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Biogenic Synthesis and Characterization of Silver Nanoparticles (AgNPs) Produced by Indigenous Microorganisms Isolated from Banana (*Musa spp*) Soils

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KEYWORDS

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Bacillus

Microorganism

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ABSTRACT

This research focused on the screening of indigenous microorganisms isolated from banana soils for their capability to synthesize silver nanoparticles (AgNPs) extracellularly. Ninety-five isolates were screened for AgNP production. The cell-free extracts of these isolates were added to silver nitrate ($AgNO_3$) aqueous solution and were observed for color changes from original pale yellow to dark brown. Ten isolates (3 bacteria and 7 fungi) were found capable of producing AgNPs. Bacterial isolates B2, B3, and B5 were molecularly identified as *Bacillus aryabhattai*, *Priestia megaterium*, and *B. megaterium*, respectively. The AgNPs produced by these bacterial isolates were circular and showed an absorbance peak at approximately 420 nm. On the other hand, the fungal isolates F2, F3, and F43 were molecularly identified as *Penicilliumcitrinum*, *P. glaucoroseum*, and *P. oxalicum*. The AgNPs produced by the *Penicillium spp* were aggregated, circular and showed absorbance peaks at 420 nm. The other four fungal isolates, F7, F24, F29, and F40, were identified as *Aspergillus flavus*, *A. terreus*, and *A. japonicum* (F29 and F40), respectively. The AgNPs produced by the *Aspergillus* spp. were circular and showed absorbance peaks between 420 nm and 450 nm. The continuous search for novel isolates that can carry out the biogenic synthesis of AgNPs remains the focus of nanotechnological research. This study confirms microorganisms of *Bacillus*, *Penicillium*, and *Aspergillus* genera can effectively biosynthesize AgNPs.

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1 Introduction

Nanotechnology is a field of science concerned with the study and control of matter with dimensions ranging from 1 to 100 nanometers (Nasrollahzadeh et al. 2019). It covers a wide range of topics, from expanding traditional device physics to completely new methods based on molecular assembly, from synthesizing novel materials with nanoscale dimensions to whether we can directly manipulate matter at the atomic scale (Jeevanandam et al. 2018). It has the potential to generate a wide range of novel materials and products with applications in medicine, electronics, energy generation, and agriculture (Rai and Ingle 2012). Nanotechnology has the potential to provide green and environmentally friendly plant disease management options. Many microorganisms are already recognized for forming inorganic material within or outside the cell to create nanoparticles. Among the many nanoparticles, silver nanoparticles (AgNPs) stand out in various fields (Yaqoob et al. 2020; Alharbi et al. 2022; Salleh et al. 2022).

Silver nanoparticles are between 1 and 100 nm in size and have unique features that aid molecular diagnostics, treatments, and devices used in a variety of medical operations (Prabhu and Poulose 2012). High electrical and thermal conductivity, surface-enhanced Raman scattering, catalytic activity, chemical stability, and non-linear optical behavior are among the physicochemical attributes of AgNPs that distinguish them from bulk Ag. In addition, AgNPs have a high surface area-to-volume ratio, have better interaction with the microorganism, and are well-known antimicrobial agents that work against a wide range of bacteria, both Gram-positive and Gram-negative (Anjum et al. 2013).

Bacteria, fungi, and plant extracts are the three major sources of biosynthesis of silver nanoparticles and are produced mostly by reduction/oxidation reactions. Nanoparticles can be synthesized by bacteria either through extracellular mechanisms or within cells. Bacteria such as *Bacillus licheniformis* (Kalimuthu et al. 2008) use the NADH-dependent nitrate reductase- mediated reduction of silver ion (Ag^{+}) to elemental silver (Ag^{0}). However, non-enzymatic reduction also occurs, such as with *Lactobacillus* A09, where Ag^{+} reduction occurs intracellularly on the surface of the bacterial cell (Van Hullebusch et al. 2003). Fungal biosynthesis of AgNPs is possible due to their ability to secrete proteins such as those of *Aspergillus flavus,* which secretes a 32-kDa reductase protein that can reduce Ag^{+} ions (Jain et al. 2011).

The antioxidant or reducing characteristics of microbial enzymes interact with the appropriate compounds to synthesize the desired nanoparticles. The three essential considerations for the biogenic synthesis of nanoparticles are the non-toxic stabilizing agent, solvent medium for synthesis, and an environmentally friendly reducing agent. Behavior, biodistribution, safety, and efficacy are some important factors that depend on the physicochemical properties of the nanoparticle. Characterization of AgNPs is significant for evaluating the functional features of the produced particles. Parameters such as size, size distribution, surface area, solubility, aggregation, and other properties of the nanoparticle need to be evaluated (Zhang et al. 2016).

Several studies have proved the potential of microorganisms as a biofactory of silver nanoparticles (Mukherjee et al. 2008; Fayaz et al. 2010; Sunkar and Nachiyar 2012). Rhizosphere soils of healthy banana plants harbor bacteria belonging to *Trichoderma, Actinobacteria,* and *Bacillus* genera (Xue et al. 2015), and fungi belonging to *Fusarium, Aspergillus,* and *Penicillium* genera (Zhou et al. 2019). The diversity of indigenous microorganisms found in banana rhizosphere soil can be a source of novel isolates that can carry out reliable biosynthesis of silver nanoparticles with certain properties such as high stability, and having the desired size and composition. Hence, this study aims to isolate, screen, and identify indigenous microorganisms found in the banana rhizosphere for the biogenic synthesis of silver nanoparticles.

2 Materials and Methods

2.1 Sample Collection and Processing

The banana soil samples were collected from the central experimental station, Pili Drive, University of the Philippines Los Baños College, Laguna. The soil of the study area which belongs to the Lipa series was classified as fine, clayey, mixed, shallow, isohyperthermic Typic Eutrudepts formed from weathering of hard tuffaceous rocks.

Three banana plants per variety were randomly selected in the field to obtain a composite sample. Plants were uprooted and roots were carefully shaken. Soil particles less than 3 mm thick adhering to the roots were considered as rhizosphere soil and were collected by gently brushing the roots. The samples were placed in sterile plastic bags and stored under cooled conditions until preparation in the laboratory.

2.2 Isolation of fungi and bacteria from banana rhizosphere soil

Ten grams of the soil was mixed with 95 ml of sterile distilled water. Prepared soil solution was taken for serial dilution (10^{-3}, 10^{-4}, $10^{-5,}$ and 10^{-6}) and cultured by spread plate method on nutrient agar (NA) for bacterial isolation and on potato dextrose agar (PDA) for fungal isolation. Extensive colony purification was done to attain single colony cultures by repetitive inoculation of the bacterial colony on NA and point inoculation on PDA for fungal isolates. The pure cultures of the isolates were transferred in NA and PDA slants. The morphological and microscopic properties of the isolates were assessed. The pure cultures of the isolates capable of biogenic synthesis of AgNPs

were submitted to Philippine Genomic Center for sequencing. Resulting sequences were aligned and compared with those from Basic Local Alignment Search Tool (BLAST).

2.3 Extracellular Synthesis of AgNPs using Bacteria

The capability of the bacterial isolates to carry out biosynthesis of AgNPs was determined by Malarkodi et al. (2013), and Adan et al. (2018) with some modifications. The isolates were inoculated freshly in 10 ml NA broth and were kept in an orbital shaker (150 rpm) for 24 hours. The same incubation condition was applied to ten ml (10 ml) of nutrient broth only, which served as the control. The cultures were centrifuged at 2000 rpm for 15 minutes and the supernatant was obtained. The supernatants (free from any kind of precipitates) were passed through sterilized membranes using a 0.22-micron pore-size filter.

Silver nitrate ($AgNO_3$) aqueous solution was added to the vials containing 5ml of the supernatant at a final concentration of 1mM $AgNO_3$. These vials ($AgNO_3$ – supernatant mixture) were incubated in an orbital shaker (150 rpm) at 35° C for 7 days under dark conditions. Control solution ($AgNO_3$ – nutrient broth) was prepared and subjected to the same incubation condition to confirm that AgNP synthesis was mediated by extracellular agents of bacterial origin. After incubation, a visual inspection was performed relative to color changes in the control, to confirm whether AgNPs were produced. Purification of AgNPs was carried out by centrifugation at 10,000 rpm for 10 min twice and the nanoparticles were collected for characterization.

2.4 Extracellular Synthesis of AgNPs using Fungi

The capability of the fungal isolates to carry out biosynthesis of AgNPs was determined by the method described by Magdi et al. (2014) with some modifications. For this, fungal isolates were cultured on PD broth at 28 °C on a rotary shaker for 96 hours. The biomasses were obtained using Whatman filter paper No. 1 and then washed with ultrapure water to eliminate any remaining components of the medium. Then, the biomass was incubated for 24 h in separate flasks containing 100 ml water. The biomass was filtered, and the resulting cell filtrate was collected and used for AgNP biosynthesis.

For the biosynthesis of AgNPs, 50 ml of cell filtrate was mixed with 10 ml $AgNO_3$ at a final concentration of 1 mM $AgNO_3$. The control was a reaction mixture without $AgNO_3$. The prepared solutions were incubated for 7 days at 28 °C and were kept in the dark during the experiment. After incubation, a visual inspection was performed relative to color changes in the control, to confirm whether AgNPs were produced. Purification of AgNPs was carried out by centrifugation at 10,000 rpm for 10 min twice and the nanoparticles were collected for characterization.

2.5 Characterization of Produced Silver Nanoparticles

The AgNPs were subjected to optical absorbance measurements using a UV-Vis spectrophotometer (MultiSkan Sky Spectrophotometer, Thermo Fisher Scientific) scanning between 250 nm and 700 nm at a 1nm resolution. Detailed characterization of the size, distribution and morphology of the nanoparticles was performed using a particle analyzer and scanning electron microscopy (Prisma E-SEM, Thermo Fisher Scientific).

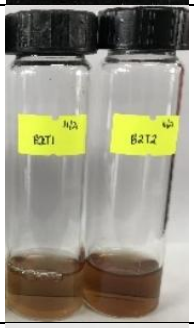
3 Results and Discussion

A total of 51 bacterial isolates and 44 fungal isolates were screened for AgNP production. Among the 95 tested isolates, only 10 isolates (3 bacteria and 7 fungi) were observed with the ability to change the color of the reaction mixture from its original pale-yellow color to dark brown (Tables 1 and 2). The change in color of the solution indicates the production of AgNPs by reduction of Ag^+ to Ag^0, mainly due to the excitation of surface plasmon vibrations in the AgNPs. The increase in color intensity of the solution was attributable to an increase in the number of nanoparticles generated, as silver ions in the aqueous solution were reduced (Elamawi and Al-Harbi 2014).

The three bacterial isolates capable of synthesizing AgNPs were B2, B3, and B5. Interestingly, all isolates belong to the class *Bacillus*. These results are in agreement with previous findings, and various previous studies confirmed that *Bacillus* strains can produce AgNPs (Saravanan et al. 2011; Deljou and Goudarzi 2016; Ahmed et al. 2020). Isolates B2, B3, and B5 (Table 3) were molecularly identified to be *Bacillus aryabhattai*, *Priestia megaterium*, and *B. megaterium*, respectively. As illustrated in Figure 1, the UV-visible absorption spectra showed absorbance peaks at approximately 420 nm. The absorption peak observed in this study is specific for AgNPs, which is similar to the results previously obtained (Priyadarshini et al. 2013; Omole et al. 2018). The scanning electron microscope micrographs (Figure 2) of the dry mass show spherical shape AgNPs with an average size of 88.10 nm, 51.08 nm, and 80.76 nm for isolates B2, B3, and B5, respectively. In addition, energy-dispersive X-ray spectra of AgNPs (Figure 3) produced by isolates B2, B3, and B5 showed peaks of silver (Ag), suggesting that the nanoparticles are indeed Ag in composition.

AgNPs in the range of 50 nm were synthesized using the bacterium *B. licheniformis*, which exhibited maximum absorbance at 440 nm in UV-vis spectroscopy (Kalimuthu et al. 2008); similarly, the synthesis of AgNPs of an approximate size of 40 nm was successful using the culture supernatant of *B. licheniformis* (Kalishwaralal et al. 2008). Equal results were obtained for silver nanoparticles with an average size of 52.5 nm using the culture

Table 1 Screening of Bacterial Isolates for Production of Silver Nanoparticles

Isolate No	Color change		Inference
	Initial	After	
Control ($AgNO_3$ only)			No change in color
B2			+
B3			+
B5			+

supernatant of *Klebsiella pneumonia* and *Escherichia coli* (Shahverdi et al. 2007). Extracellular biosynthesis of highly stable AgNPs from bacterial strain *B. megaterium* (NCIM 2326) was obtained (Saravanan et al. 2011), and the extracellular formation of AgNPs of approximate size 42 nm to 92 nm and with UV-Vis absorption at 450 nm was also found from *Bacillus* sp. (Das et al. 2014).

The seven fungal isolates capable of synthesizing AgNPs were F2, F3, F7, F24, F29, F40, and F43. These isolates belong to only two fungal classes, which are *Penicillium* and *Aspergillus*. Several studies confirmed that *Penicillium* (Hemath Naveen et al. 2010; Ma et al. 2017; Shareef et al. 2017; Taha et al. 2019) and *Aspergillus* strains (Gade et al. 2008; Jain et al. 2011; Li et al. 2011) can produce AgNPs.

Table 2 Screening of Fungal Isolates for Production of Silver Nanoparticles

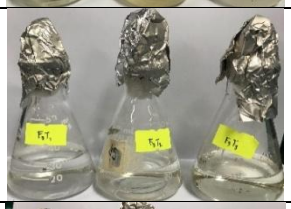
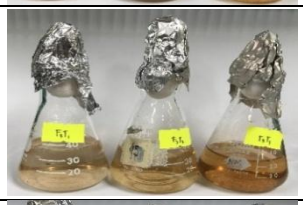
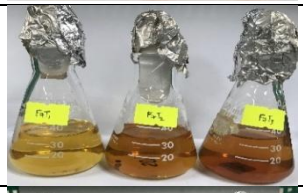
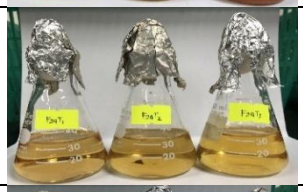
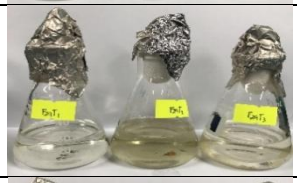
Isolate No	Color change		Inference
	Initial	After	
Control ($AgNO_3$ only)			No change in color
F2			+
F3			+
F7			+
F24			+
F29			+
F40			+
F43			+

Table 3 Molecular identification of the microorganisms capable of synthesizing AgNPs

Isolate Code	Species	Accession Number	Similarity	E Value
Bacteria				
B2	*Bacillus aryabhattai*	MT605510.1	99.34%	0
B3	*Priestia megaterium*	MW363319.1	98.95%	0
B5	*Bacillus megaterium*	JF343138.1	99.50%	0
Fungi				
F2	*Penicillium citrinum*	MT820334.1	92.15%	8e-109
F3	*Penicillium glaucoroseum*	MT530148.1	89.63%	4e-138
F7	*Aspergillus flavus*	MW805395.1	97.25%	0
F24	*Aspergillus terreus*	MT530046.1	90.62%	5e-153
F29	*Aspergillus japonicus*	EU645679.1	92.73%	4e-125

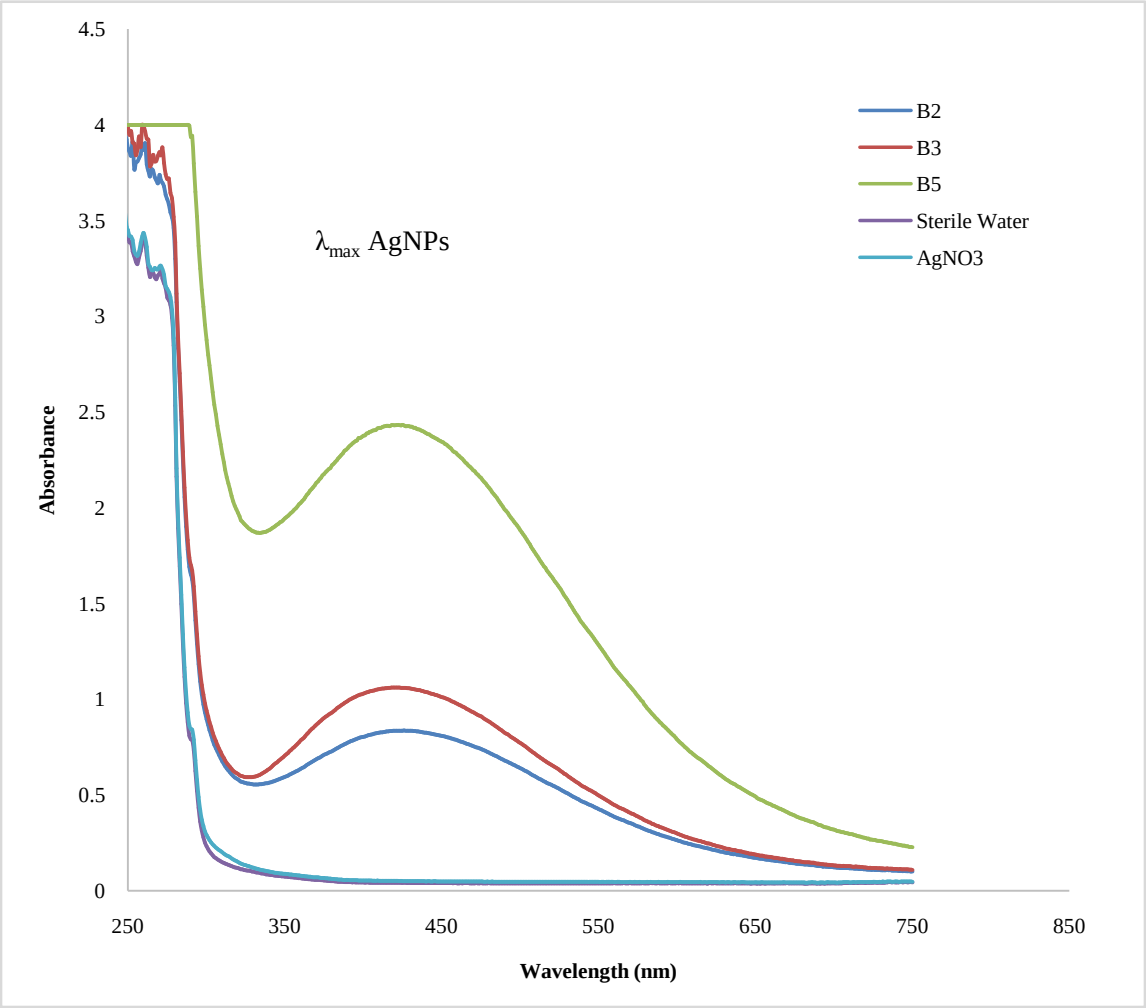


Figure 1 The UV-Vis spectra recorded for the reaction of the different bacterial cell filtrates and $AgNO_3$ solution

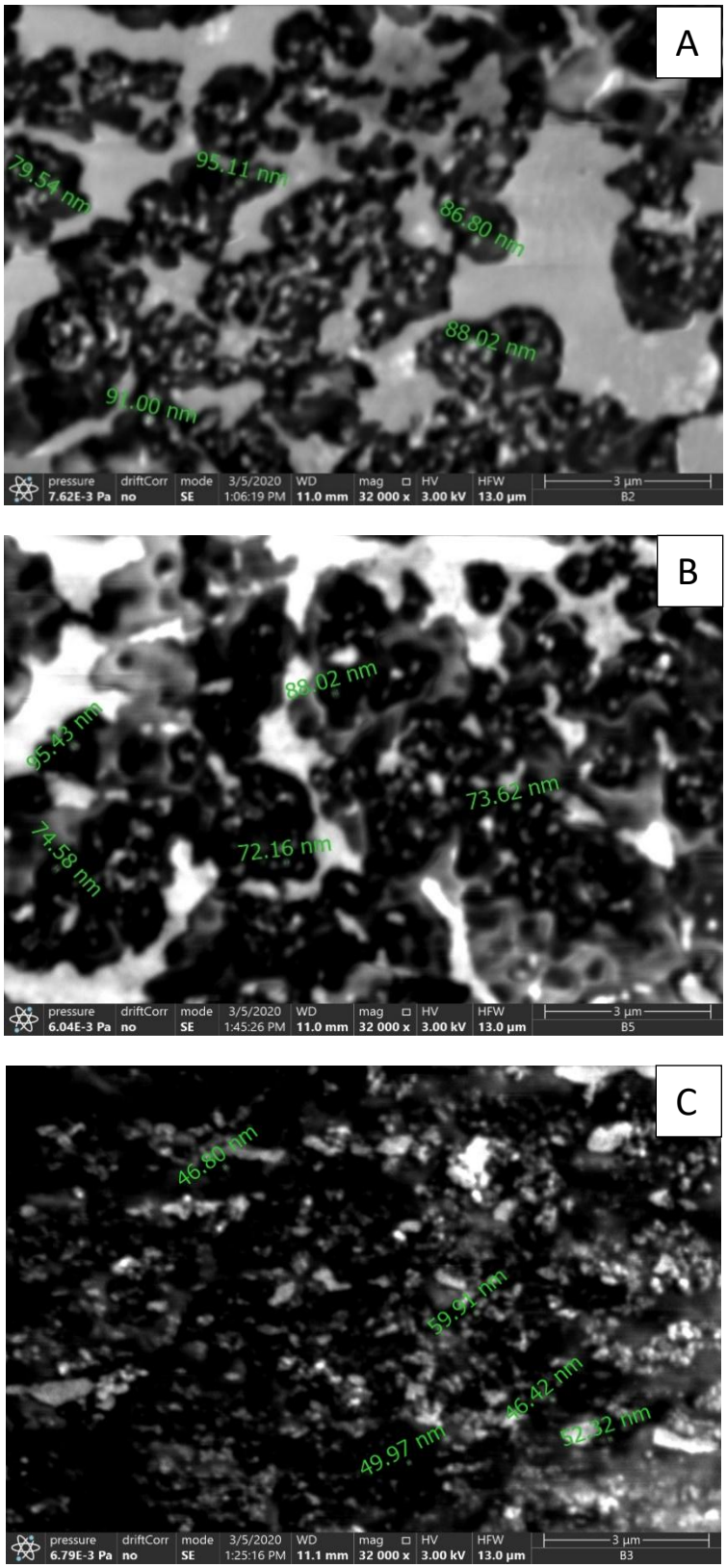
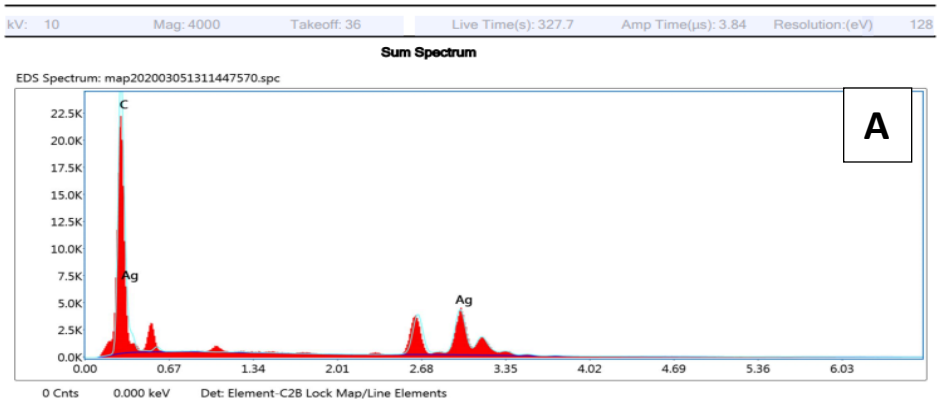
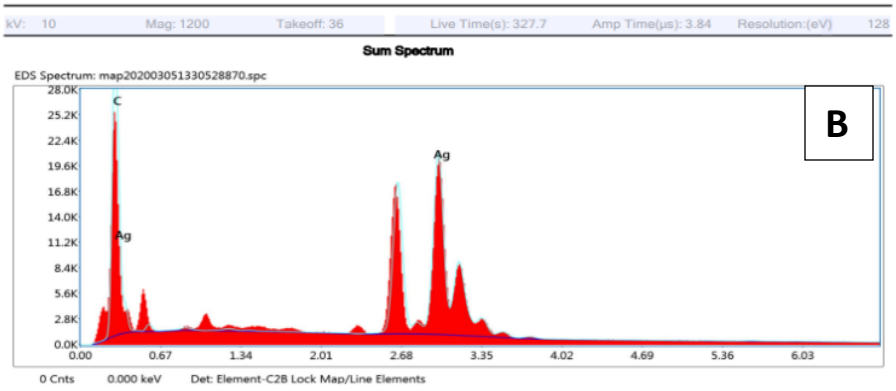


Figure 2 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) B2, b) B3 and c) B5.



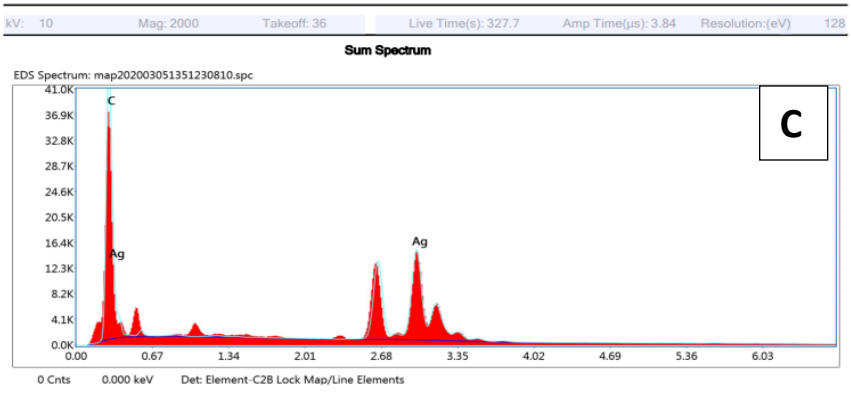
Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	34.17	82.34	3.67
AgL	65.83	17.66	3.47



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	11.53	53.93	4.28
AgL	88.47	46.07	2.88



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	20.61	69.98	3.95
AgL	79.39	30.02	3.02

Figure 3 EDX Spectra of AgNPs produced by isolates a) B2, b) B3 and c) B5

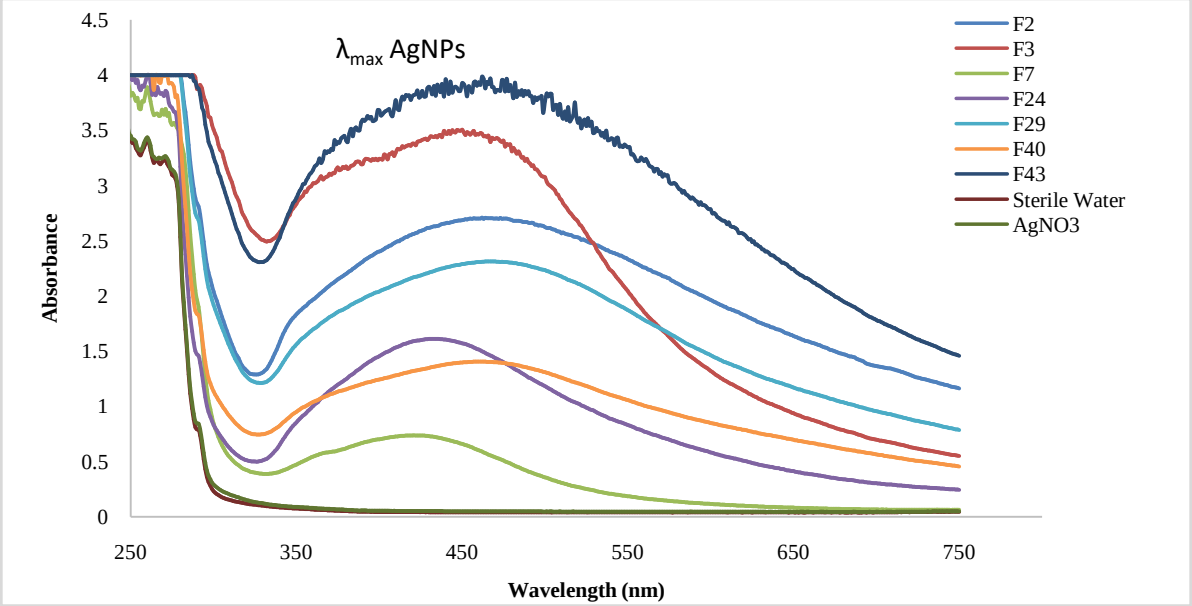


Figure 4 The UV-Vis spectra recorded for the reaction of the different fungal cell filtrates and $AgNO_3$ solution

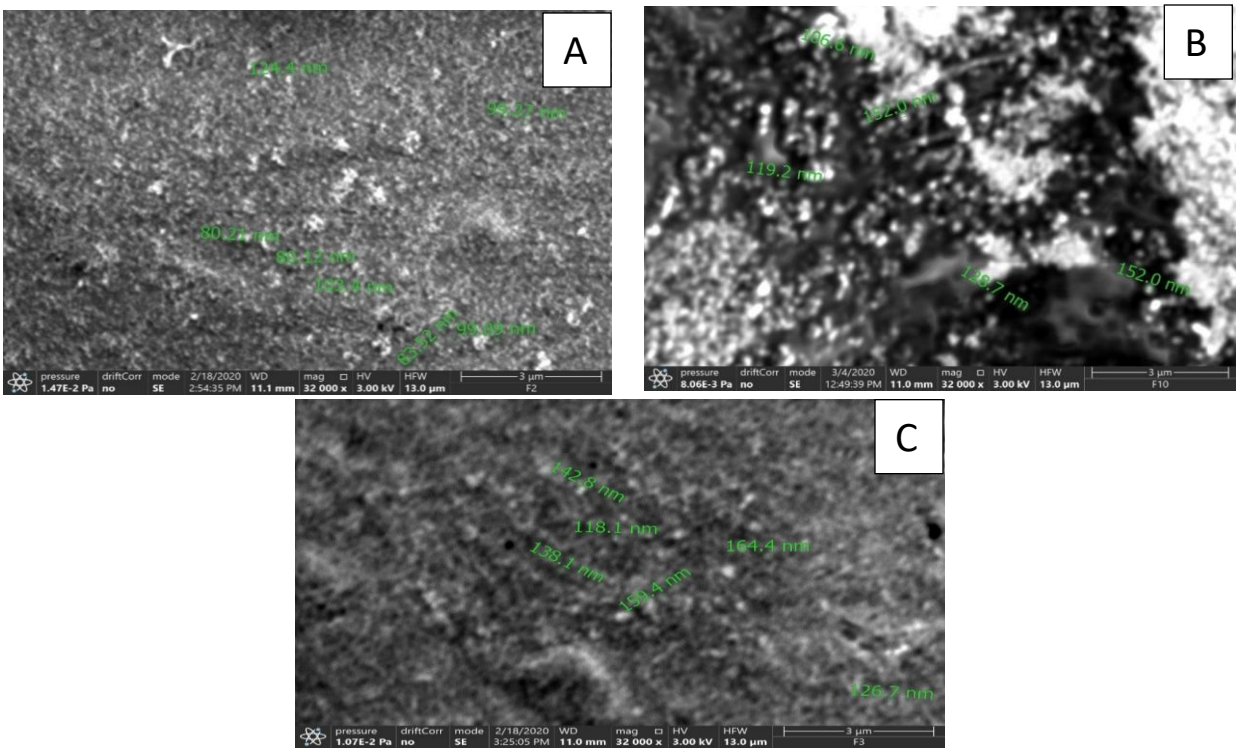


Figure 5 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) F2, b) F3 and c) F43

Isolates F2, F3 and F43 were molecularly identified as *P. citrinum*, *P. glaucoroseum*, and *P. oxalicum*, respectively. As shown in Figure 4, the UV-visible absorption spectra showed absorbance at approximately 450 nm. The scanning electron microscope micrographs (Figure 5) of the dry mass show aggregated spherical-shaped AgNPs with an average size of 88.31 nm, 141.58 nm, and 131.7 nm for isolates F2, F3, and F43, respectively. The likelihood of aggregation is high for small-sized particles because of the large surface area and attractive force between the particles (Honary et al. 2013). In addition, energy dispersive X-ray spectra of AgNPs (Figure 6) produced by isolates F2, F3, and F43 showed peaks corresponding to silver (Ag), suggesting the presence of Ag in the nanomaterial.

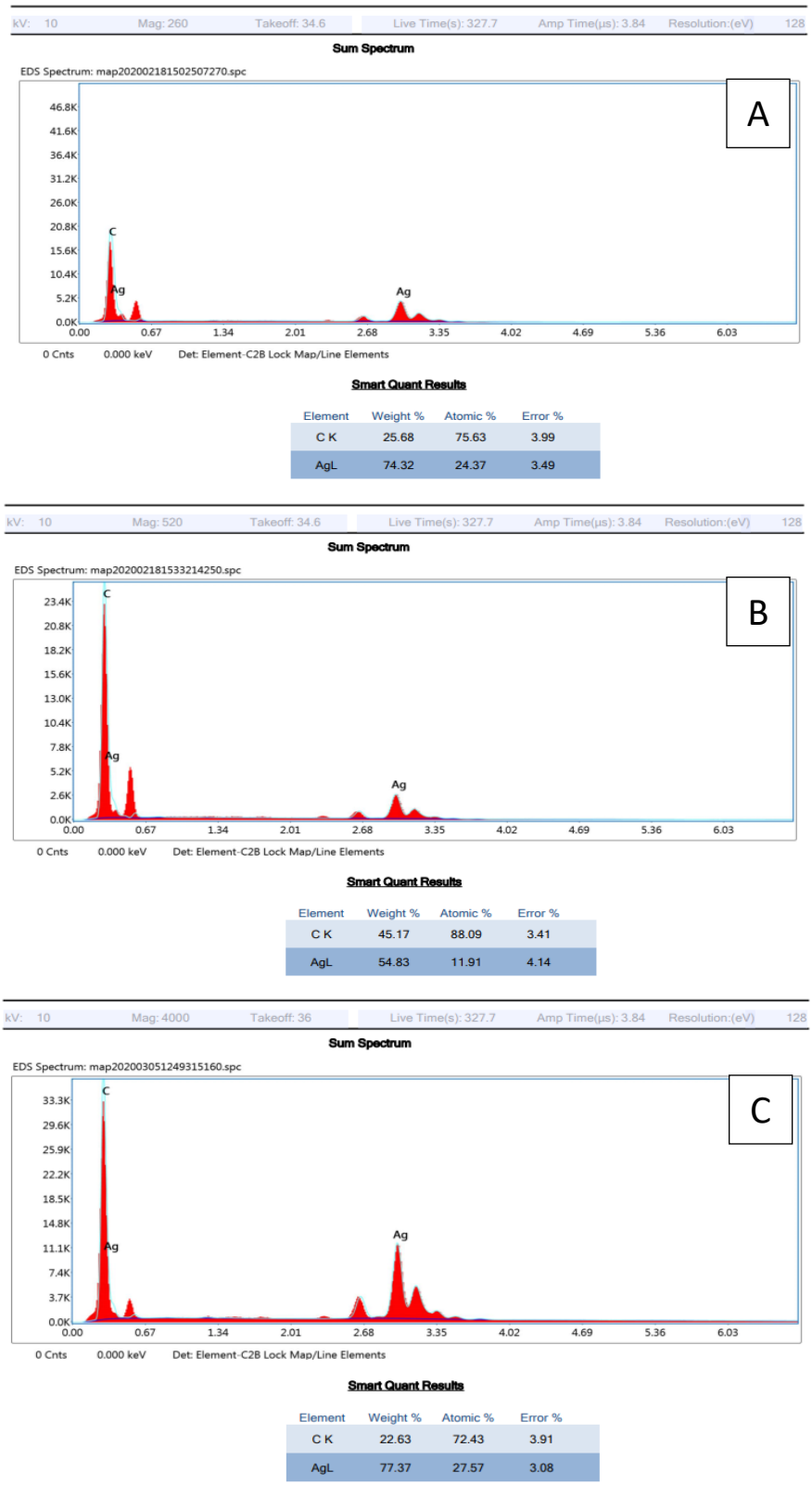


Figure 6 EDX Spectra of AgNPs Produced by isolates a) F2, b) F3 and c) F43.

The *Penicillium* family is an emerging nanofactory for the biosynthesis of green nanomaterials. There are 25 *Penicillium* species reportedly used for the biosynthesis of nanomaterials (Barabadi et al. 2019); notably, AgNPs were predominantly produced by exploiting *Penicillium* species. In another study, the crude cellular extract of *P. oxalicum* GRS-1 produced spherical AgNPs with sizes ranging from 10 to 40 nm (Rose et al. 2019). In addition, AgNPs produced from *P. oxalicum* have a characteristic strong broad peak at 456 nm and are also spherical (Bhattacharjee et al. 2017). Moreover, the production of spherical-shaped and well-dispersed AgNPs with an average particle size of 2 to 5 nm from *P. citrinum* was confirmed (Danagoudar et al. 2020), which exhibited an absorption band around 400 to 420 nm and a particle size of 90 to 120 nm (Honary et al. 2013).

Isolates F7, F24, and F29 were molecularly identified as *Aspergillus flavus, A. terreus*, and *A. japonicus,* respectively. F40 was morphologically similar to F29, hence assumed also to be *A. japonicus*. As shown in Figure 4, the UV-visible absorption spectra showed absorbance at approximately 420 nm to 450 nm. The scanning electron microscope micrographs (Figure 7) of the dry mass show aggregated spherical shaped AgNPs with an average size of 124.62, 143.54, 159.86, and 85.92 nm, respectively. Moreover, EDX spectra of AgNPs (Figure 8) produced by isolates F7, F24, and F29 showed peaks corresponding to silver (Ag), suggesting the presence of Ag in the nanomaterial.

Various previous studies confirmed the capability of some *Aspergillus* species in the production of silver nanoparticles. In a study done by Li et al. (2011), polydispersed spherical particles ranging in size from 1 to 20 nm were produced using culture supernatants of *A. terreus*. They also reported that reduced nicotinamide adenine dinucleotide (NADH) was an essential reducing agent for biosynthesis and that AgNP production may be an enzyme-mediated extracellular reaction process. In another study, the fungus *A. flavus* produced monodispersed AgNPs that showed an absorption peak at 420 nm and an average size of 8.92 nm (Vigneshwaran et al. 2007). Extracellular synthesis of cubic

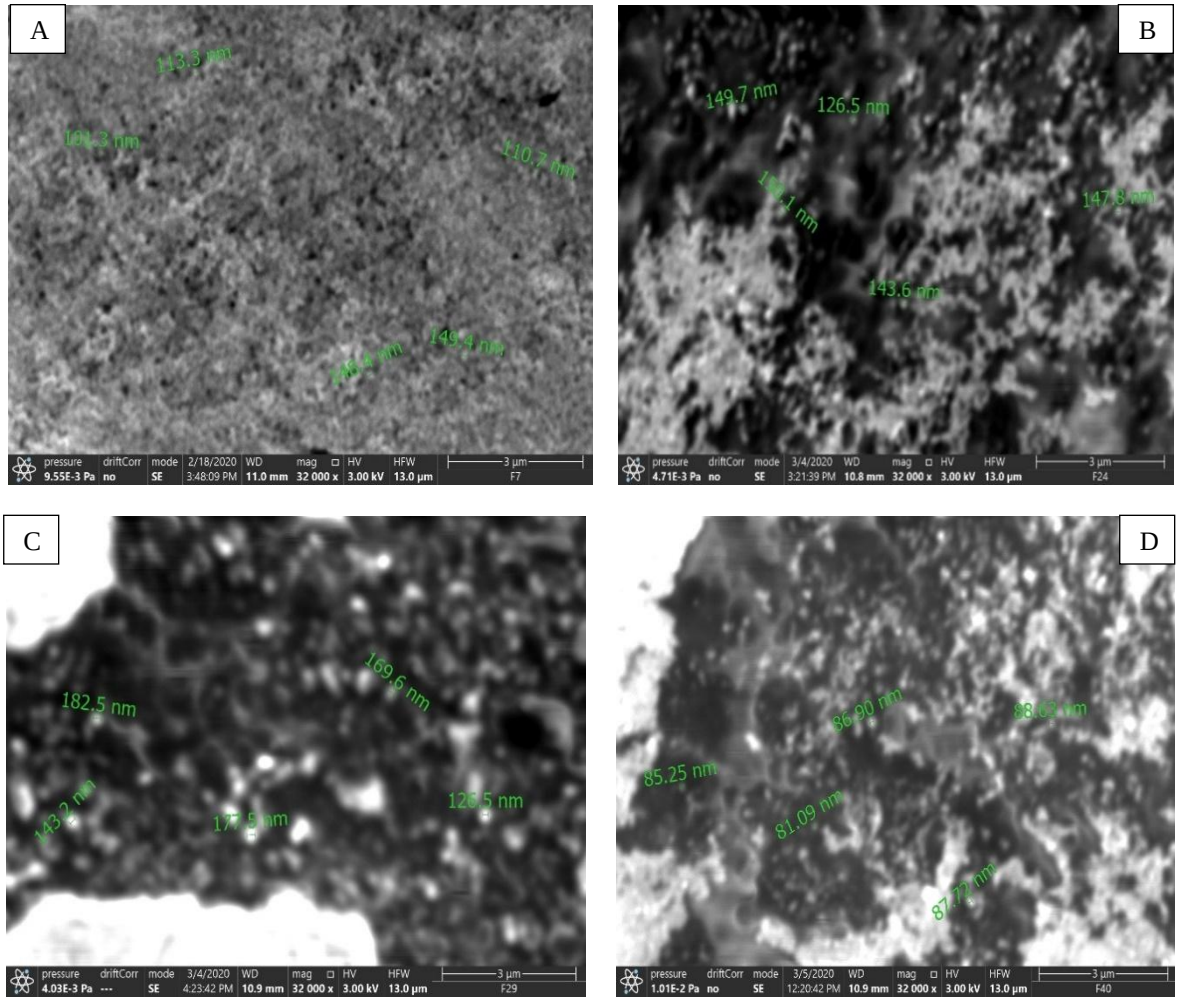


Figure 7 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) F7, b) F24 c) F29 and d) F40

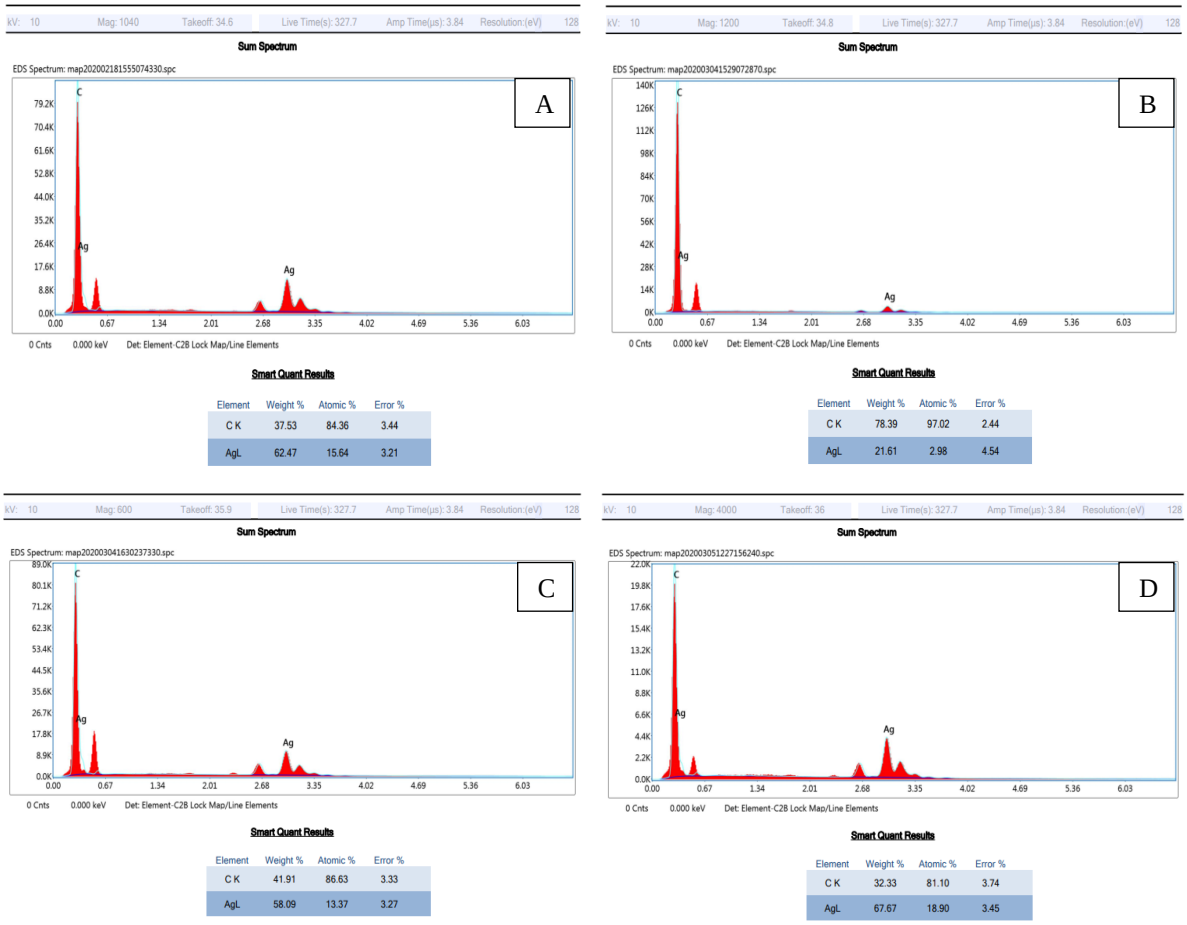


Figure 8 EDX Spectra of AgNPs produced by isolates a) F7, b) F24 c) F29 and d) F40.

structured AgNPs showed a characteristic absorbance peak of 420 nm and an average size of 33.5 nm using *A. flavus* (Sulaiman et al. 2015). Extracellular AgNPs were synthesized from cellular extracts of an *Aspergillus* consortium, which consisted of *A. niger*, *A. michelle*, and *A. japonicus* (Samuel and Guggenbichler 2004).

Conclusions

Results of the study responded to the challenge of investigating new isolates that are capable of reliable and efficient synthesis of AgNPs. A total of ten isolates belonging to the bacterial class *Bacillus* and fungal classes *Penicillium* and *Aspergillus* were found capable of producing AgNPs. The AgNPs produced have characteristic absorption peaks at 420 nm to 450 nm, mostly spherical and of varied sizes. EDX spectra showed peaks belonging to silver (Ag), suggesting the presence of Ag in the nanoparticles.

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