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# Laccase Facilitated Synthesis of Silver Nanoparticles for Biomedical, Biocontrol and Dye Degradation Applications

Kazeem, M.O[1]*, Titilope, A. P.[1], Agbaje, A. B.[2], Ogunleye, G. E.[3]

[1]Department of Microbiology, Faculty of Life Sciences, University of Ilorin, Nigeria
[2]Department of Biological Sciences, Faculty of Natural and Applied Science, Al-Hikmah University, Ilorin, Nigeria
[3]Department of Biological Sciences, Faculty of Applied Sciences, Koladaisi University, Ibadan, Nigeria.

*Corresponding Authors Email: kazeem.mo@unilorin.edu.ng

## ABSTRACT

Green synthesis of various metallic nanoparticles has gained significant attention in recent years due to their numerous applications. Laccase synthesis of silver-nanoparticles (AgNPs), characterization and applications were investigated. The synthesized AgNPs were characterized using UV-visible spectroscopy, FTIR, SEM and EDS. Antimicrobial activity of the synthesized AgNPs against *Escherichia. coli, Staphylococcus aureus*, *Colletotrichum sp*., *Fusarium sp.*, *Penicillum* and *Aspergillus niger* was studied. Disease suppression capability of AgNPs on chili pepper and pear fruits were monitored by visual observation. A yellow to brown colour change and surface plasmon resonance peak at 407 nm indicated AgNPs formation. The FTIR spectra of the AgNPs reveals the appearance of distinctive bands, SEM analysis showed a particle size (20 nm-100 nm) with spherical morphology while EDS reveals a strong signal in the silver region and confirm the formation of AgNPs. The AgNPs showed growth inhibition of bacteria (33-42%), fungi (12-33%) and dyes degradation (25-63%). Furthermore, effective disease suppression was observed on pear and chili pepper fruits. This study described the synthesis of AgNPs using laccase from *A. carbonarious* and emphasizes its role in disease suppression and industrial dye degradation.

**Keywords:** Laccase, $AgNO_3$ Nanoparticles, Biomedical, Biocontrol, Dye degradation.

## INTRODUCTION

Nanotechnology is a rapidly growing science focusing on the synthesis and application of materials ranging from 1 to 100 nanometers, with significant applications in various human life fields. Nanomaterials, with their high surface-area-to-volume ratio, high reactivity, and enhanced catalytic and biological properties, are suitable for various applications in biomedicine and agriculture (Upadhyay *et al*., 2020). The attention has been drawn to precious metals like silver, gold, and platinum, as well as magnetic oxides like $Fe_3O_4$ nanoparticles (Jamkhande *et al*., 2019).

The conventional methods for synthesizing nanoparticles often involve toxic chemicals, leading to waste generation and potential environmental pollution (Ahmed *et al*., 2016). Biogenic nanoparticle synthesis using plants, microbes, and their products is gaining global attention due to its rapid, stable, reproducible, and eco-friendly nature compared to physical and chemical methods (Lateef *et al*., 2016).

The recent advancement in nanotechnology involves enzyme-mediated synthesis of nanoparticles. Active enzymes typically catalyze nanoparticle formation, but under certain conditions, they can be denatured to release amino acids, which serve as reducing

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and stabilizing agents. Enzymes like ligninase, cellulase, nitrate reductase, and sulfite reductase are crucial in the synthesis of nanoparticles, acting as reducing and capping agents. (Elegbede *et al*., 2018; Singh *et al*., 2017). Recently, the synthesis of AgNPs using crude keratinase produced by a feather-degrading *Serratia ficaria* isolated from a peacock feather was reported (Sudha *et al.*, 2023). Laccase, a blue copper oxidase, has significant industrial potential due to its ability to catalyze the oxidation of phenolics, aromatic amines, and other electron-rich substrates. Laccase, a key lignolytic family member, exhibits broad substrate specificity and has shown potential in industrial applications like biobleaching (Unuofin *et al*., 2022), detoxification (Blánquez *et al*., 2019), and biosensor (Kadam *et al*., 2022). Laccase are also used as animal feed digestibility improvement, enhancement of texture and flavor of bakery products and clarification in beverage industry (Alberto *et al*., 2020; Backes *et al*., 2021).

The aim of this study is to explore the application of crude laccase from *Aspergillus carbonarius* F5 produced in our laboratory (Arekemase *et al.*, 2022), for the synthesis of AgNPs and examine their antimicrobial, biocontrol and dye degrading potential. This is an effort to further increase the prospect of application of laccases in nanobiotechnology.

## MATERIALS AND METHODS

### Sample Collection

Chilli peper (*Capsicum annuum*) and pear (*Pyrus communis L.*) fruits showing symptoms of disease were procured from Ipata Market, Ilorin, Kwara State, Nigeria. The bacteria isolates were obtained from the culture collection of the Microbiology Department, University of Ilorin.

### Isolation and Characterization of Spoilage Fungi

Fungi were recovered from the fruit samples using the serial dilution method. The stock solution was made by combining one gram (1g) of contaminated pear and chilli pepper fruits with 10 mL of distilled water. Following serial dilution, the appropriate diluent was plated on PDA plates and incubated at 30 °C for 5 days. Colonies were stored as pure culture at 4 °C. Fungal isolates were described based on colony and cellular morphology. The morphology was based on colour, size, texture while microscopic examination was done based on reproductive and vegetative structure using lactophenol cotton blue stain.

### Laccase Production

Crude laccase was produced from *Aspergillus carbonarius* F5 as previously described by Arekemase *et al.* (2022) with slight modification. Briefly, two agar plugs of 5 mm from a 5 day old *A. carbonarius* F5 culture was inoculated into 250 mL Erlenmeyer flask containing 100 mL of basal medium containing (g/L):Yeast extract, 2 g; $CuSO_4$, 2.0 mM; $MnSO_4$, 1 mg; $MgSO_4$. $7H_2O$, 0.2 g; $KH_2PO_4$, 1 g; and $CaCl_2$ 0.2 g, pH 6.0. Additionally, 2 g of acid pretreated *Delonix regia* pods was added to the flask, as carbon substrate and incubated at 30 °C, under shaking at 150 rpm for 7 days. Culture was harvested and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was removed and saturated with 80 % ammonium sulphate. The resulting pellet was dissolved in 10 mM phosphate buffer (pH 6.5) and stored at 4 °C until further use.

### Laccase Assay

Laccase activity was carried out in a reaction mixture containing 1 mL of 10 mM guaiacol, 2 mL of 100 mM sodium acetate buffer, and

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1 mL of crude enzyme. The mixture was incubated at 30ºC for 10 min and the absorbance was read at 450 nm using UV spectrophotometer (Vis spectrophotometer S32A) (Arekemase *et al*., 2022). Laccase activity at 10.53 U/mL was obtained in this study.

**Biosynthesis of AgNPs**

The Laccase produced from *A. carbonarius* F5 as described above was used for the synthesis of the AgNPs. Here, 3 mL of crude enzyme was added with 150 mL of 1 mM $AgNO_3$ solution and crude laccase enzyme (6 mL) was dispensed into reaction vessel containing 15 mL of 1 mM $AgNO_3$. A control experiment containing 50 mL of $AgNO_3$ and 3 mL of distilled water was also set up. The reaction was incubated at room temperature (28 ± 2 °C) with intermittent shaking. The development of colour change to brown as a result of formation of AgNPs was observed visually.

**Characterization of AgNPs**

The synthesized AgNPs was characterized by measuring the absorbance spectrum using UV–Vis spectrophotometer (752 UV-Vis Spectrophotometer) with the wavelength ranging from 400 - 700 nm. The biosynthesized AgNPs' functional groups are determined using Fourier Transform Infrared (FTIR) spectroscopy. (Bhat *et al.*, 2011). The surface morphology of the biosynthesized nanoparticles was elucidated through Scanning Electron Microscopy (SEM) and the elemental composition was evaluated using the Energy Dispersion X-ray Spectroscopy (EDS).

**Antifungal Activities of Biosynthesized AgNPs**

The antifungal activities of the *A. carbonarious* F5 Laccase syhthesised AgNPs (AF5L-AgNPs) were tested against the pathogenic fungi such as *Colletotrichum sp*., *Fusarium sp.*, *Penicillum* and *Aspergillus niger*. The fungal culture plug of 5 mm diameter was placed on PDA impregnated $AgNO_3$ (100 mL PDA and 10 mL AgNPs), while the control plate is devoid of nanoparticles. The plates were incubated for 48 hours at 30°C, and the fungal radial growth was monitored to assess the growth inhibition. using equation 1.

$$\% \text{ Growth inhibition} = \frac{Dcontrol - Dtest}{Dcontrol} x\ 100 \ldots\ldots\ldots\ldots Eq\ 1$$

where *D* represents diameter of fungal growth.

**Antibacterial Activities of Biosynthesized AgNPs**

The antibacterial activity of AF5L-AgNPs was tested against clinical isolates of *Escherichia coli* and *Staphylococcus aureus*. The bacterial strains were grown on nutrient agar and incubated at 37°C for 24 hours. The bacteria were then added to a nutritional broth, cultured at 37°C for 24 hours, and adjusted to the 0.5 McFarland standard. Then, 1 mL of the AF5L-AgNPs was added to test tubes containing 8 mL of peptone and 1 mL of the bacterial suspension, and incubated at 37°C for 24 h. The control comprised 1 mL of bacterial solution and 8 mL of peptone water without AF5L-AgNPs. The optical density of bacteria at 600 nm was measured using a UV-Vis spectrophotometer to monitor their growth. The percentage inhibition of growth was calculated using the methods of Salem *et al.* (2014) as illustrated in equation 2.

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$$\% \text{ Growth inhibition} = \frac{ODcontrol - ODtest}{ODcontrol} x\ 100 \ldots\ldots\ldots\ldots Eq\ 2$$

Where O*D* represents optical density of bacterial growth.

**Dye Degradation Activities of Biosynthesized AgNPps**

The degradation of blue and black dye using AF5L-AgNPs were monitored through decolorization, with the reaction incubated at 30°C for 24 hours and the absorbance measured using a UV-Vis spectrophotometer. In a 50 mL Erlenmeyer flask, 1 mL of AF5L-AgNPs was combined with 9 mL of dyes. The control experiment involved a mixture of 1 mL pure water and 9 mL of dye. The flask was incubated under shaking at 100 rpm. The reaction's absorbance was measured at 619 nm with a UV-Vis spectrophotometer. The percentage degradation of the dyes as a result of the nanoparticles was determined using equation 3.

$$\% \text{ dye degradation} = \frac{Acontrol - Atest}{Acontrol} x\ 100 \ldots\ldots\ldots\ldots Eq\ 3$$

where *A* represents the absorbance value.

**Evaluation of Biosynthesized AgNPs as Biocontrol and Preservative**

Two methods were used to evaluate AF5L-AgNPs' antifungal activity on fresh healthy pear and chilli fruits. Fresh fruits were first sanitized with ethanol and sodium hypochlorite before being air-dried. The AF5L-AgNPs and crude laccase were applied separately to the surface of the fruits using a sterile brush while the control was left untreated. The fruits were air-dried, stored at room temperature (30 ± 2 °C) for 5 days, and monitored daily for disease symptoms. The second procedure involved purposely injuring the fruits with a sterile needle after they had been surface sterilized. The AF5L-AgNPs were then applied to the wound area and left to air dry for 30 minutes. A fungal pathogen was then applied on the wounded site. The fruits were kept at room temperature for five days and were monitored daily for any signs of disease.

## RESULTS AND DISCUSSION

**Identification of Fungal Pathogen**

Two fungi each were isolated from pear and chilli pepper fruit. After characterization it was clear that the pear fruits were attacked by *Aspergillus niger* and *Penicilium digitatum* while the chilli were attacked by *Colletotrichum gloesporioides* and *Fusarium sp.*The microscopic view of the fungi is shown in Figure 1.

**Biosynthesis and Characterization of Biosynthesized AgNPs**

The color of $AgNO_3$ changes from yellow to brown due to laccase reduction, indicating the formation of silver nanoparticles. The color change in metallic nanoparticles is attributed to the coherent excitation of surface plasmoson resonance (SPR). The brown colour of the biosynthesized AgNPs was previously reported (Alharbi *et al.*, 2023). This was confirmed by UV–vis spectroscopy (Figure 2). The SPR of the silver nanoparticles remained between 400 and 450 nm.The SPR reveals AgNPs absorbing radiation intensely at 407 nm due to electron transition, with the maximum absorption wavelength indicating AgNP synthesis. The obtained value falls within the established range of 391-460 nm for AgNPs (Alharbi *et al.*, 2023; Lateef *et al.*, 2015)

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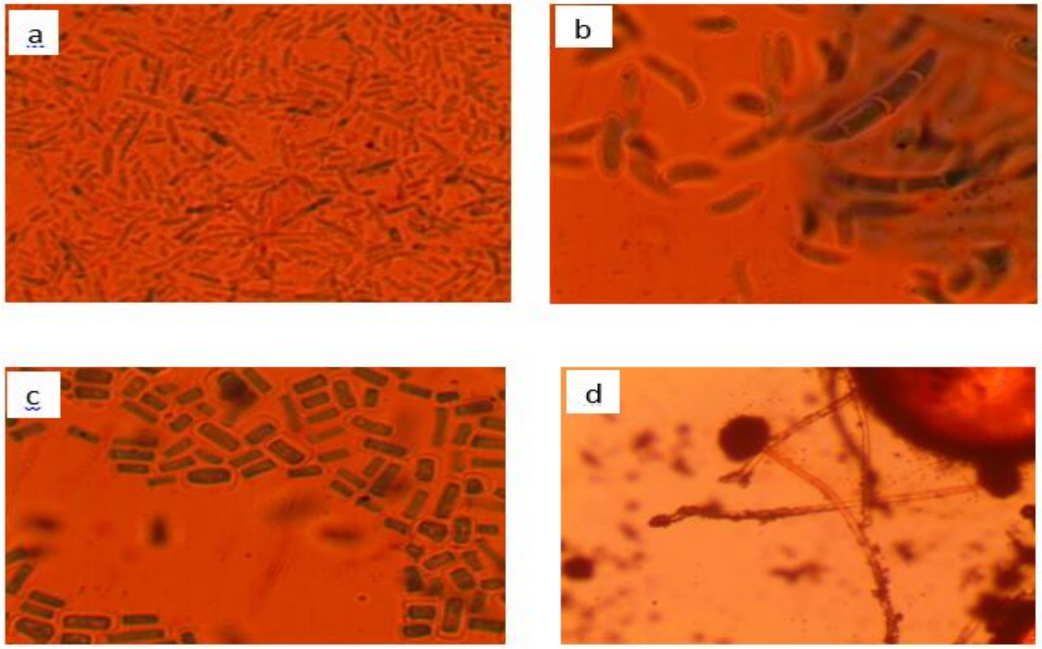


**Figure 1:** Microscopy of fungi pathogens at x 100 objective *Collectrichum gloesporioides* (a), *Fusarium sp.* (b), *Penicillium digitatum* (c), *Aspergillus niger* (d)

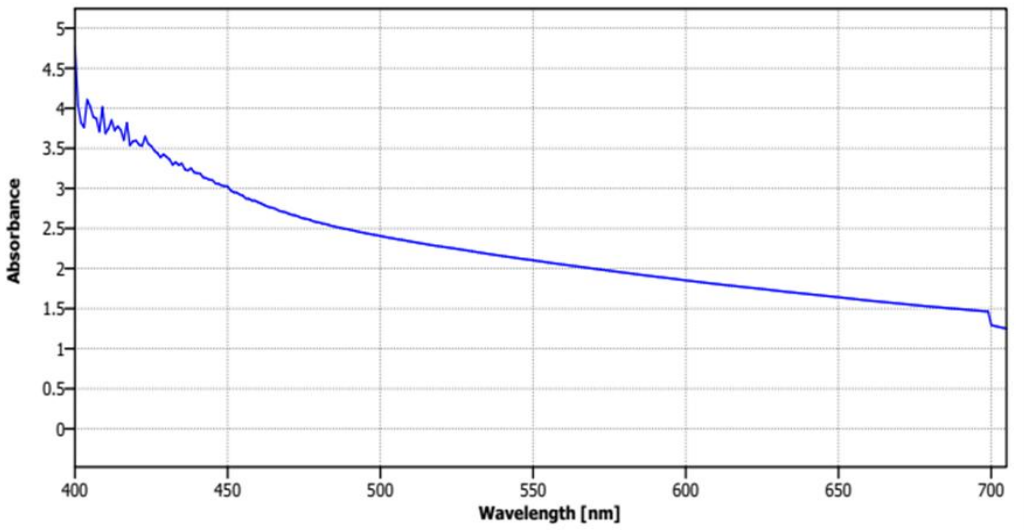


**Figure 2:** UV-Vis absorption spectra of biosynthesized AF5L-AgNps

The FTIR spectra shows the functional groups in the synthesized AgNps (Figure 3). The FTIR spectra of the synthesized AgNPs reveals that the synthesized AgNPs contain some compounds. The stability of the AgNPs can be attributed to the presence of these functional groups. Thirteen absorption peaks were observed starting from 3785cm$^{-1}$ to 712.66 cm$^{-1}$. The absorption peaks at 3785 - 3406 cm$^{-1}$ could be attributed to OH-stretching of hydroxyl groups, alcohols and N-H secondary amides. The absorption band

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at 2919 and 2855.82 $cm^{-1}$ indicated the C-H-stretches of aldehydes. The peak at 2354 $cm^{-1}$ corresponded to the –COOH overtone acid group. The peaks at 1711.88$^{-1}$ and 1601.41 $cm^{-1}$ are attributed to the C=O stretch of carboxylates. The peak at 1442.53 $cm^{-1}$ is assigned to the C=C bend of aldehyde and the absorption peak at 1372 $cm^{-1}$ indicated symmetrical C-H bend of alkane. The peak at 1280 $cm^{-1}$ corresponded to the vibration of carboxylic esters. The peak at 1038 $cm^{-1}$ is attributed to the presence of C-O functional group of esters and phenol. The absorption peak at 712.66 $cm^{-1}$ is attributed to the C-CL stretch of monochloride.The phenolic groups act as reducing agents while the C-H and N-H groups (proteins) act as stabilizers. Similar results was also reported by (Elegbede *et al.*, 2018) with biosynthesis of AgNPs using xylanase.

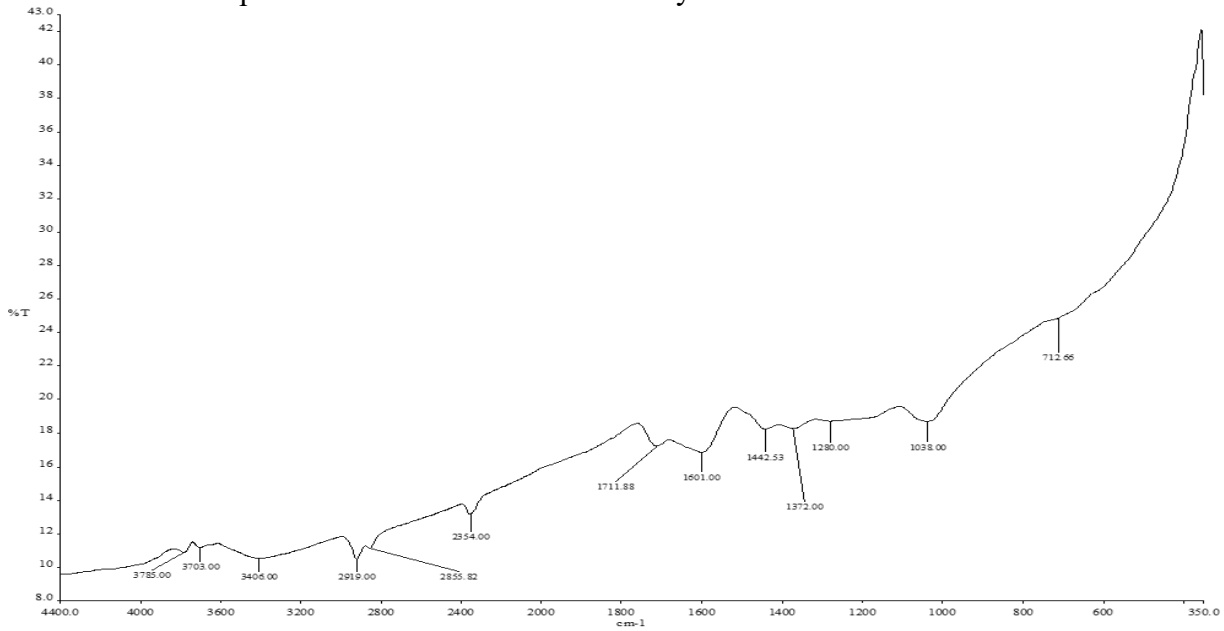


**Figure 3:** FTIR spectra of biosynthesized of biosynthesized AF5L-AgNPs.

The SEM images (Figure 4a-c) show the morphological properties of the AgNPs. The AgNPs were visualized at 7000, 8000 and 9000 magnification, respectively. At 7000 and 8000 magnification the AgNPs appeaed as ordered and cluster structures. However, the AgNPs structure appeared as clauded mass when observed under 9000 magnification.The images reveal that the nanoparticles were spherical in shape and the sizes ranges between 20 nm-100 nm. The AgNPs of spherical shape and varying sized have been previously reported (Lateef *et al.*, 2015). The EDS was used to ascertain the elements present in the synthesized silver nanoparticles (Figure 4d). A strong signal was obtained at the energy level of 1.5keV for silver, some weak signals from C and O were also observed. The major emission energy at 1.5keV indicates that silver has been correctly identified.

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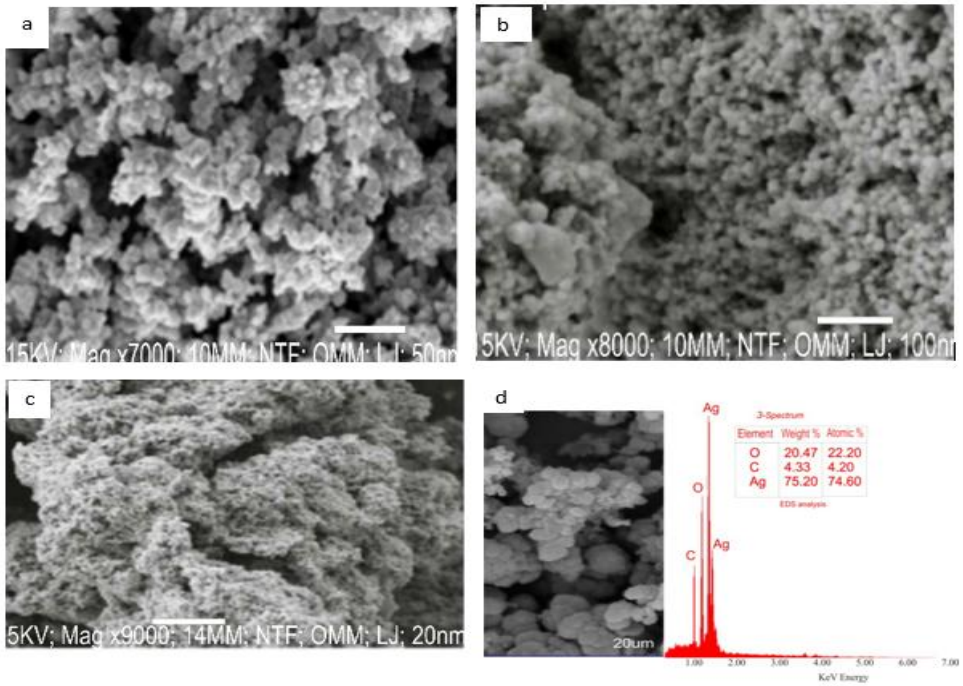


**Figure 4**: SEM analysis of biosynthesized AF5L-AgNPs 50 nm with Mag x 7000 (a), 100 nm with Mag x 8000 (b), 20 nm with Mag x 9000 (c), EDS spectrum of biosynthesized AF5L-AgNPs (d)

**Antibacterial, Antifungal and Dye degradation potential of AF5L-AgNPs**

The synthesized nanoparticles showed antibacterial activity by inhibiting growth of *E. coli* by 33% and *S. aureus* by 42% (Figure 5). It exhibited antibacterial activity against both the Gram-positive and Gram-negative bacteria. From the results, it was observed that the synthesized nanoparticles exhibited high zones of inhibition in both cases by significantly reducing the growth of *A. niger, P. digitatum*, *C. gleosporioides,* and *Fusarium spp*. The inhibitory properties of nanoparticles have been linked to the commencement of an attack on the cell wall, which kills fungal spores, culminating in the seepage of internal contents and, eventually, cell death. AgNPs, when small, can effectively infiltrate bacterial cells in liquid mediums, potentially enhancing their antibacterial effect. AgNPs exhibit antibacterial properties due to their interaction with bacterial cell constituents containing sulphur and phosphorus, causing cell death through cell division and respiratory chain attack (Salem *et al*., 2014).

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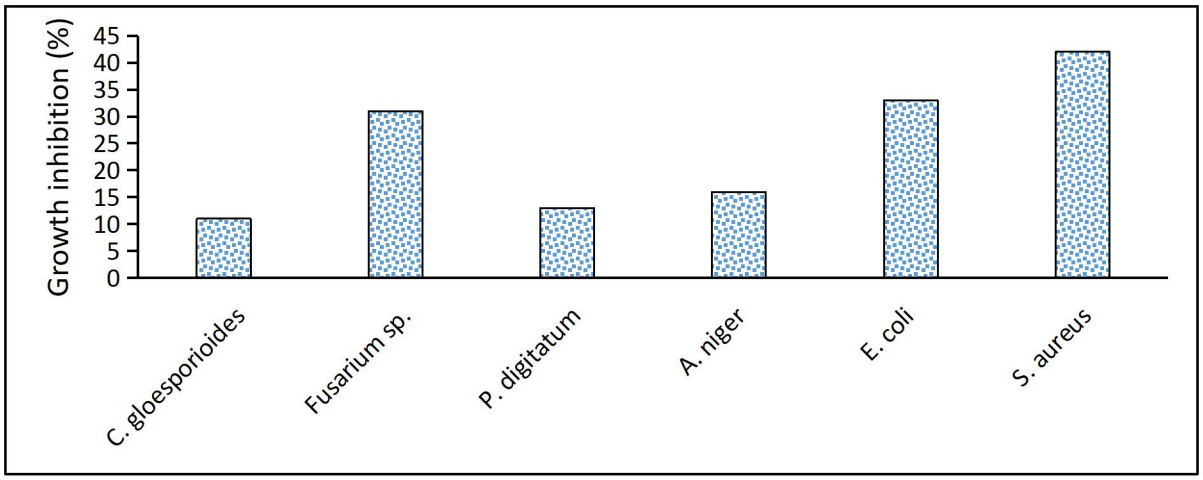


**Figure 5:** Antimicrobial potential of biosynthesized AF5L-AgNPs

**Dye Degradation Potential of AF5L-AgNPs**

The biosynthesized AF5L-AgNPs degraded blue and black dye to varying degrees (Figure 6). The biosynthesized AF5L-AgNPs decolorized tested dyes, with blue dye showing the best degradation, while black dye showed the least degradation after 24 hours of reaction. After 24 hours of reaction, the biosynthesized AF5L-AgNPs decolorized the tested dyes by 63% for the blue dye and 21% for the black. Nanoparticle biodegradation of dyes have been previously reported (Gola *et al.*, 2021; Marimuthu *et al.*, 2020). Nanoparticles catalyze dye degradation through redox reaction, acting as electron transfer mediators between dye biomolecules and particles. Nanoparticles offer greater efficiency in wastewater treatment compared to other physical and chemical methods that concentrate or transfer colors between phases (Elegbede *et al.*, 2018).

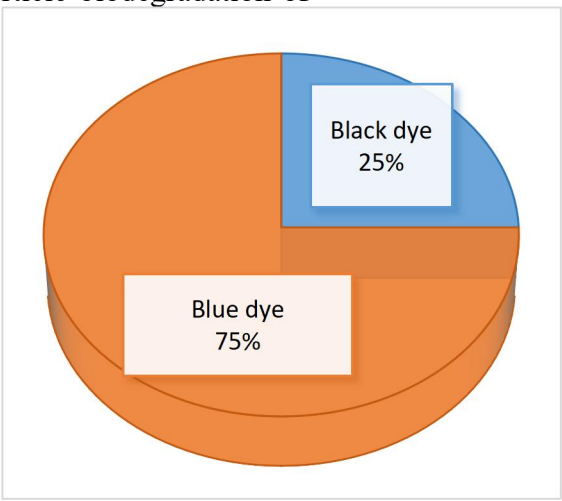


**Figure 6:** Dye degradation potential of the biosynthesized AF5L-AgNPs

**Evaluation of AF5L-AgNPs in biocontrol of fruit disease**

The biosynthesized AF5L-AgNPs inhibited disease development on chilli pepper and pear fruit (Figures 7 and 8). This finding clearly demonstrated that the AgNPs could prevent pear and chilli pepper fruit from spoilage by *C. gloeosporioides* and *A. niger*, respectively.

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Fruit treatment by the coating technique maintained both chilli pepper and pear fruit for a 5-day storage period. Meanwhile treatment by injection of AF5L-AgNPs lowered disease severity on the injured fruits compared to the fruits that were not treated with the nanoparticle. Crude laccase treatment effectively reduced disease severity and incidence in pepper and pear fruits, ensuring intact, unspoiled fruits compared to the untreated. The reduction of disease may be attributed to increased cell permeability and intracellular material leakage resulting from nanoparticle treatment (Li *et al.*, 2022).

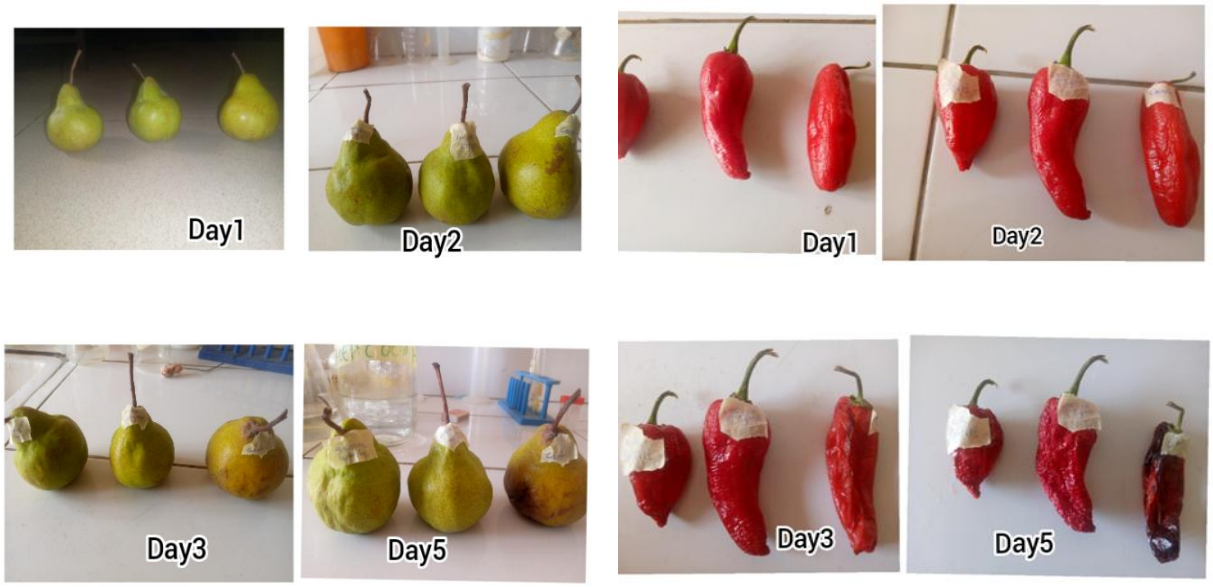


**Figure 7:** Disease suppression on fruits coated with biosynthesized AF5L-AgNPs
Key: From the left Fruit + AF5L-AgNPs, Fruit + Laccase, Fruit only

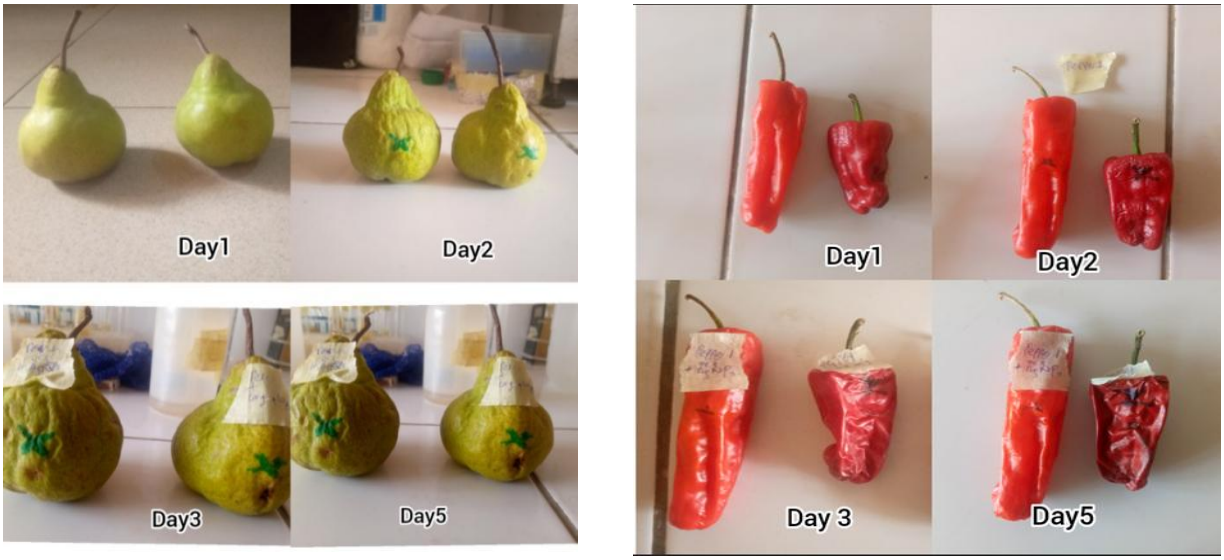


**Figure 8.** Disease suppression on wounded fruits injected with biosynthesized AF5L-AgNPs
Key: from the left, Fruit +pathogen + AF5L- AgNPs, Fruit + pathogen only

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## CONCLUSION

The study utilized laccase from *A. carbonarious* F5 to synthesize AgNPs with a spherical shape and sizes ranging from 20 nm to 100 nm. The UV, SEM, FTIR, EDS analysis shows the characteristic features of the manufactured AF5L-AgNPs. The AF5L-AgNPs also displayed antibacterial and antifungal activities against selected pathogenic isolates of *E. coli*, *S. aureus*, *A. niger*, *Fusarium sp*. *C. geosporioides* and *P. digitatum.* The nanoparticle demonstrated the ability to decolorize and degrade both black and blue dye. The nanoparticle effectively preserves pear and pepper fruits, reducing disease severity and controlling spoilage, demonstrating its potential in managing postharvest disease in stored fruits. As a result, this work thoroughly substantiated the potential nanobiotechnological application of *A. carbonarious* F5 laccase, demonstrating its innumerable usefulness in biomedical, industrial, and agricultural domains. Further research on the mechanism of AF5L-AgNPs' antifungal activity and determining AgNPs residue level on fruits is recommended for safety reasons.

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