, total of 44 rhizospheric soils from different plants were collected, suspended in normal saline (1:10 ratio) and serially diluted to 1:10 and 1:100. From original and diluted suspensions, 100µL of the sample was dispensed on cetrimide agar plates and incubated at room temperature. After 24-48hrs of incubation, colonies were monitored for their size, pigment production and physical characteristics and processed for culture purification and finally microscopically examined after Gram staining.

Preliminary Screening of Nanoparticle Production

For initial screening of nanoparticle production a method adopted by Quinteros *et al*., 2016 was used. In summary, a loop full culture of each isolate was inoculated in 10mL Trypticase Soy Broth (TSB) and incubated at 37^{o}C at 200rpm for 24 hrs. Samples were later centrifuged at 10,000g for 15 minutes at 4°C and Cell Free Supernatant (CFS) was collected in a separate tube and stored at -20^{o}C till further use. Later CFS of each isolate was mixed with 1mM, 5mM and 10mM silver nitrate solution at the v/v ratio of 10%, 30% and 50%, respectively. Simple broth was mixed with mentioned concentrations of silver nitrate solutions at respective v/v ratio as control. All tubes were wrapped with aluminum foil and placed in a dark box and incubated at room temperature with 100rpm on orbital shaker for 48hrs. Next day change in color and pellet formation was observed.

Molecular identification

Molecular identification of three best isolates encoded SMS13, SMS100 and SMS124 (selected on the basis of nanoparticle production) was carried out by amplifying 16SrRNA gene by Polymerase Chain Reaction (PCR) and DNA sequencing. Genomic DNA of selected isolates was extracted using wizard genomic DNA purification kit (Promega) and PCR amplification was performed to amplify 16SrRNA of selected strains using universal 16S rRNA primers (Forward FD1 5'- AGAGTTTGATCC TGGCTCAG -3', Reverse RP1 5'-CGGTTACCTTGT TACGACTT-3'). Briefly, the 35µL reaction mixture containing 1x KCl buffer, 7U of Taq DNA polymerase, 2mM of dNTPs, 4mM of MgCl2, 2µM forward and reverse primers each and 7µL sample DNA was prepared. The mixture was then amplified as initial denaturation at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C/30 sec, annealing at 60°C/min, extension at 72°C/min and final extension was performed at 72°C for 10 min. Amplicons were then subjected to DNA sequencing at Macrogen, South Korea. Electropherograms of 16S rRNA sequences were analyzed using Chromas to check the quality of sequencing. DNA sequencing of PCR amplicons of selected isolates were subjected to non-redundant nucleotide Basic Logic Alignment Search Tool (BLAST) to find the homologous sequences in the database.

Growth Curve and Production of Nanoparticles

Selected *Pseudomonas* isolates were incubated in TSB at 37^{o}C, 150rpm for overnight. Next day 20µL starter culture from each isolate was dispensed in three separate flasks containing 100mL TSB and incubated at 37^{o}C with 150rpm. After each hour (0hr-10hrs and 24hrs) two separate sets of 2mL samples were drawn. One set was used to check optical density (OD) at 600nm using TSB as blank. The other set was centrifuged at 10,000g for 15 minutes at 4^{o}C and CFS was used for nanoparticles production as defined earlier.

UV-Vis Spectroscopy

Silver nanoparticles were freshly prepared using CFS of three selected isolates and absorbance was measured from 200-800nm using double beam UV-spectrophotometer (Shimadzu UV-1800). Baseline corrections were carried out using TSB as blank as conducted in earlier studies (Quinteros *et al*., 2016; Singh *et al*., 2018).

Scanning Electron Microscope (SEM)

Freshly prepared silver nanoparticles from CFS of selected isolates were subjected to Scanning Electron Microscopy (SEM) coupled with Energy Dispersive X-ray spectroscopy (EDX) as suggested by Puchalski *et al*., 2007 at Centralized Science Laboratories, University of Karachi. SEM was performed to assess morphology of synthesized nanoparticles using JEOL electron microscope (JSM-6380A). Total 10µL of each sample was smeared on separate grids and were left to air dry. A thin layer of gold was coated up to 300Å to make the samples conductive in JEOL sample coater (JFC-1500). Samples were then observed at different magnifications to analyze size and shape of nanoparticles. Final images were taken at 15000, 20000 and 40000X.

Energy Dispersive X-Ray Spectroscopy (EDX)

Elemental composition of silver nanoparticles was carried out by the Energy Dispersive X-ray spectroscopy (EDX) detector (JEOL-EX-54175jMU) attached with the SEM machine.

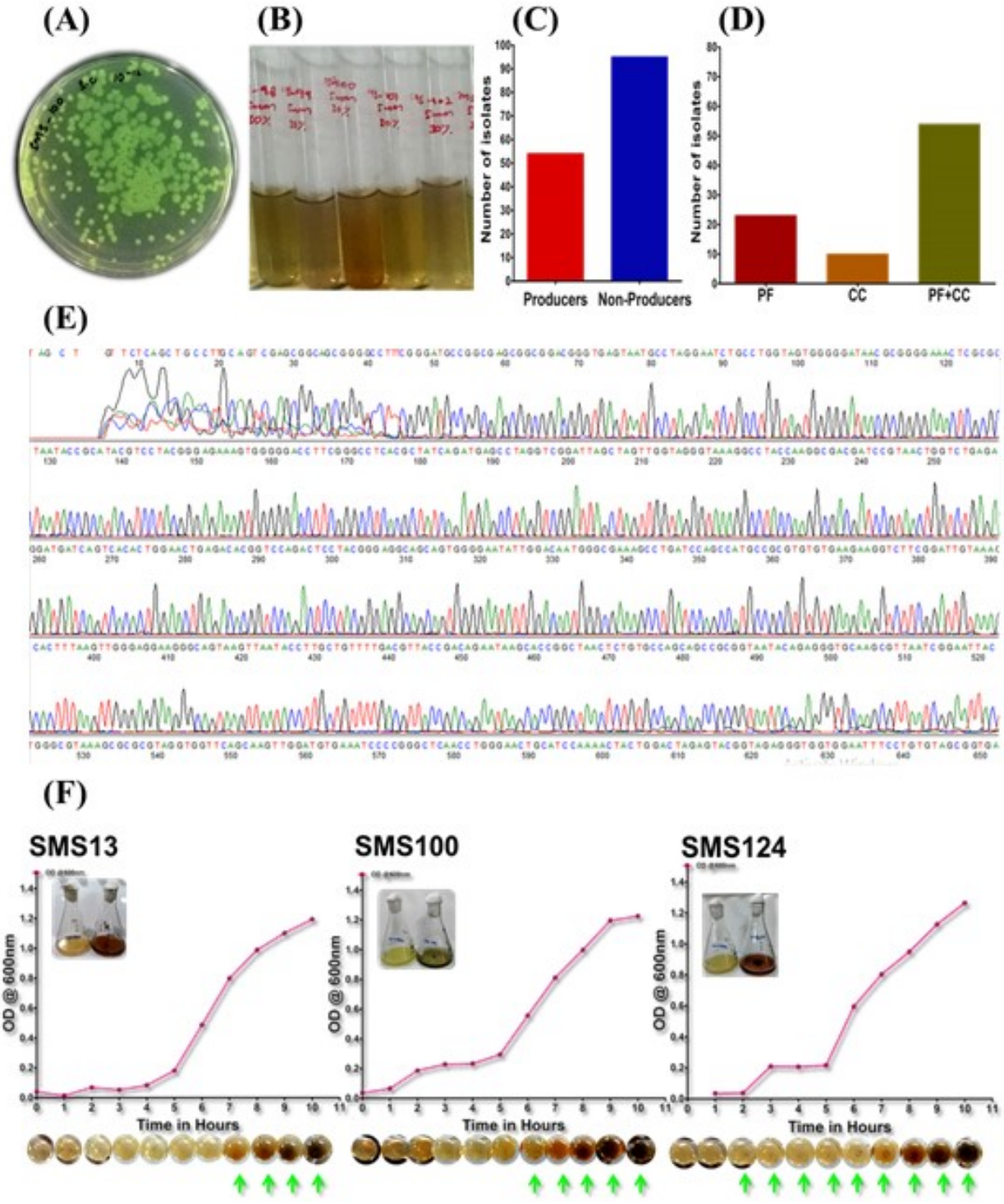

Fig. 1: Isolation, Identification and Nanoparticle Production from *Pseudomonas*. Representative photograph of cetrimide agar plate showing isolated green pigmented colonies *Pseudomonas* (A) microscale screening of nanoparticle production (B) histograms showing frequencies of isolates showing nanoparticle producers and non-producers (C), isolates showing only colour change, pellet formation and both colour change and pellet formation (D), representative electropherograms of 16srRNA gene of SMS13 (E), growth curve of the selected isolates with bulk productions of nanoparticle are shown in inset of respective graphs of isolates as labeled (F). Nanoparticle production is terms of colour change and pellet formation, aligned with the time scale, are shown at the bottom of respective graphs of the isolates.

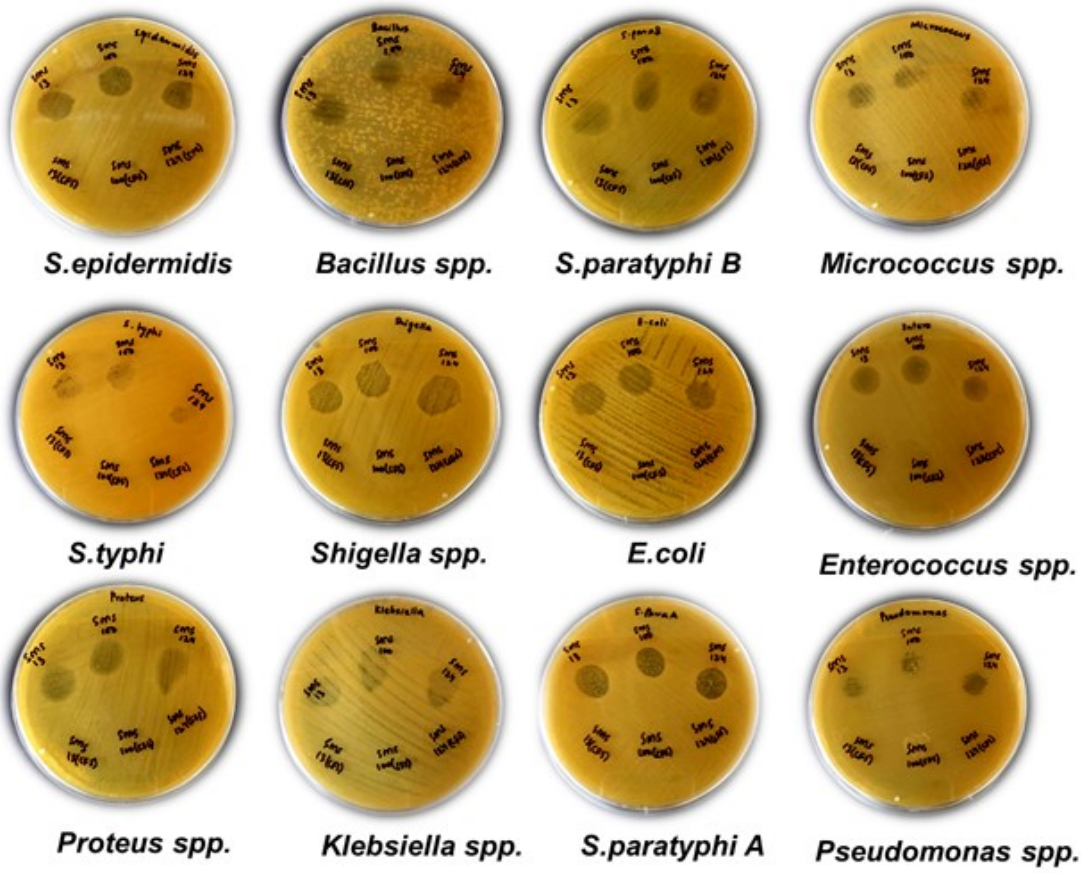

Fig. 2: Antibacterial activity of Ag-Nanoparticle produced from *Pseudomonas*. Spot assay of nanoparticles and CFS of SMS13, SMS100, SMS124 (as labeled) against twelve different clinically isolated bacteria. Inhibition of the growth, specifically at the spots of nanoparticles but not at the CFS represents antibacterial potential of nanoparticles.

Antimicrobial Activity

In vitro antimicrobial screening of nanoparticles produced by three best producers (SMS13, SMS100 and SMS124) was carried out against twelve different clinical bacterial isolates using spot assay. The indicator organisms were first inoculated in 2ml nutrient broth and allowed to grow under shaking condition at 100rpm, 37^{o}C for 2 hrs. After 2hrs, bacterial lawn was developed on heart infusion agar plates using sterile cotton swab. On each plate, 10µL of freshly prepared nanoparticle suspension from selected isolates and their respective CFS (control) was spotted. The plates were then left undisturbed until sample was completely absorbed and incubated at 37°C overnight.

RESULTS

In total, 155 strains of *Pseudomonas spp.* were isolated from rhizosphere collected from 44 different plants distinguished on the basis of colonial sizes (large circular, medium, small and pin pointed) and pigments (green, red, white and transparent) production. Each isolate was given an isolate code as SMS#. Microscopic examination showed that 149 isolates were Gram-negative rods in scattered confirmation as expected from *Pseudomonas* isolates. Six of the isolates were found to be Gram positive and excluded from the subsequent investigations (fig. 1A).

Initial screening of *Pseudomonas* isolates for the synthesis of silver nanoparticles was done by mixing CFS of each isolate with different concentration of $AgNO_3$ at different v/v ratio and nanoparticle production was assessed on the basis of colour change and pellet formation. Producers of the silver nanoparticles were identified, when CFS or respective isolate with silver nitrate solution showed both colour change of the medium and pellet formation (fig. 1B). Based on this, of 149 isolates, 54 (~36%) were found to be producers whereas 95 (~64%) were considered as non-producers. Amongst these, SMS13, SMS100 and SMS124 were selected since being found to be most prolific in colour change and dense pellet formation (fig. 1C, D). These isolates were then taxonomically identified using 16srRNA sequencing as strains of *P. aeruginosa* based on over 99% sequence identity with 16srRNA gene of *P. aeruginosa* FC1385 (fig. 1E).

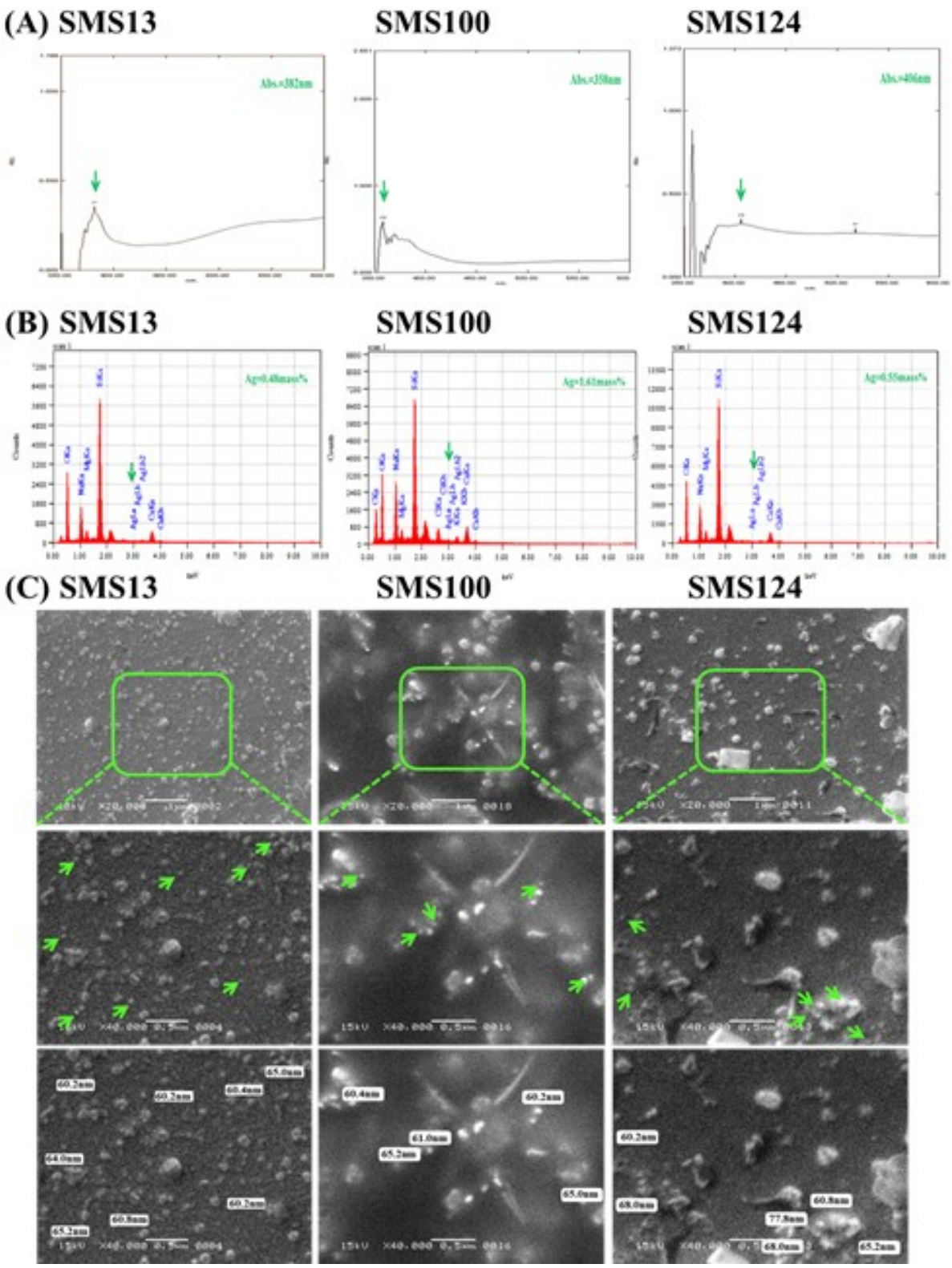

Fig. 3: Characteristics of Ag-Nanoparticles Produced from *Pseudomonas*. UV-vi spectroscopy (A), EDX based elemental analysis (B) scanning electron micrographs of nanoparticles (C) produced by selected *P.aeruginonsa* strains SMS13, SMS100 and SMS124 (as labeled) are shown here. Green box in the scanning electron micrographs are zoomed in the second row, where respective nanoparticles are shown with green arrows and sizes of the same are estimated in third row.

Growth kinetic experiment was run for the three best producers in order to identify the onset of nanoparticle production during the growth phases. The log phase of all three isolates started between 5th and 6th hours of the starter culture inoculation and attained stationary phase at 9th hour of inoculation. Start of nanoparticle production of all isolates was noticed at 2nd hour with hourly increase (up to 10 hours) in the production as exhibited by colour change and/or pellet formation (fig. 1F).

Biological activity of silver nanoparticles from SMS13, SMS100 and SMS124 was assessed against clinical indicator organisms like *Pseudomonas spp.*, *S. paratyphi A*, *S. epidermidis*, *Enterococcus spp., K. pneumoniae*, *E. coli*, *P. mirabilis*, *Micrococcus spp.*, *S. typhi*, *S. dysenteriae, B. subtilis* and *S. paratyphi B* by spot assay. Interestingly, silver nanoparticles produced by all three isolates inhibited the growth of all the screened indicator organisms as reflected by zone of inhibition at and/or around the dispensed spot. In contrast, the pure CFS of any of the isolate did not show any antibacterial activity against the screened organisms (fig. 2).

UV-Vis spectroscopy (200-800nm) showed slight variations in the maximum absorbance for nanoparticles produced by three selected isolates as the maximum absorbance of SMS13, SMS100, SMS124 was detected at 382nm, 358nm and 406nm, respectively. SEM of silver nanoparticles synthesized from SMS13 and SMS100 showed that their diameter varies from 60-65nm whereas silver nanoparticles of SMS124 were in the size range of 60-77nm. The presence of silver was detected at variable with average mass% of silver found in the fields with nanoparticles from SMS13, SMS100, and SMS124 was 0.48%, 1.61% and 0.55%, respectively (fig. 3A-C).

DISCUSSION

Pseudomonas are known to be present in diverse environmental niches such as fresh water, sewage, food, coastal marine, surfaces of plant tissues and rhizosphere (Mercado-Blanco *et al*., 2016; Mulamattathil *et al*., 2016; Qessaoui *et al*. 2019). Isolation of multiple strains of *Pseudomonas* (n=149) from limited rhizosphere soil samples suggests highly ubiquitous and taxonomically enriched distribution of the bacteria especially in rhizosphere, where it promotes plant health and growth (Qessaoui *et al*. 2019).

Approximately 36% of isolates were found to be nanoparticles producers considering both criteria of pellet formation and colour change. These numbers could be improved if either of those characteristics were considered individually as reported by Pourali and co-workers in which they have taken 37 different bacterial soil isolates and found all isolates were to be the producer of nanoparticles by considering only colour change as indicator (Pourali *et al*., 2016). Since many *Pseudomonas* spp. are known to produce pigments (DeBritto et al., 2020) that may influence the colour change in CFS, we employed presence of both pellet formation and colour change for the identification of nanoparticle producer. In addition to many other bacterial species as mentioned earlier, nanoparticle production has been previously reported by several strains of *Pseudomonas* (Kumar and Mamidyala, 2011; Ramalingam *et al*., 2014; Quinteros *et al*., 2016; Singh *et al*., 2018). The ability to produce nanoparticles in turn provides bacteria a mechanism to detoxify metal ion by extra cellular bio-mineralization, complex formation and precipitation or intracellular bioaccumulation (Hulkoti and Taranath, 2014; Iravani S, 2014).

Production of nanoparticles were observed at the end or beginning of the log phase with maximum production attained at stationary phase. This is consistent with the observation that production of nanoparticle is dependent on the pH of the environment (Chitra and Annadurai, 2014). Production of several metabolites and waste produced during the exponential phase of bacterial growth may result in the change in pH of the medium to the optimum required for the nanoparticle production (Chen *et al*., 2017). In addition it has been proposed that a bacterial enzyme namely nitrate reductase activity may facilitate nanoparticle production (Krukemeyer e*t al*., 2015; Quinteros *et al*., 2016). Therefore, it is also possible that as the cellular density increases the quantity of the enzyme may reach to the thresholds that result in the sudden production of nanoparticles. This in turn point that nanoparticle production by *Pseudomonas* isolates could be analogous to the production of several metabolites in cellular density dependent manner, also dubbed as quorum sensing (Gurney *et al*., 2020).

Silver nanoparticles are capable of absorbing electromagnetic radiations in visible region from 350 to 460nm via excitation of localized surface plasmon resonance property (Syafiuddin *et al*., 2017). Consistently, in this study, silver nanoparticles synthesized from *Pseudomonas* isolates showed absorption within their respective standard range. Ideally, transmission electron microscopy is employed to study the size, shape and dimensions of nanoparticles (Anjum, 2016), however, due to the technical limitation, SEM is employed in the present study. Scanning electron micrograph of nanoparticles validated the size of the proposed particles are in between 60nm to 77nm, a standard considered for the nanoparticles (Punjabi *et al*., 2015). It is important to note that in addition to these particles, several large size particles were also observed pointing to the possibility of the assemblies of nanoparticles of different dimensions. Studies have shown that silver nanoparticles produced by different bacteria including *Pseudomonas* may assemble together in

variable shapes including spherical, rod, hexagonal, octagonal and flower like (Punjabi *et al*., 2017). The chemical nature of nanoparticles as assessed by Energy Dispersive X-ray spectroscopy (EDX) verified the existence of silver in the particles though in a relatively less amount. Further optimization of the medium and/or selection of the much better isolates may further improve the nanoparticle production both in terms of quantity and quality.

Our findings revealed that synthesized silver nanoparticles were effective in inhibiting the bacterial growth of several clinical isolates. Since *Pseudomonas* species are known to produce antimicrobial peptides and/or proteins (bacteriocins), it is possible that the observed bacterial growth inhibition is the result of bacteriocins produced by *Pseudomonas* isolates rather than nanoparticles (Behrens *et al*., 2020). However, since no antibacterial activity was observed in CFS of any selected strains against any of the screened isolates, therefore, it is reasonable to conceive the observed activity is the manifestation of the antibacterial properties of nanoparticles. Nevertheless, the appearance of antibacterial activity against all the isolates may also be due to oligodynamic action of the silver in the CFS (Prasher, 2018).

The present study holds caveats that could further strengthen and improve the findings. For instance a relatively large scale bacterial isolation and inclusion of more niches may result in the identification of better *Pseudomonas* strains for nanoparticle synthesis. Similarly, usage of transmission electron microscope may provide the dimension, shape and size of nanoparticles at better resolution. Moreover, expressional analysis of nitrate reductase production could also be included in the growth curve studies in order to assess its potential role in nanoparticle synthesis in quantitative terms.

CONCLUSION

To the best of our knowledge, the present study is the first in relation to investigate the potential of indigenous strains of *Pseudomonas* for nanoparticle synthesis and characterization of promising isolates and nanoparticles. This serves as a template for relatively expanded studies on other bacterial genera to develop a relatively eco-friendly and cost effective approach for green nanoparticle synthesis.

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