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RESEARCH

Characterization of Silver Nanoparticles Biosynthesized using Keratinase from *Aspergillus* species and their Antibacterial Activity against Clinical Pathogens

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Abstract
Silver nanoparticle (AgNP) synthesis using biologically sourced agents has recently gained interest from researchers due to its environmentally friendly, cost-effective and non-toxic nature. This study evaluated the antibacterial potential of AgNPs biosynthesized using crude keratinase produced by *Aspergillus* recovered from dumpsite. *Aspergillus* species were cultured in a minimal medium containing feather (1%) and yeast extract (0.2%) for the bioproduction of keratinase. Silver nitrate (1 mM) was bio-reduced using keratinase and characterized using UV–visible spectroscopy, Fourier transform infrared (FTIR) spectroscopy and transmission electron microscopy (TEM). The antibacterial potentials of the AgNPs were investigated on multidrug resistant-clinical pathogens (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Yersinia enterica*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, *Citrobacter freundii*, *Enterobacter cloacae* and *Klebsiella oxytoca*) using disk diffusion method. Three fungal isolates *A. niger* (F1), *A. oryzae* (F2) and *A. aculeatus* (F3) were recovered with *A. niger* (F1) and *A. aculeatus* (F3) showing high keratinase activities of 48.52 U/ml and 46.04 U/ml, respectively. Absorbance peaks of 289 nm and 295 nm were observed for *A. niger* (AgNPs-F1) and *A. aculeatus* (AgNPs-F3), respectively. The spectral peaks from the FTIR revealed the presence of O–H stretch of amide (3616.59 and 3474.41) in biosynthesized AgNPs. Particle sizes ranging from 3.04 to 12.39 nm and 15.47 to 32.83 nm were observed for AgNPs-F1 and AgNPs-F3, respectively. AgNPs-F1 and AgNPs-F3 showed antibacterial against 80% and 70% of test isolates with *Staphylococcus aureus*, *Yersinia enterica*, *Klebsiella pneumoniae*, *Enterobacter cloacae* and *Klebsiella oxytoca* as the most susceptible pathogens.

Keywords *Aspergillus* species · Silver nanoparticle · Keratinase · Multidrug-resistant bacteria · Clinical pathogens

1 Introduction

Nanotechnology has gained interest from various fields like biology, medicine, physics, chemistry and materials science [1]. Advancements in nanotechnology have cleared the path for new possibilities in nanomedicine, enabling nano-antibiotics and nanoparticle production [2]. Nanotechnology stands out as a fascinating research field focused on manipulating materials at a characteristic scale typically measured in nanometers [3], displaying unique physicochemical properties as opposed to larger particles of similar material, leading to increased reactivity. Their small size and large surface area enhance their effectiveness, making them particularly advantageous for various biological applications [4].

Synthesis of these nanoparticles could be achieved by biological, physical and chemical methods [5]. Both

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chemical and physical methods are at a disadvantage with much concern about their toxicity and low yield. However, the biological (green synthesis) method is considered better than these conventional techniques owing to its reduced toxicity and eco-friendly character. Furthermore, there are high nanoparticle yields with minimal downstream processing effort [6]. Utilizing green nanoparticles offers several benefits, including low energy consumption, high efficiency and moderate operating conditions [7], with high relevance in medicine, catalysis, electrochemistry, agriculture, biotechnology, textile coating, cosmetics, food and environment [8.9]. The desirable characteristic of biogenic nanoparticles is their ability to function as a template capable of reducing and stabilizing nanoparticles [10].

Nanoparticles are available in different forms and they include zinc oxide (ZnONPs), silver (AgNPs), platinum (PtNPs), copper (CuNPs), iron oxide (Fe_3O_4NPs), gold nanoparticles (AuNPs) and selenium nanoparticles (SeNPs) with excellent reports on some as good antimicrobial agents especially in the area of drug resistance [11, 12]. An increase in microbial resistance against the common conventional antibiotics in recent times has prompted research using bactericidal nanomaterials [13]. The efficiency of AgNPs extends to both Gram-negative and Gram-positive bacteria with much attention on multidrug-resistant (MDR) bacteria. When combined with organic compounds or antibiotics, AgNPs exhibit synergistic effects, increasing their antibacterial efficacy against harmful pathogens which include *Staphylococcus aureus* and *Escherichia coli* [14]. Antimicrobial properties of AgNPs are increasingly being used in augmentation of the therapeutic activity of drugs like Nystatin, Amphotericin B, Fluconazole and composite scaffolds for precise drug release control and for targeted drug delivery owing to their reduced toxicity and excellent biocompatibility [15].

Biosynthesized AgNPs when used at a low concentration are safe for humans and also considered safe inorganic antibacterial agents having demonstrated strong antimicrobial activity against a broad range of pathogens [16]. The monovalent ionic silver (Ag +) deposited by AgNPs influences their activity as it diffuses into microbial cells, destroying the cell membrane, respiratory enzymes and electron transport components to limit growth [17]. Several studies have explored fungi, yeast, actinomycetes, bacteria and microalgae as a sustainable approach to synthesizing nanoparticles with unique chemical and physical properties [18–20].

Fungi are becoming increasingly important as biological agents in the synthesis of AgNPs due to their availability, abundance and extensive tolerance to metals. They have shown the ability to release a plethora of extracellular enzymes that function as stabilizing agents [21, 22]. The utilization of fungi is valuable in that a significant amount of enzymes and cellular protein can be harnessed for the downstream synthesis of AgNPs [23]. Among the fungi genera, of importance are *Aspergillus* [24], *Fusarium* [25], *Acinetobacter* [26], *Penicillium* [27] and *Rhizopus* [28].

To further explore the potential of fungi in biomedical applications, this study aims to biosynthesize and characterize AgNPs bio-reduced by crude keratinase produced from *A. niger* and *A. aculeatus* isolated from dump sites and its resulting antibacterial efficacies on multidrug-resistant clinical pathogens.

2 Materials and Methods

2.1 Fungi Isolation and Identification

The fungi used in this study were isolated using the method reported by Sonbol et al. [29]. The composed medium includes the following: KH_2PO_4 (1.36), $MgSO_4.7H_2O$ (0.20), NaCl (2), $(NH_4)_2SO_4$ (1), $FeSO_4{\cdot}7H_2O$ (0.01), CMC (3.00), feather powder (20), yeast extract (1) and agar powder (20) in g/L. The pure isolates were identified using cellular morphology and molecular techniques.

2.2 Collection of Clinical Test Isolates

Ten (10) pathogenic isolates used for this research were obtained from the Microbiology Unit at the University of Ilorin Teaching Hospital, Ilorin, Kwara State. The isolates used were *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Yersinia enterica*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, *Citrobacter freundii*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* and *Klebsiella oxytoca.*

2.3 Culture Media and Chemical

Potato dextrose agar, nutrient agar and Mueller Hinton agar (Hi media, UK) were the media used in this research. Each of these media was prepared according to the manufacturer's instructions. Analytical grade silver nitrate ($AgNO_3$) used in this study was purchased from Sigma-Aldrich, USA.

2.4 Keratinase Production and Quantification Assay

This was carried out as described by Amuthavalli and Ravi [30]. Minimal media containing 1% feather and 0.2% yeast extract (pH 7.5) was inoculated with fungal isolates. The culture was incubated at room temperature (25 ± 2 °C) under agitation (100 rpm) for 72 h. Flasks containing 19 mL minimal medium were inoculated with 1 mL from the initial growth medium. The flasks were incubated at room temperature under agitation (100 rpm) for 14 days. At intervals of 2 days (48 h), incubating flasks were centrifuged at 5000 rpm for 20 min and the supernatant (crude

extracellular keratinase) was kept at 4 °C for further analysis. The modified method by Mamangkey et al. [31] was explored to determine the keratinase activity in this study. Approximately 1.5 g of the feather powder was dissolved in 2 mL of phosphate buffer saline-PBS (pH 7.5) and incubated with 0.5 mL of the crude enzyme. Only PBS and feather powder were utilized for the control experiment. The reaction mixtures were incubated under agitation (100 rpm) at 40 °C for 3 h. To eliminate precipitated keratin, the reaction was terminated using 2 mL of 10% trichloroacetic acid (TCA) and centrifuged at 5000 rpm for 15 min. One unit of the keratinase activity was thereafter quantified using the equation described below:

$$U = 4 \times n \times A_{280}/(0.01 \times 10)$$

where n is the dilution rate, 4 is the final reaction volume (ml) and 10 is the incubation time (min).

2.5 Biosynthesis of Silver Nanoparticles Using Extracellular Crude Keratinase

For AgNP biosynthesis with keratinase, the method reported by Tariq et al. [32] was adopted with slight modification. Exactly 9 ml of 1 mM silver nitrate ($AgNO_3$) solution was inoculated with 1 mL crude keratinase and vortexed. The reaction solution was then exposed to bright conditions at room temperature, and the reduction of silver ions to silver atoms occurred progressively over time. Control experiments were conducted using a silver nitrate solution without supernatant (crude keratinase) under the same conditions. Extracellular AgNP synthesis was observed with a colour change from colloidal yellow to dark brown.

2.6 Characterization of the Synthesized Silver Nanoparticles

AgNPs mediated by keratinase were analyzed using different analytical techniques. These include UV–Vis spectrophotometer, FTIR and TEM. Absorbance spectra of supernatant aliquots were analyzed in a UV–Vis spectrophotometer (Analytika, Specord 200) to determine the absorbance and corresponding wavelength of the silver nanoparticles. Spectral peaks were observed using FTIR Model Nicolet 6700 spectrophotometer (400–4000 cm^{-1}) and interpreted to identify the functional groups present. Morphology and mean sizes of the formed AgNPs were observed and evaluated by TEM (JEM-ARM200F-G Japan (JEM-1010) model). The particle size distribution of silver nanoparticles was evaluated using ImageJ 1.45 Software 1493.

2.7 Antibiotic Susceptibility Profile of Clinical Pathogens

The antibiotic susceptibility profile of the clinical pathogens used as test isolates was determined using conventional antibiotics for bacteria treatment following Kirby-Bauer's disc diffusion method [33]. The antibiotics used were Ceftriaxone (CAZ), Cefuroxime (CRX), Amoxicillin-clavulanic acid (AUG), Ciprofloxacin (CPR), Gentamycin (GEN), Ofloxacin (OFL), Cefixime (CXM), Erythromycin (ERY), Septrin (SEP), Nitrofurantoin (NIT), Streptomycin (STR) and Zinnat (ZIN). This was performed by making bacterial lawns on Mueller Hinton agar (MHA) plates using a cotton swab after which the plates were carefully impregnated with the antibiotics discs and incubated at 37 °C for 18–24 h. For each of the antibiotics tested, the inhibition zones observed were measured and recorded.

2.8 Antibacterial Screening of Biosynthesized Silver Nanoparticles

The biosynthesized AgNPs were tested against the clinical isolates using the disc diffusion method [33]. Sterile filter paper disks with 5-mm diameter impregnated with synthesized AgNPs were aseptically placed on MHA plates seeded with the clinical pathogens. The plates were then incubated at 37 °C for 18–24 h. The clear zone observed was measured and recorded accordingly.

3 Results and Discussion

3.1 Fungi Isolation

Three *Aspergillus* (F1, F2 and F3) were isolated and identified in this study. The production and activity of keratinase from purified fungi are shown in Fig. 1. The highest keratinase activity recorded in this study was 48.52 U/ml by *A.* F1 at day 9, while F3 had 46.04 U/ml at day 11. F2 showed the least keratinase activity across all days with 13.52 U/ml as the highest. The two best keratinase producers (F1 and F3) were morphologically and molecularly confirmed as *Aspergillus niger* (F1) and *Aspergillus aculeatus* (F3) (Fig. 2). An earlier study by Timorshina et al. [34] has reported *Aspergillus* species as a promising keratin substrate degrader after obtaining high keratinolytic activity from the fungi.

3.2 Biosynthesis of Silver Nanoparticles

The ability of different keratinase-producing *Aspergillus* (*A. niger* and *A. aculeatus*) to synthesize AgNPs is shown in Fig. 3. The appearance of dark brown colouration after 48 h was observed, with the resulting products coded as

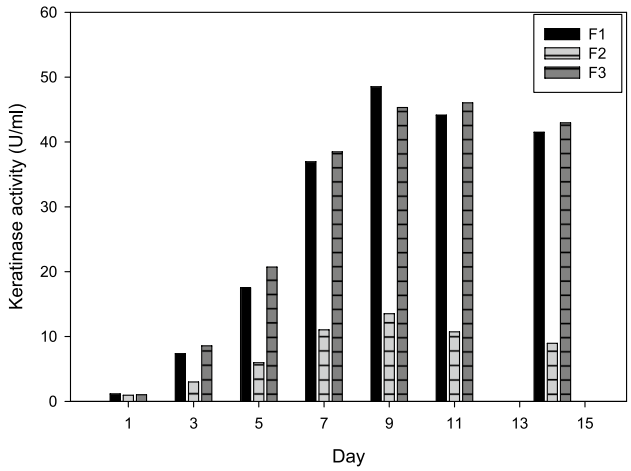


Fig. 1 Keratinase activity by fungal isolates (*A. niger* F1, *A. oryzae* F2 and *A. aculeatus* F3)

AgNPs-F1 and AgNPs-F3. As stated by Ahmad et al. [35], AgNP formation was observed visually with the appearance of a dark-brown colouration. In agreement with this observation was a study by Sudha et al. [36] reporting the bio-reduction and synthesis of AgNPs with crude keratinase produced by *Sarratia* sp. with the appearance of brown colour. The colour formation has been documented to be a result of surface plasmon resonance excitation of metal nanoparticles [37].

3.3 Characterization of the Biosynthesized Silver Nanoparticles

The synthesis of AgNPs was characterized using UV–visible spectrophotometer in the range of 250–700 nm with a peak absorbance of 0.86 A at 440 nm for AgNPs-F1 while a peak absorbance of 1.21 A at 385 nm was recorded for AgNPs-F3 (Fig. 4). Hashem et al. [38], Abada et al. [39] and Chengzheng et al. [40] all reported peaks at 420 nm, 385 nm and 440 nm, respectively, which were similar to the peak obtained in this present study. Dual peaks, one at a lower absorbance at 270 nm and higher absorbance at 410 reported by Chinnappa et al. [41]. Peak absorbance for synthesized nanoparticles has been reported to range from 350 to 450 nm [42, 43].

Fourier transform infrared spectroscopy (FTIR) analysis revealed possible biomolecules that were involved in the

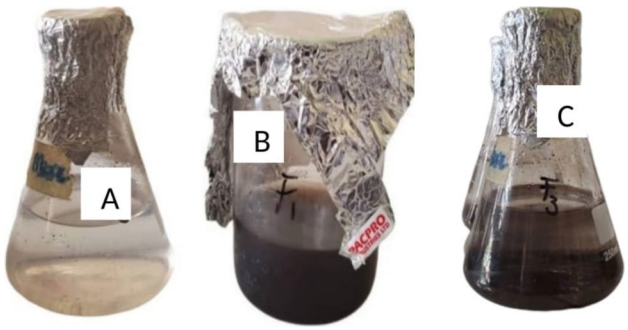


Fig. 3 **A** $AgNO_3$ solution. **B** Synthesized AgNPs using crude keratinase from *A. niger* (AgNPsF1). **C** Synthesized AgNPs using crude keratinase from *A. aculeatus* (AgNPsF3)

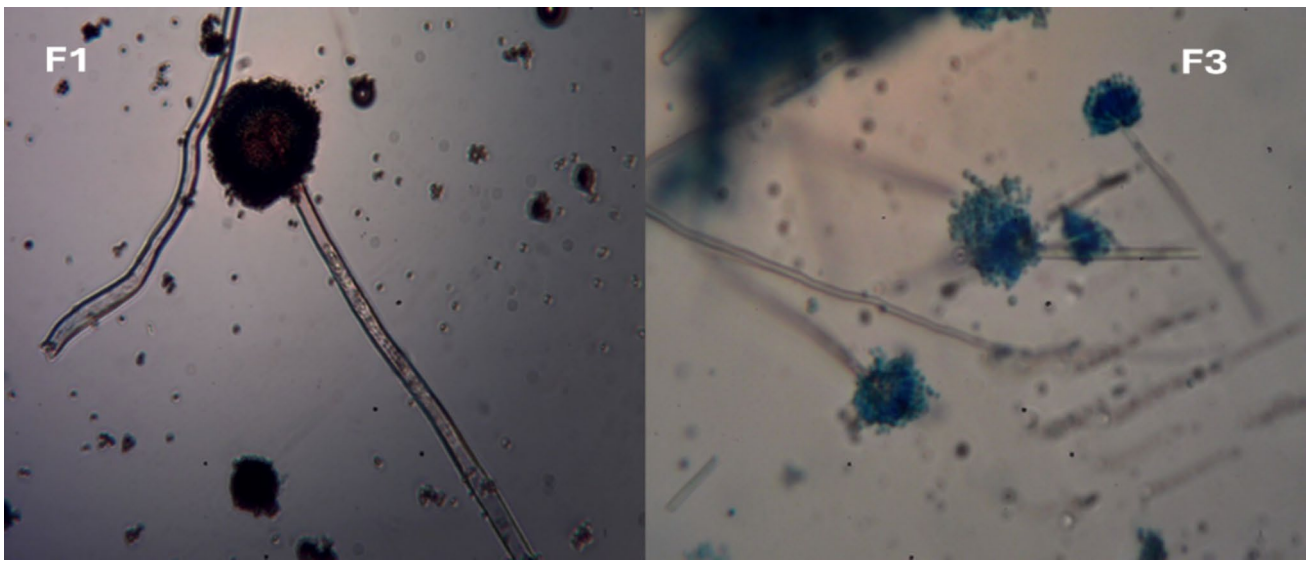


Fig. 2 Micrograph of *A. niger* (F1) and *A. aculeatus* (F3)

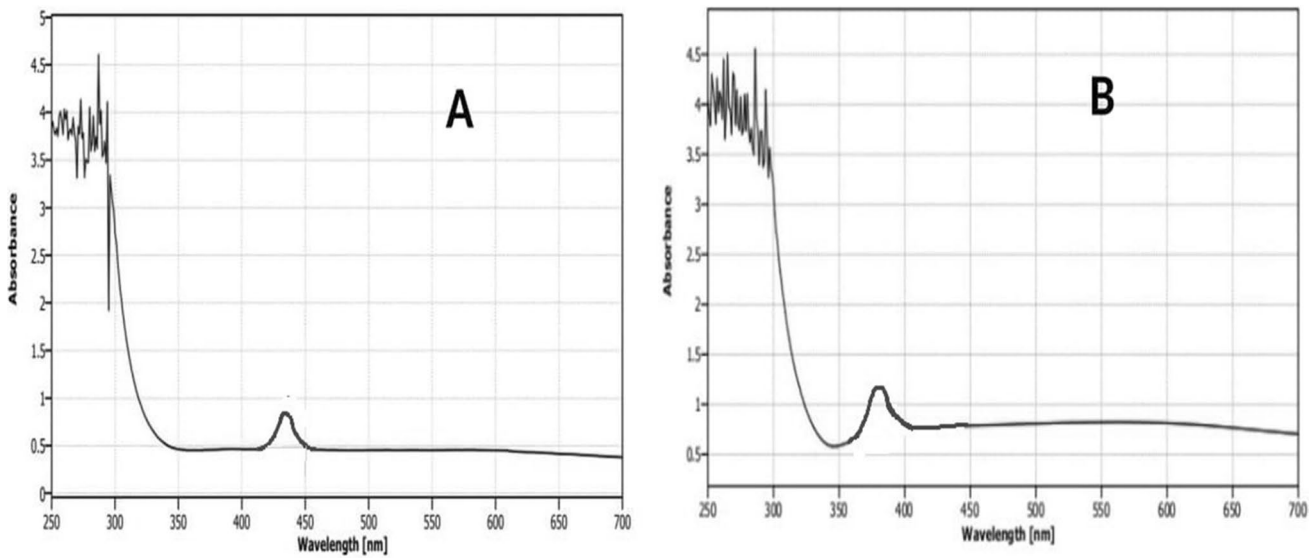


Fig. 4 UV–Vis spectroscopy of synthesized AgNPs using crude keratinase from **A** *A. niger* and **B** *A. aculeatus*

bioreduction of silver salt and stabilization of AgNPs. Figure 5 record 7 spectral peaks for AgNPs-F1 and AgNPs-F3. AgNPsF1 peaks were 3616.59, 3224.27, 2059.10, 1641.15, 1273.55, 1150.40 and 672.30 cm^{-1}, while for AgNPs-F3, 3474.41, 2055.54, 1641.16, 1315.04, 1125.07, 669.13 and 419.00 cm^{-1} were interpreted as spectral peaks of –NH or –OH group with vibrations of some free amides. The peaks detected at a wave number of 3616.59, 3474.41 and 3224.27 cm^{-1} were ascribed to the O–H amide group stretching and phenol/carboxylic group stretching vibrations while peaks at 2059.10 and 2055.54 cm^{-1} indicate CH_2 stretching. These groups containing O–H bonds can stabilize AgNPs by forming hydrogen bonds or coordinating with the metal surface hence preventing aggregation and improving the dispersion of nanoparticles in aqueous solutions [44, 45]. Peaks at 1641.15 and 1641.16 cm^{-1} are the bending vibration of the amide I band of the protein (C = C group) [46]. The carboxyl group C–O stretching vibrations showed peaks of 1315.04 and 1273.55 cm^{-1}. Spectral peaks at 1150.40 and 1125.07 cm^{-1} indicate C–O stretch vibrations belonging to the phenol, ether, or ester functional groups. Similarly, spectral peaks at 672.30 and 669.13 cm^{-1} suggest the

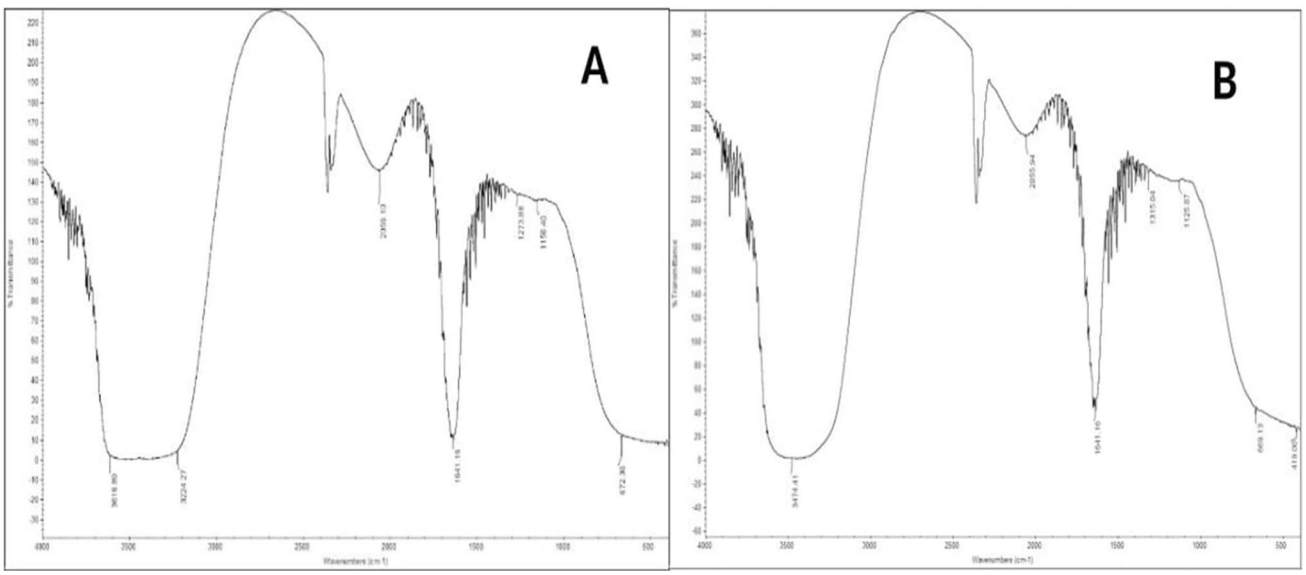


Fig. 5 FTIR spectrum of synthesized AgNPs using crude keratinase from **A** *A. niger* and **B** *A. aculeatus*

presence of OCN bend of an amide IV band. A detectable peak indicating the presence of C = O of the amide VI bands was observed at 419.00 cm^{-1}. Similar peaks were reported by Abeer Mohammed et al. [47].

The TEM image (Fig. 6) shows that the AgNPs were monodispersed spherical shaped well observed in AgNPsF1. The size of the AgNPs-F1 and AgNPs-F3 ranged from 3.04 to 12.39 nm and 15.47 to 32.83 nm, respectively. The size range reported for AgNPs-F1 was corroborated by Wan et al. [48] who reported a range of 5–12 nm, while the large size reported by AgNPs-F3 also corroborated that of Jalilian et al. [49] also reported spherical AgNPs with a 50 nm size.

3.4 Antimicrobial Assay of Silver Nanoparticles

The antibacterial activities of AgNPs-F1, AgNPs-F3 and conventional antibiotics against the clinical pathogens are shown in Fig. 7. Assessment of the conventional antibiotics showed that 70% of the pathogens were sensitive to CPR and GEN, and 60% were sensitive to OFL and NIT/ZIN. The test pathogens were multi-resistant to CAZ, CRX, CXM, SEP and STR. Among the test isolates, *E. coli*, *Y. enterica* and *K. oxytoca* were the most resistant pathogens. The study recorded improved antibacterial activities of keratinase-synthesized AgNPs over conventional antibiotics. The results obtained revealed that 80% of the test isolates were susceptible to AgNPs-F1 with the two exceptions (20%) by *S. liquefaciens* and *C. freundii*. In addition, 70% showed susceptibility to AgNPs-F3 with three exceptions (30%) by *E. coli*, *P. aeruginosa* and *B. subtilis*. AgNPSF1 had a greater activity compared to AgNPsF3 which had a little activity against the tested pathogens. The exhibition of antibacterial activity by AgNPs against clinical pathogens as observed in this present study agrees with the findings of Lateef et al. [50]. This activity by AgNPs in this study demonstrated the effectiveness of AgNPs to combat clinical pathogens.

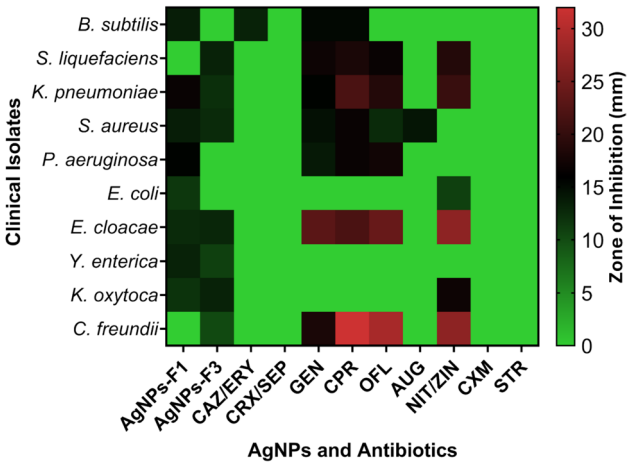


Fig. 7 Comparison of antibacterial screening of keratinase-synthesized AgNPs and antibiotics. Keys are as follows: Ceftriaxone (CAZ), Cefuroxime (CRX), Amoxicillin-clavulanic acid (AUG), Ciprofloxacin (CPR), Gentamycin (GEN), Ofloxacin (OFL), Cefixime (CXM), Erythromycin (ERY), Septrin (SEP), Nitrofurantoin (NIT), Streptomycin (STR), Zinnat (ZIN)

According to the antibiotics susceptibility test result, all the pathogens were resistant to four or more antibiotics used in this study making them multidrug-resistant isolates. Drug-resistant bacteria are emerging pathogens with resistance profiles that pose a great challenge in treating and controlling their spread [51]. More et al. [52] suggest that antimicrobial activity exhibited by silver nanoparticles is a factor of the continuous release of silver ions implicated in

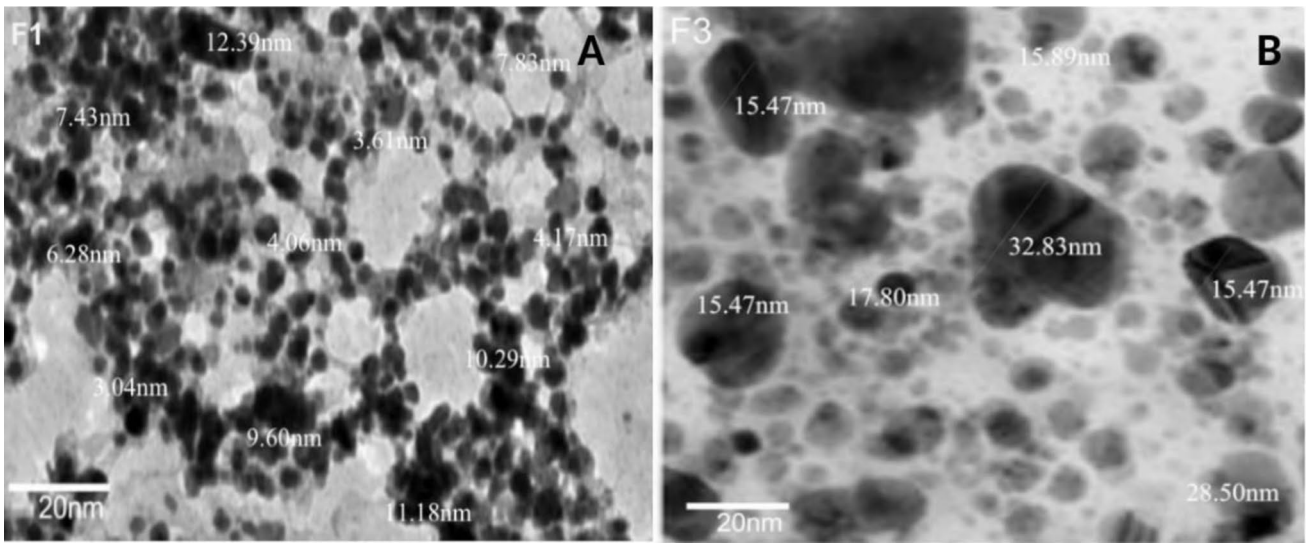


Fig. 6 TEM micrograph of the synthesized AgNPs using crude keratinase from **A** *A. niger* and **B** *A. aculeatus*

the bactericidal process. Silver ions have a strong affinity for sulfur-containing proteins and are attracted by electrostatic forces. They easily adhere to cell walls and the cytoplasm. Ji et al. [53] documented that silver nanoparticles inhibit the respiratory chain dehydrogenase, hence inhibiting metabolic activity and growth of bacterial cells. It was also reported that it affects the integrity of *E. coli* cells through depolarization and destabilization of the cell membrane. The small size of metallic nanoparticles guarantees that a substantial surface area of the particles interacts with the bacterial cells. Such a large contact surface will likely increase the effectiveness of bacterial elimination. [54]. The effectiveness of silver nanoparticles against microbes has been suggested and attributed to firm adhesion of AgNPs to microbial cell walls and plasma membranes, leading to enhanced cell permeability [55], or from the disruption of the plasma membrane, causing leakage of cellular contents [56]. Keratinase-based synthesized AgNP is promising as an alternative antimicrobial compared to chemical-based synthesized AgNP. As observed in this study, it has shown stronger antimicrobial activity against clinical pathogens. It is also a sustainable green method that is eco-friendly with no toxic byproducts compared to chemical synthesis. Studies have also shown that biosynthesized AgNPs can be produced in controllable size and shape and do not harm healthy human cells compared to chemical-based synthesis [57, 58].

4 Conclusion

This study demonstrated that keratinase-synthesized AgNPs have significant antibacterial properties against multidrug-resistant clinical pathogens. The result showed synthesized AgNPs especially AgNPs-F1 had better activity than standard antibiotics utilized in this study. The effectiveness of AgNPs against *K. oxytoca*, *E. coli* and *Y. enterica* which were found to be most resistant has further attested AgNPs as new and alternative antimicrobial agents to combat the alarming issue of antimicrobial resistance (AMR) across the globe. It is also important that more research on the synergistic potentials of antibiotics and keratinase-synthesized AgNPs is considered and also scaled for in vivo testing to promote robust clinical applications.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12668-024-01770-w.

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Author Contribution F.O. Conception, visualization, practical work, manuscript writing, resources O.O Visualization, Resources, supervision P.F Supervision, editing R.T Editing, practical work Z.B Editing O.B Editing, software, data interpretation, Plagiarism check B.A Arrangement, Editing.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Research Involving Humans and Animals Statement No human or animal model was used in the study.

Ethical Approval Not applicable.

Conflict of Interest None.

Informed Consent None.

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