Asian Journal of Biochemistry, Genetics and Molecular Biology

Volume 15, Issue 2, Page 46-59, 2023; Article no.AJBGMB.106457
ISSN: 2582-3698

Production of Copper Nanoparticles by *Aspergillus niger* and Evaluation of their Biomedical Applications

Syed Bilal Hussain [a*], Maria Aslam [a], Asad Aslam [a], Sadaf Noor [a] and Muhammad Zubair [b]

[a] Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan.
[b] Department of Forestry and Range Management, FAS and T, Bahauddin Zakariya University, Multan, Pakistan.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGMB/2023/v15i2332

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/106457

Original Research Article

Received: 21/07/2023
Accepted: 28/09/2023
Published: 06/10/2023

ABSTRACT

Nanotechnology is a well advanced area and different types of nanoparticles are now produced. The most revolutionary topic of the twenty-first century is nanotechnology. Recently, metallic nanoparticles (NPs) have gained a lot of attention. In this study a straightforward, safe, and economical method is used for synthesizing copper nanoparticles (CuNPs) by using cell free filtrate of *Aspergillus niger.* Fungi secretes enormous amount of enzymes which act as reducing as well stabilizing agents. Synthesized CuNPs were characterized by different techniques. Ultraviolet (UV)-visible spectrophotometer showed a characteristic peak at 380nm, Dynamic Light Scattering (DLS) analysis indicated that CuNPs have average size of 570nm with 0.480 PDI. SEM (Scanning Electron Microscopy) revealed CuNPs are of cubic shape. As per FTIR (Fourier Transform Infrared Analysis) two functional groups i.e., -O-H- and C=C- on the surface of CuNPs. Genetic analysis of

**Corresponding author: E-mail: drsbhussain@gmail.com;*

Aspergillus niger revealed the presence of nitrate reductase enzyme that may involve in reduction of metal into NPs. Furthermore, CuNPs showed significant antibacterial action against *Escherichia coli, Bacillus subtilis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* with 17mm, 20mm, 17mm and 20mm respectively. CuNPs also showed antioxidant potential with 59.41µg/ml.
Fungi secretes valuable enzymes with inherent ability to reduce metal ions into NPs. This reduced the cost of NPs synthesis and also synthesized NPs exhibit greater biomedical applications.

Keywords: CuNP; fungi; reductase enzyme; antibacterial activity; antioxidant activity.

1. INTRODUCTION

Nanotechnology is the field of science that involves the production of nanoparticles**.** Utilizing tiny atoms or molecules in order to produce incredibly tiny but useful particles known as nanoparticles is essentially a combination of science and engineering [1]. These are categorized as particles that differ from other extensive material because of their size range of 1 nm to 100 nm Nanotechnology is a highly developed field that alters people's ways of thinking by allowing them to handle tiny particles on a vast platform to achieve a variety of objectives [2]. It emerged in the twenty-first century and has attracted a lot of interest globally. Since nanotechnology manipulates multiple Nano size materials in the fields of physics chemistry, and biology in addition to atoms and molecules, it has become increasingly important during the past 15 years [3]. According to their shape, size, and chemical makeup, nanoparticles are broadly categorized into a number of classes. The most frequently used NPs (Nanoparticles) types, according to their physical and chemical characteristics are, carbon based, semiconductor NPs, ceramic NPs, polymeric NPs, lipid based NPs and metal based NPs [4]. Two commonly used approaches for synthesis of NPs are top down and bottom up. Among all these NPs metal NPs are widely used and most important these days [5].

The latest approach towards nanoparticles is the production of metal NPs like Copper, Palladium, Gold, Silver, Platinum etc. Metal nanoparticles exhibit unique magnetic, catalytic, optical, and electrical capabilities. Different chemical and physical processes are used to produce metal nanoparticles. They are frequently employed in biotechnology [6]. Metallic nanoparticles can be applied in a variety of fields, including electronic devices, skin care products, protective coatings, packing, and biotechnology [7-11]. The biological method is used quite frequently because it is less expensive, more effective, environment friendly, and simple to use because it doesn't require expensive or toxic substances [12]. Now a day's different materials are used for the production of NPs like filtrates of plant, fungus, algae, bacteria, virus and yeast. However, the production of NPs from fungus is latest approach [13]. As different fungi have been using for the production of NPs. Fungi are more efficient Nano factories than bacteria and plants because, they are capable of producing large amounts of enzymes and proteins [14]. According to various studies, fungi are relatively easy to synthesize large numbers of nanoparticles [15]. Since they release a lot of the enzymes required for the process. Reductase enzyme is present in fungus that believed to reduce metal into metallic nanoparticle. Copper nanoparticle (CuNPs) are produced from *Aspergillus niger* that is filamentous ascomycota having spectate hyphae. Easily grown on all media and have higher amount of biomass than others. CuNPs produced from *A. niger* show excellent biological activities [16].

2. MATERIALS AND METHODS

2.1 Isolation and Purification of Fungus

From soil sample, *Aspergillus niger* (*A. niger*) was isolated. Soil samples were collected from the area around plant roots, where most of the activity of microbes is centered. The soil specimens, that weighed approximately 4g, were collected using a sterile spade and clean polythene plastic bags. From June to August of 2022, samples of soil were taken at various sites of Bahauddin Zakariya University in Multan. Samples were diluted by serial dilution.

2.1.1 Serial dilution

A serial dilution is the process of gradually reducing a solution's concentration in accordance with an appropriate dilution rate. In biological processes, it is usual practice to decrease the total number of cells present in a culture in order to accelerate the process [17]. Carefully weigh 1g of soil and added it to a test

tube containing 10 ml of filtered water so as to remove fungus spores from the soil samples. Six test tubes containing 9 ml each of sterile diluents (0.9% saline or distilled water) and the soil sample wereused. A clean pipette was filled with 1 ml of a thoroughly mixed sample. The sample was added to the first tube after it has been filled to a total volume of 10 ml. By adding and removing the pipette multiple times, the dilution properly mixed. The liquid was removed from the 10^{-1} dilution and poured into the second tube [18]. The overall diluting factor in the second tube has changed to 10^{-2}. After that, the remaining portion of the tube had the same treatment. The result is the ultimate dilution of the fungus and cells. Fungal cultures are then grown on SDA (Sabouraud Dextrose Agar) plates using the final dilution. Media was produced for the fungus to be cultured [19]. For preparing the media, added 17.1 g of SDA with 250 ml of filtered water, weighed it, mixed it, and finally autoclaved it. Cooled it between 37ºC and 40ºC. Media was poured into petri plates, where it was then solidified. Wire loops that have been sterilized are used for fungal spore culture. Plates were cultured and then stored in an incubator for seven days at 37ºC. For the purpose of fungal purification, sub-culturing was done. Pure culture was stored 4ºC.

2.2 Identification of Fungus

Aspergillus niger was identified by different methods like morphological characterization and biochemical assay.

2.2.1 Morphological identification

For 7 days, macroscopic characteristics such colony size, colour, and shape were observed. In order to determine microscopic characteristics, a typical coverslip approach was used. The slides were prepared, microscopy was carried out [20]. Pure fungal spores were collected using a needle. They were forced onto a slide and tortured until enormous structures were completely destroyed. After adding a drop of water onto the slide, it was covered with a coverslip. The excess moisture was absorbed using tissue paper. The slide was observed under a microscope to see the characteristic structures of fungus.

2.2.2 Biochemical assay

Fungus culture was biochemically identified using the catalase assay. Some of fungal colonies were picked from culture plate and dipped into 4 ml of 3% H_2O_2 solution for the catalase assay using a sterilized wire loop for 8 to 10 seconds to watch the bubbling pattern. Three runs of the experiment were done to get the mean value [21].

2.3 Preparation of Biomass

For the preparation of cell free extract to use for the preparation of nanoparticles, biomass of fungus was prepared. For this purpose this purpose, the Sabouraud Dextrose Broth (SDB) media was made in a 100ml flask. 7.5g of SDB and 250 ml of autoclaved water were added before being swirled. After that, prepared media was autoclaved. Media autoclaved cooled at room temperature [22]. *A. niger* spores were mixed in media using sterile wire loop. The medial flask was removed from the rotatory shaker after being shaken at a speed of 120rpm for 7 days while maintaining a temperature of 37°C.

2.4 Preparation of Filtrate

Utilizing Whatman White Filter Paper No. 1, biomass was collected. The media was eliminated by washing the biomass three times in deionized water, leaving only the fungal biomass [23] .The flask was cleaned and then filled with the washed biomass. In 100 ml of deionized water, 5 g of fungal biomass was introduced. For one day of incubation, the mixture was randomly shaken at 12 rpm. Following that, the Whatman filter paper method and the 0.5 m syringe filter were used, respectively, to filter the biomass. CuNPs were synthesized using the produced filtrate.

2.5 Synthesis of CuNPs

The synthesis of CuNPs involves a variety of criteria. In order to produce the largest number of nanoparticles possible, the synthesis of CuNPs was optimised by adjusting the pH, time, concentration, and ratio. To evaluate the effect of pH on the formation of CuNPs, the pH of the reaction mixture was kept constant between 7 and 8. At pH 7, the majority of color shift was seen. Different concentrations of copper sulphate were maintained. The ratio between the changing quantities of the fungal filtrate and the copper sulphate solution was altered using various ratios that were established as (fungal filtrate: $CuSO_4$ solution) 1:1. For the synthesis of CuNPs, 5ml of filtrate and 5ml of salt solution were combined at a ratio of 1:1. As a control, 2-3

ml of fungal filtrate free of $CuSO_4$ was left in the shaker while tubes were randomly stirred for 24 hours at 120 rpm.

2.6 Drying of CuNPs

After one day of incubation, CuNPs were centrifuged three times at 12000 rpm for 5 minutes. They were then washed with distilled water. Three times through these steps, only NPs in the form of pellets remained after three washings. The pellets were oven dry at 100°C for one day. Before being transferred to 1.5 ml Eppendorf, NPs finely powdered.

2.7 Genetic Characterization of CuNPs Synthesizing *Aspergillus niger* Strain

2.7.1 Apparatus

The Gel Electrophoresis System apparatus (Cleaver Scientific, UK), NanoDrop spectrophotometer (BIODROP) and PCR thermal cycler machine (BIO-RAD T100) were used to analyze the presence of gene responsible for the nitrate reductase enzyme production.

2.7.2 Genomic DNA isolation from *Aspergillus niger*

To extract DNA from isolated fungal samples, already utilized procedures were modified somewhat [19]. The mycelia of the fungus were removed carefully through filtration utilizing a sterilized funnel and filter paper after 48–60 hours of *A. niger* culture on SDB. Using a cleaned scalpel mycelia were scraped off the filter paper's surface before being dried. Dried mycelia were crushed into a fine powder using liquid nitrogen. 50mL falcon tubes were filled with 2-3 g of either fresh or crushed mycelia and 5 mL of extraction buffer. Used a sterilized pestle and mortar to fully ground new mycelia in the extraction buffer. The falcon tube was then filled with 5 mL of a 3 M sodium acetate buffer (pH 5.2), which was then incubated at -20 degrees Celsius for 10 minutes. At 4°C and 8,000 rpm, the tube was rotated for five minutes (6,080 g). The resulting solution was collected in a new falcon tube, and an equal volume of ice-cold isopropanol was added next. The tube was held at ambient temperature for 5 minutes and then spun again at 8,000 rpm (6,080xg) at 4°C for an additional five minutes. The pellet was washed with 70% (v/v) chilled ethanol for five minutes at 4°C by rotating at another 8,000 rpm in 400 ml of TE buffer or double-distilled deionized water (D_3H_2O). The quality of the extracted DNA was evaluated on a 1% (w/v) agarose gel. The quantity of DNA was determined using NanoDrop (BioDrop). The DNA was then preserved at 4°C [24].

2.7.3 Polymerase Chain Reaction (PCR)

A PCR thermal cycler was used to amplify the desired niaD gene responsible for the nitrate reductase enzyme. The primers were designed using Primer 3 Plus. The sequence of primers and their details are provided in Table 1. The niaD gene, which may produce the reductase nitrate enzyme in *A. niger*, was detected using a specific primer. This enzyme might aid in the transformation of metal into nanoparticles. 20 µl of reaction mixture were prepared for the PCR procedure. 2 µl of reverse and forward primers, 3µl of nuclease-free water, 3µl of genomic DNA, and 10µl of 1X master mix were added to the reaction mixture. Gel documentation was used to analyze the PCR products. On a 2.5% agarose gel, amplified PCR products were separated using horizontal gel electrophoresis. DNA banding patterns observed using gel documentation. As a molecular marker, a 100 bp DNA ladder was employed.

2.8 Physical Characterization of NPs

The CuNPs were assessed using UV visible spectrum, Fourier transform infrared (FTIR), and dynamic light scattering (DLS) analyses, SEM and XRD.

Table 1. Primer sequence and its details

	Sequence (5'to 3')	Length (bp)	Tm (ºC)	GC%	Product size (bp)
Forward primer	-GGTTACTGGGGAGAACGACA-	20	59.03	55.00	243
Reverse primer	-CTCAGAAACGTCGATGCCAG-	20	59.01	55.00	243

2.8.1 UV-Visible spectrophotometry

A UV spectrophotometer was used to determine the absorbance of CuNPs [25]. 1 ml of CuNPs were collected at various concentrations after one day of incubation, with only the wavelengths between 200 nm and 800 nm being changed. Micro-plate spectroscopy on a 96-well plate for the UV spectrum was used to find CuNPs. Distilled water used as a blank. sample data were taken and the resulting graph was produced on an Excel spreadsheet.

2.8.2 FTIR (Fourier Transform Infrared Analysis)

FTIR analysis was done using an FTIR Spectrum listed as a sequence of wavelengths from 1000 to 3500 cm-1 In order to identify the functional groups that bind with CuNPs. FTIR analysis was carried out using an interferometer. Potassium bromide was diluted CuNPs at a ratio of 1:100. On the sample holder, one drop of the solution [26].

2.8.3 DLS (Dynamic Light Scattering Analysis)

Dynamic light scattering (DLS) was used to examine the size distribution and colloidal stability of CuNPs [27]. Presented the mathematical strategy required to calculate the Z-average and extract size data from the correlation function. The charge on the surface of CuNPs was determined using dynamic light scattering analysis and a particle size analyzer at 25°C with a 90° detection angle.

2.8.4 XRD (X Ray Diffraction)

X-Ray diffraction was used to investigate the crystalline structure of Cu nanoparticles. The XRD study was performed on a Philips XRD 3100 diffractometer (Philips Electronics Co., Eindhoven, Netherlands) at 45 kV and 30 mA. To produce X-Rays with a 1.54060 wavelength, copper K radiation and a graphite monochromatic radiation were used. Iron nanoparticles were placed in a glass container that was tilted from 20° to 60° [28].

2.8.5 SEM (Scanning Electron Microscopy)

The solution of the nanoparticles must first be dried before being applied on the sample container and sputter-coated with a conducting substance, such as gold, for SEM analysis. The sample was then scanned with a precisely focused electron beam. The nanoparticles must be resistant to vacuum, and the electron beam could harm the polymer. Findings from dynamically scattered light are equivalent to the average size information from the SEM [29].

2.9 Antibacterial Activity of CuNPs

Infections by bacteria can result in fatal health problems. We used NPs as an option to treat serious bacterial infections. Gramm positive and gramm negative microorganisms were used to investigate the antibacterial effectiveness of CuNPs [30]. Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli*, as well as gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, were the focus of CuNPs' antibacterial effect**.** Nutrient broth was used for growing various cultures of bacteria. 1.3 g of nutritious broth was dissolved in 100 mL of distilled water, and then autoclaved. Tubes were incubated for 24 hours at 37ºC**.** The agar well diffusion method was used to assess the antibacterial activity of CuNPs. Nutrient agar petri plates were prepared by mixing 7 g of nutrient agar with 250 ml of distilled water in flask measuring 500 ml, then autoclaved and cooled at room temperature. A 5mm sterilized steel borer was used to drill wells into agar plates after agar solidified. Cotton swabs that had been sterilized were used to disseminate bacterial culture onto agar plates. 100 ul of a CuNPs solution (1 mg/ml) were added to designated wells. The drug ciprofloxacin served as a standard. Petri plates were placed in incubator for 24 hours at 37 ºC. After 24 hours of the incubation period, the zones of inhibition around each well were assessed.

2.9.1 Minimum Inhibitory Concentration (MIC) and Minimum Inhibitory Concentration (MBC)

Using a common micro-dilution method, the Minimum Inhibitory Concentration (MIC) of CuNPs produced by the fungus *A. niger* was evaluated. A 96-well round-bottom microtiter plate was used to measure MIC. Three bacteria, *Staphylococcus aureus*, *Salmonella enterica* and *Pseudomonas aeruginosa*, were tested for MIC. Bacterial strains were mixed in Luria burtani medium, and each strain's optical density was assessed using a spectrophotometer set to 600 nm. The preparation of two fold repeated dilutions of each strain involved adding 100µl of nutrient broth to each well. "Broth + Microbial Culture" was chosen as the negative control, while "NPs + Microbial Culture" was chosen as the positive control. Pre-incubation absorbance

was measured at 600 nm. The absorbance at 600 nm was measured 20 hours after incubation. Minimum Bactericidal Concentration (MBC) refers to the lowest concentration of NPs that could be effective in killing 99.9% of bacteria. Following MIC, a particular dose section of MIC was set for MBC. MBC was assumed to observe bacterial strains growing or not [31].

2.10 Antioxidant Activity

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenger assay was used to compare the antioxidant activity of CuNPs to ascorbic acid, which acted as the positive control [32]. 1 ml of different concentrations (10-100 µg/ml) CuNPs and ascorbic acid were mixed with 1 ml of a 0.5 mM DPPH in methanol solution, and the mixture was then incubated for 30 minutes at room temperature in the dark. A UV-Vis spectrophotometer was then used to detect the reaction mix's absorbance at 517 nm. The %age inhibition was calculated as follow.

$$\%age\ inhibition = 1 - (A_o - A_s/A_c) \times 100$$

Where A_o is absorbance of blank, A_s is absorbance of test sample and A_c is absorbance of control.

IC50 was also calculated to find the concentration of CuNPs.

3. RESULTS

3.1 Morphological Identification

Fungal colonies on SDA media had a smooth look after 7 days of incubation, were primarily straw to pale yellow in color on the opposite edge of the colony and had a white color that changed to a dark brownish black colors from the first day (Fig. 1). Fig. 2 depicts the fungal strains that were recognized through microscopic examination. These strains had septate hyphae, globular vesicles, long, unbranched, smooth conidiophores, and black conidia.

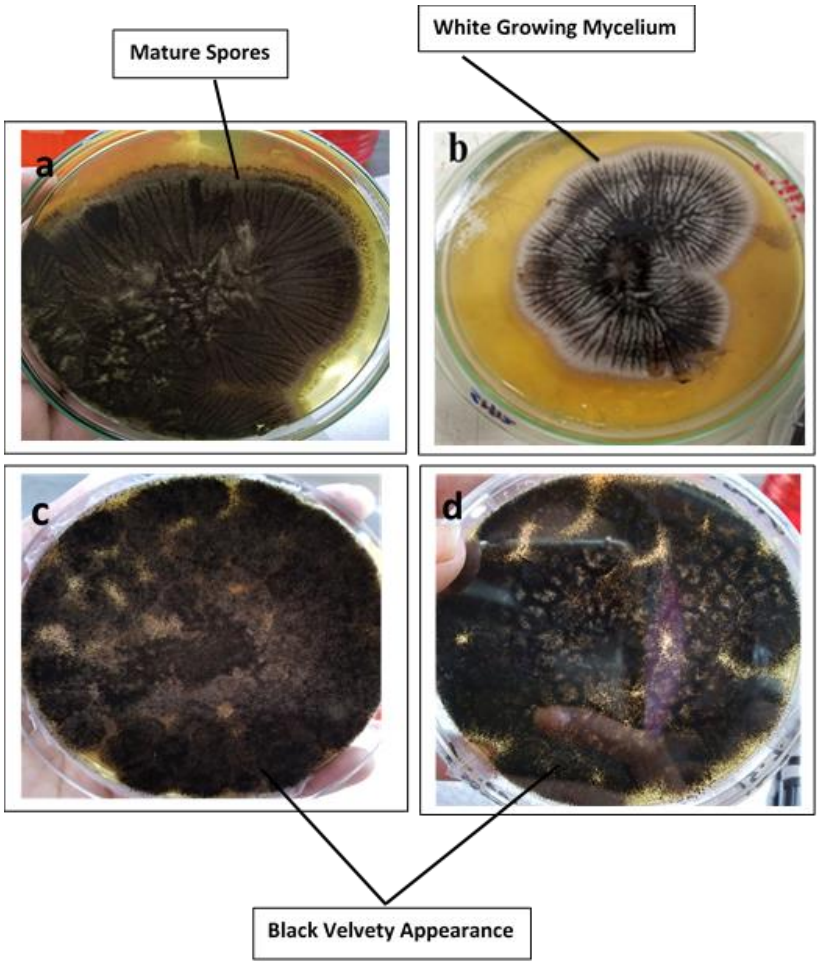

Fig. 1. Morphological identification of fungal isolates

3.2 Biochemical Assay

For the catalase test, fungal strains showed bubbling patterns. Enzyme catalase, which is present in fungi, assists hydrogen peroxide break down into water and oxygen gas. Bubbles formed in the presence of oxygen gas, indicating the fungus was functionally active (Fig. 3).

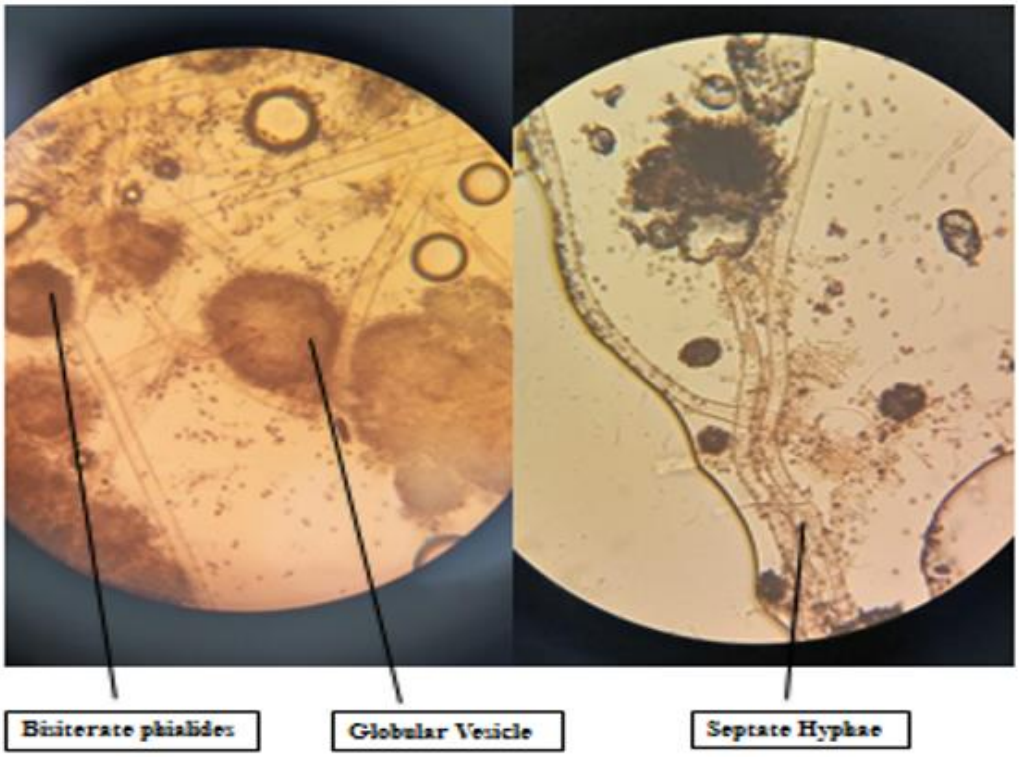

Fig. 2. Microscopic identification of fungal isolates

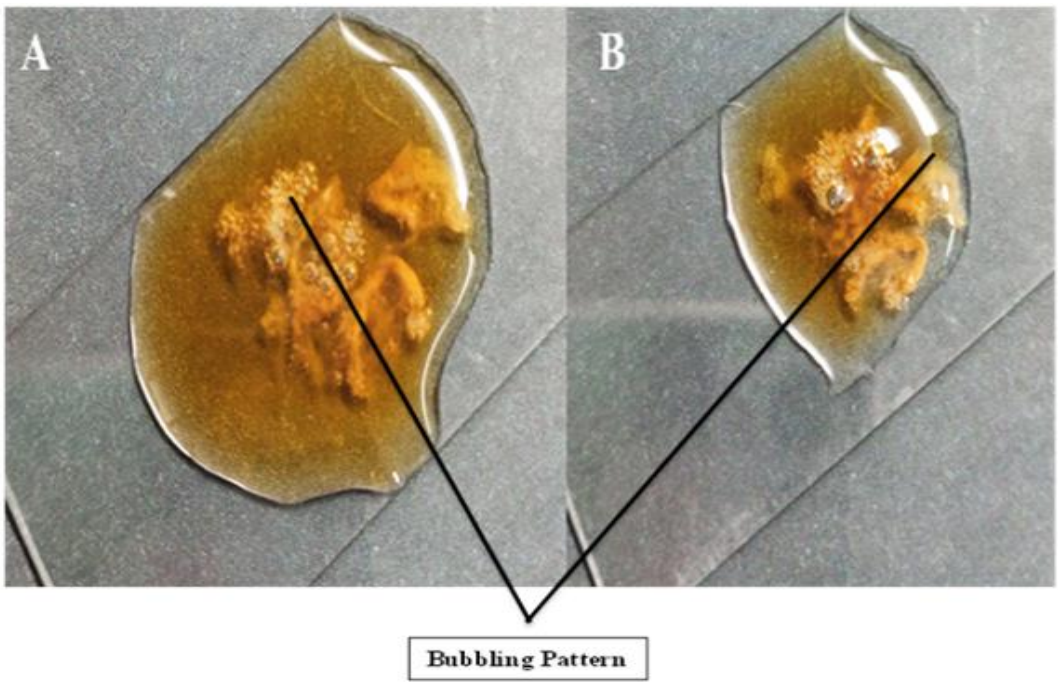

Fig. 3. Biochemical assay of fungal isolates

3.3 Synthesis of CuNPs

The screening demonstrated that only two of the four *Aspergillus niger* strains, SN1 and SN2, generated CuNPs. For the synthesis of CuNPs from copper sulphate salt, *A. niger* filtrate acted as a reducing agent. This process reduced the copper, and the formation of CuNPs was confirmed by a change in colour from colourless to blue. As illustrated in Fig. 4 the blue colour of copper nitrate changed to brown.

3.4 Chain Reaction (PCR) Amplification

PCR products of niaD gene are shown in the Fig. 5. The size of gene was 243 as compared to ladder.

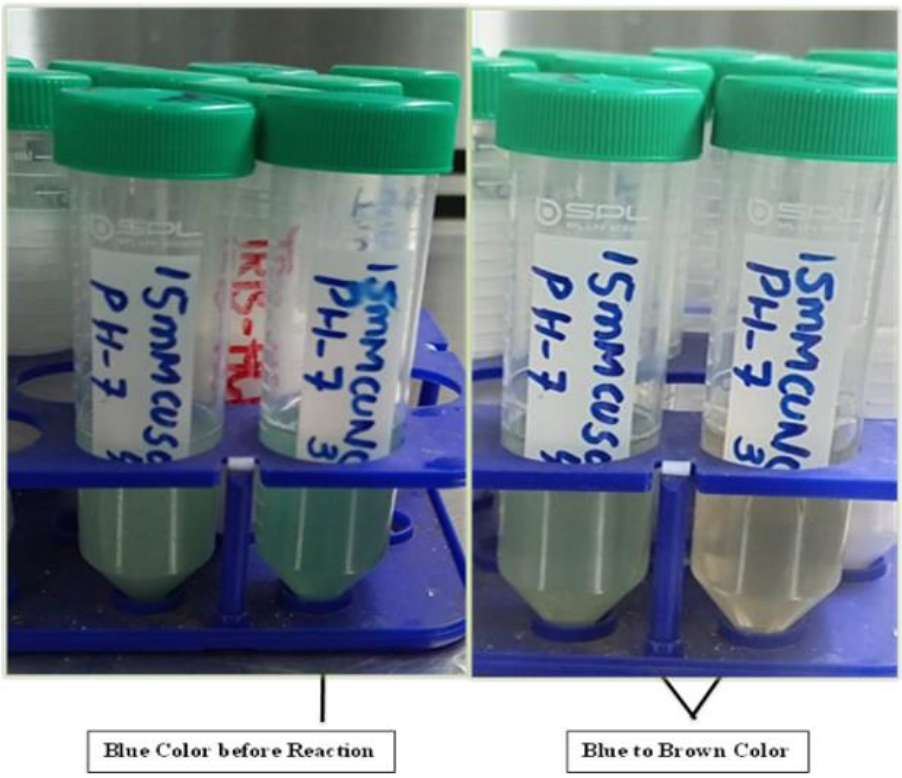

Fig. 4. Color changes indicates the production of nanoparticles

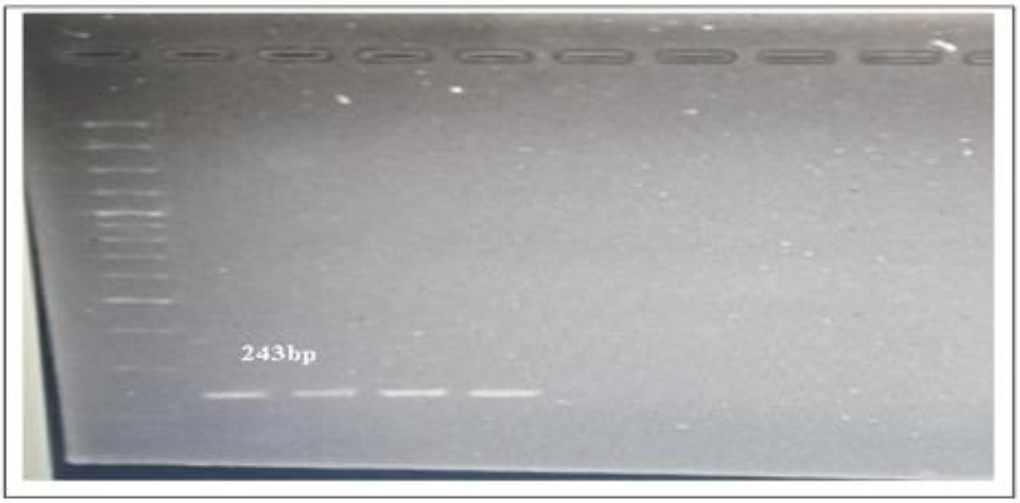

Fig. 5. PCR products of niaD gene

3.5 Physical Characterization of CuNPs

3.5.1 U.V. visible spectrum analysis

UV-visible analysis confirmed that *A. niger* produced CuNPs. Between 200 and 600 nm in the UV-visible spectrum, CuNPs were studied. The absorbance of biosynthesized CuNPs can be seen in Fig. 6 to range broadly between 350 nm and 500 nm, with the largest absorbance peak occurring at 380 nm. CuNPs showed matching large absorption peaks as a result of surface Plasmon resonance (SPR).

3.5.2 Dynamic Light Scattering (DLS)

Adopting a dynamic light scattering method Fig. 7 depicts the size distribution of CuNPs that is identical to that of other materials. For separated CuNPs with an average NP size of 570 nm and the greatest frequency, the model peak was readily apparent in the 500 nm–800 nm region. Using dynamic light scattering analysis, the polydispersion index (PDI) of CuNPs was observed to be 0.480.

3.5.3 FTIR (Fourier Transform infrared analysis)

Biosynthesized copper nanoparticles produced by FTIR displayed absorbance peaks. The highest peak at 3330.07cm^{-1} and the peaks that measure the stabilized CuNPs were obtained. The bending of the O-H bond is expressed by the O-H functional group on the phenolic ions in this way. The range of the FTIR spectrometry wave was 1000-3500cm^{-1}. The stretching of the -C=C bond demonstrated the presence of a (alkene) functional group in copper nanoparticles near the strong absorption peak of 1636.91 cm-^{1}. The results shown in Fig.8. These peaks serve to identify the functional bonds involved in the capping and binding of CuNPs.

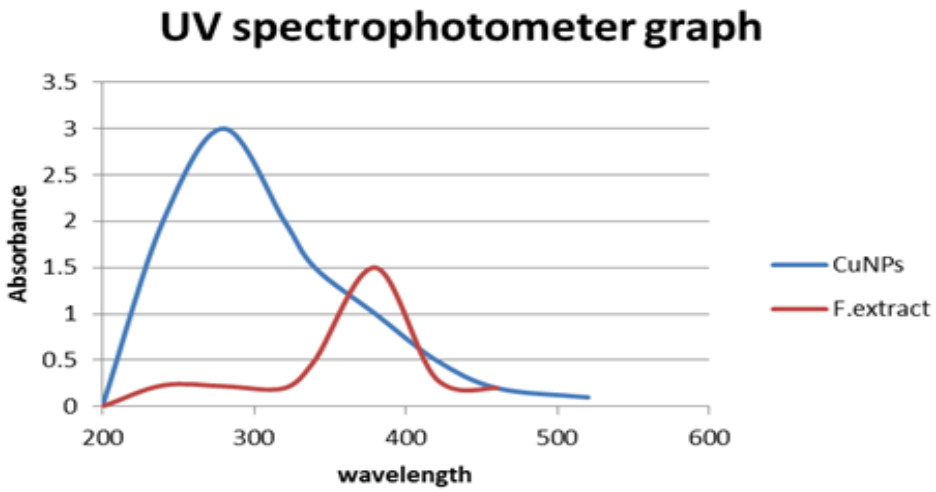

Fig. 6. U.V analysis of copper nanoparticles

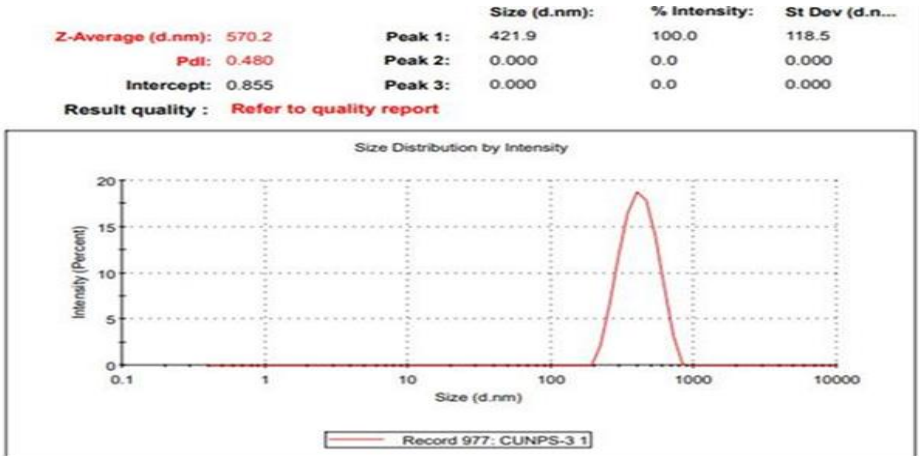

Fig. 7. DLS analysis of copper nanoparticles

3.5.4 SEM (Scanning Electron Microscopy)

The diameters of CuNPs were analysed by scanning electron microscopy. The image that was displayed was created by the scanning equipment using an electron light beam with a resolution of 5.25 A°. This offers a very extensive demonstration of the precise size of the CuNPs. One nm-sized nanoparticles can be detected by it. A JSM-6380 SEM of the kind was used to take a scanning electron micrograph. Before the test, the samples were filtered and dried.

3.5.5 X ray diffraction analysis

XRD analysis is used to evaluate the newly synthesized nanoparticles' purity, crystallized structure, and phase development. This result validates the synthesized CuNPs nanoparticles' face-centered cubic (fcc) configuration. The presence of unused peaks besides to the Bragg peaks that are typical of fcc copper nanoparticles shows that the capping and reducing agent crystallises on the nanoparticles' outermost layer. Images of the CuNPs generated by the *A. niger* fungal extract are shown in Fig. 10.

3.6 Biological Applications

3.6.1 Antibacterial activity

Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, and Bacillus subtilis were employed to investigate the antibacterial properties of the myco synthesised CuNPs. Fig. 11 (A-D) and Table 2 presents results of the antibacterial activity of CuNPs. Results indicated that zones of inhibition (ZOI) enhanced with increasing CuNPs concentration and that CuNPs had moderate antibacterial efficacy against bacteria. The 20 mm substantial inhibitory zone of *Bacillus subtilis* showed that it was more sensitive to CuNPs than other bacteria.

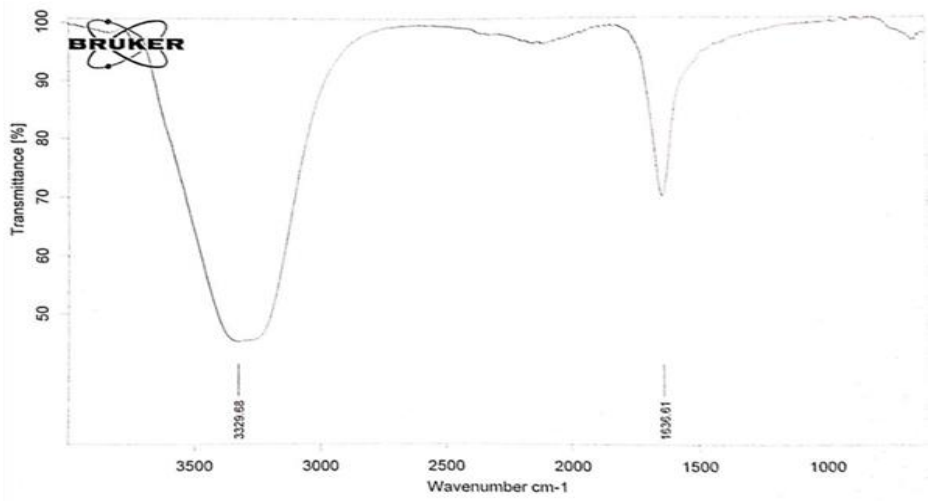

Fig. 8. Fourier transform infrared analysis of CuNPs

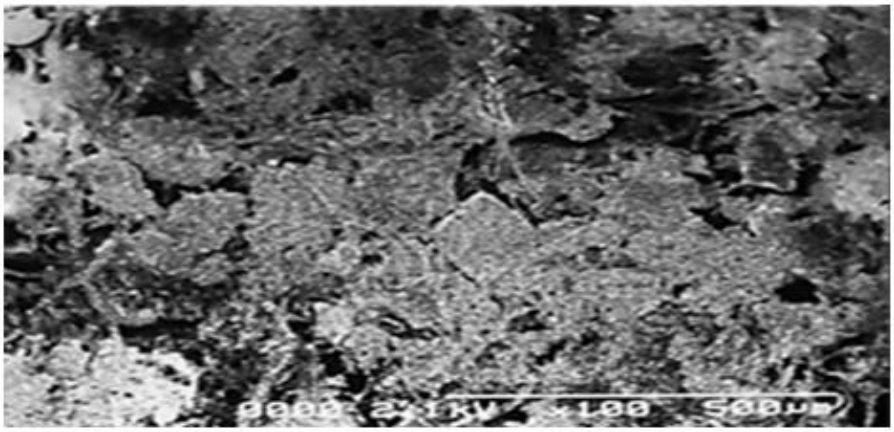

Fig. 9. SEM results of copper nanoparticles

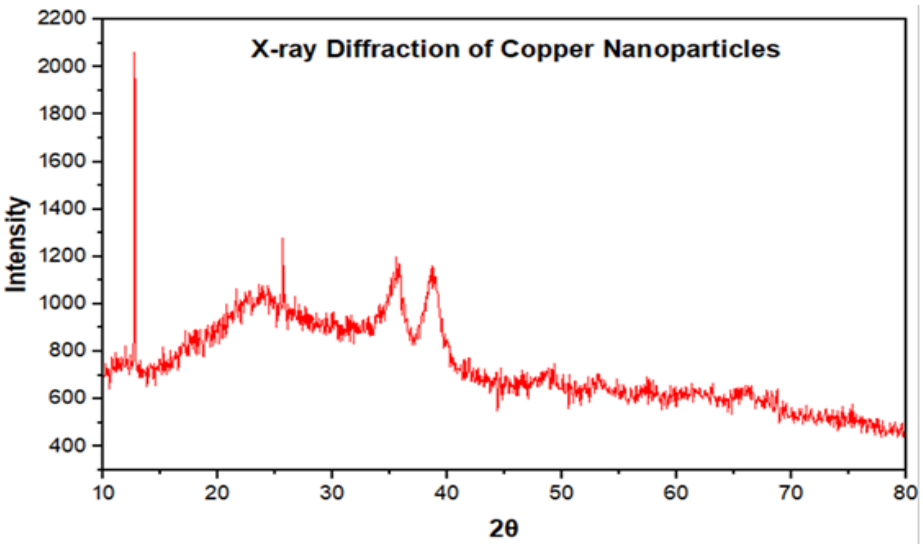

Fig. 10. XRD graphs of copper nanoparticles

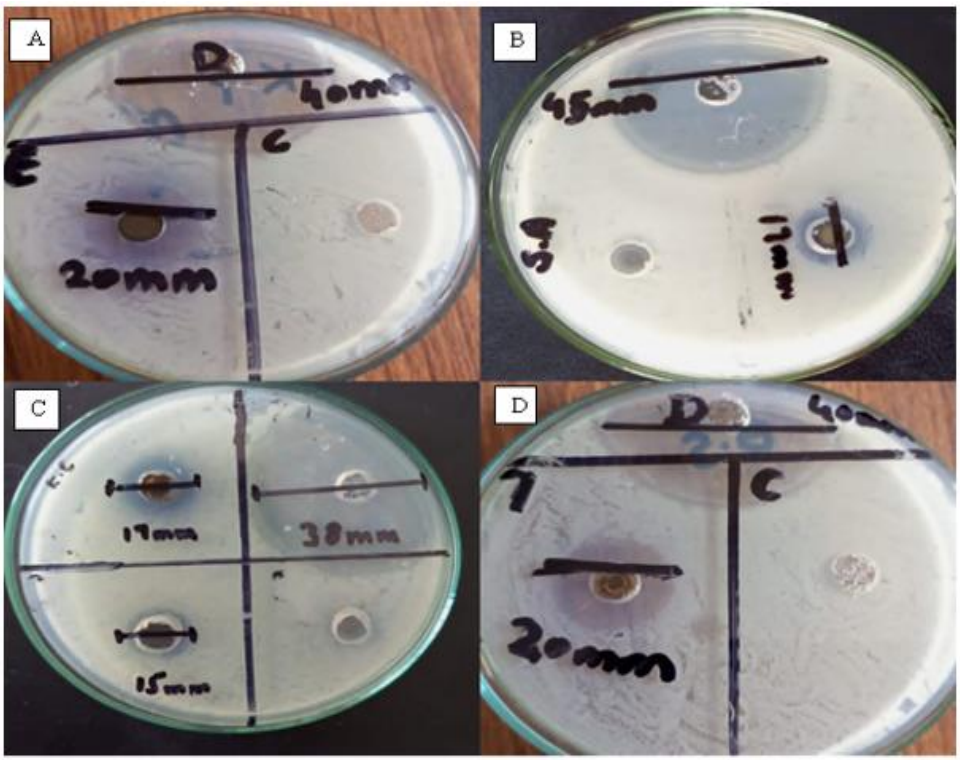

Fig. 11. Antibacterial action of CuNPs against bacteria

MIC and MBC Using a common micro-dilution method, MIC of CuNPs produced by the fungus *A. niger* was identified. Three bacteria, *S. aureus*, *S. enterica*, and *Pseudomonas aeruginosa*, were tested for MIC. Pre-incubation absorbance was measured at 600 nm. The absorbance at 600 nm was measured 20 hours after incubation. MIC values and MBC values shown in Table 3.

3.6.2 Antioxidant activity

At a concentration of 10-100µg/ml, the antioxidant values of nanoparticles and the standard (ascorbic acid) were computed. The IC50 value, which was calculated and acquired after comparison to the standard, clearly showed the anti-oxidant efficacy of CuNPs (Table 4).

Table 2. ZOI of CuNPs against different bacteria and standard drug

Bacterial Strain	Zone of Inhibition (Drug)	Zone of Inhibition (CuNPs)
Bacillus subtilis	40 mm	20 mm
Staphylococcus aureus	45mm	17 mm
Escherichia coli	38 mm	17 mm
Klebsiella pneumonia	40 mm	20 mm

Table 3. MIC and MBC of CuNPs, where positive sign showing no growth

	Salmonella enterica		***Pseudomonas aeruginosa***		***Staphylococcus aureus***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Drug (Ciprofloxacin)	5mg/5ml	+	5mg/5ml	+	5mg/5ml	+
CuNPs	5mg/5ml	+	5mg/5ml	+	5mg/5ml	+

Table 4. Antioxidant activity of CuNPs as compare to standard

Concentration	% Inhibition of Standard	IC50 (µg/ml)	% Inhibition of CuNPs	IC50 (µg/ml)
100	32.094		21.30	
75	40.241		32.41	
50	49.939		42.35	
25	62.13		57.15	
10	71.734	43.27	67.13	59.41

4. DISCUSSION

Nanoscale materials, which are larger than an atom but smaller than a micron, are the main focus of nanotechnology. There are numerous ways to produce, modify, and use these chemicals. Although there are many physical and chemical ways to make nanoparticles, it has become also possible to use biological things. In recent years, myco-genesis of nanoparticles has emerged as a significant technique for using fungus to produce nanoparticles with the desired size and form by means of an intracellular or extracellular process. Several studies have demonstrated that the NADH-dependent nitrate reductase enzyme is necessary for the conversion of metallic ions into metallic nanoparticles [33]. As fungi consist of a range of proteins and enzymes that function as reducing agents, they can always be employed to produce metal nanoparticles under similar circumstances, fungi frequently grow more quickly than bacteria. Although the production of metal nanoparticles by bacteria is widespread, fungi are better because their mycelia offer a large surface area for interactions. Microbe-derived nanoparticles are safe and eco-friendly, and they have a variety of uses in the textile, medical, delivery of drugs, physiological detector, and other sectors [34]. In this study, presented the result of two distinct fungal strains whose molecular identification was already completed. Between the four strains, SN1 and SN2 both produced nanoparticles. The other two did not produce NPs because, there may be isoforms of enzyme reductase having mutation at some extent even though they all carried the niaD gene. We developed practical, secure, and straightforward biological methods for making CuNPs, which are crucial for bio-nanotechnology. In that method, copper nanoparticles synthesized using *A. niger* extract. The process is improved by adjusting the time, salt pH concentrations, and filtrate-to-salt ratio. When copper sulfate solution (15 mM) was mixed to fungal filtrate in similar ratio and cultured for 24 hours at a pH of 7, copper nanoparticles were among the most numerous particles formed. First, UV-visible spectrum analysis is used to detect CuNPs that have been produced. CuNPs generated by myco-synthesis have a broad absorbance range of 200 nm to 600 nm. CuNPs show distinct peaks in the 200 nm – 800 nm wavelength range. The use of ultraviolet spectroscopy confirms that NPs are stable and exist extracellularly. FTIR research identified the functional groups responsible for nanoparticle stability. Absorption at 3331.0 cm^{-1} and 1635.98 cm^{-1} confirmed the presence of phenol and alcoholic functional groups due to O-

H stretching, while vibrating stretch of the C = C bonds suggested the presence of an alkene functional group, as established by DLS analysis was used to determine the CuNPs' size. poly-dispersed CuNPs with an average diameter of 570 nm and poly-dispersion index (PDI) of 0.480. CuNPs are studied using biological characterization as well as other physical characterization techniques. Both antioxidant and antibacterial activity occurred. The antibacterial activity of CuNPs was investigated in this study, and different bacteria displayed varied zones of inhibition. Inhibitory zones for *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Klebsiella pneumoniae* were 20 mm, 17 mm, 17 mm, and 20 mm, respectively IC50 values are used to calculate the antioxidant activity of CuNPs. The niaD gene was identified in both strains of *A. niger* using a PCR equipment. In A. niger, there are several reductase enzymes that can reduce metal to metal nanoparticles. The niaD gene, which codes for the nitrate reductase enzyme and may be involved in the reduction process, is the focus of this study's search for its presence. The NADPH-reductase enzyme, which is linked to the niaD gene and participates in the whole reduction process, provides electrons to copper ions during the production of CuNPs and transforms them into neutral copper.

5. CONCLUSION

Copper nanoparticles produced by fungus *A. niger*. It is an efficient, safe, and simple biological approach. Metal is transformed into metallic NPs by reducing enzymes called nitrate reductases in fungi. The niaD gene's presence is verified in this work using a PCR thermal cycler. The amplification of the reductase enzyme confirmed the existence of the gene expressing it. U.V. Visible Spectroscopy (with a peak at 380 nm), DLS (with a peak at 570 nm and PDI of 0.480, XRD, FTIR, and SEM are used to analyze the synthesized CuNPs. CuNPs inhibited the growth of *Klebsiella pneumoniae, Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli*. CuNPs had an IC50 for their antioxidant activity of 59.41 µg/ml.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Pandit C, et al, Biological agents for synthesis of nanoparticles and their applications. J. King Saud Univ. Sci. 2022; 34(3):101869.
2. Giljohann DA, et al, Gold nanoparticles for biology and medicine. Spherical Nucleic Acids. 2020: 55-90.
3. Gao Y, Wu Y, Recent advances of chitosan-based nanoparticles for biomedical and biotechnological applications. Int. J. Biol. Macromol. 2022; 203: 379-388.
4. Salem SS, Fouda A, Green synthesis of metallic nanoparticles and their prospective biotechnological applications: an overview. Biological Trace Element Research. 2021;199:344-370.
5. Ameen F, et al, A review on metal-based nanoparticles and their toxicity to beneficial soil bacteria and fungi. Ecotoxicol. Environ. Saf. 2021;213:112027.
6. Shnoudeh A.J, et al, Synthesis, characterization, and applications of metal nanoparticles, in Biomaterials and bionanotechnology. Elsevier.2019;527-612.
7. Kumar H, et al., Metallic nanoparticle: A review. Biomed. J. Sci. Tech. Res. 2018; 4(2):3765-3775.
8. Nagar N, Devra V, Activation of peroxodisulfate and peroxomonosulfate by green synthesized copper nanoparticles for Methyl Orange degradation: a kinetic study. Journal of environmental chemical engineering. 2017; 5(6): 5793-5800.
9. Shikha J, et al, Synthesis and size control of copper nanoparticles and their catalytic application. Transactions of Nonferrous Metals Society of China. 2015;25(12):. 3995-4000.
10. Nagar N, Devra V, Oxidative degradation of Orange G by peroxomonosulfate in presence of biosynthesized copper nanoparticles—a kinetic study. Environmental Technology & Innovation. 2018;10:281-289.
11. Nagar N, Jain S, Devra V, Synthesis, Characterization of copper nanoparticles and their catalytic application. International Journal of Advance Research in Science and Engineering. 2017;6(1): 666-675.
12. Nagar N, Devra V, Green synthesis and characterization of copper nanoparticles using Azadirachta indica leaves. Materials Chemistry and Physics. 2018;213:44-51.
13. Zhao X, et al., Fungal silver nanoparticles: synthesis, application and challenges. Crit. Rev. Biotechnol. 2018;38(6):817-835.

14. Rajeshkumar S, Sivapriya D, Fungus-mediated nanoparticles: Characterization and biomedical advances. Nanoparticles in Medicine. 2020:185-199.
15. Guilger-Casagrande M, Lima Rd, Synthesis of silver nanoparticles mediated by fungi*:* A review*.* Front. Bioeng. Biotechnol. 2019; 7:287.
16. Al-Zubaidi S, Al-Ayafi A, Abdelkader H, Biosynthesis, characterization and antifungal activity of silver nanoparticles by Aspergillus niger isolate. J. Nanotechnol. 2019;1(1):23-36.
17. Alsohaili SA, Bani-Hasan B.M, Morphological and molecular identification of fungi isolated from different environmental sources in the Northern Eastern desert of Jordan*.* JJBS. 2018; 11(3).
18. Su SS, Lae KZW, Ngwe H, Isolation and identification of pseudomonas aeruginosa from the clinical soil. University of Yangon Res J. 2018;8: 271-275.
19. Yaragalla S, Rajput A, Identification of fungal growth from the internal organs of preserved human cadavers. Am J Microbiol Res. 2017;5(1):25-7.
20. Fitriarni D, Kasiamdari RS, Isolation and identification of endophytic fungi from leave and stem of Calopogonium *mucunoides.* Journal of Tropical Biodiversity and Biotechnology. 2018;3(1): 30.
21. Hadwan MH, New method for assessment of serum catalase activity. Indian Journal of Science and Technology. 2016;9(4):1-5.
22. Kim JC, et al, Tenebrio molitor-mediated entomopathogenic fungal library construction for pest management*.* Journal of Asia-Pacific Entomology. 2018;21(1): 196-204.
23. Shaligram NS, et al, Biosynthesis of silver nanoparticles using aqueous extract from the compactin producing fungal strain*.* Process biochemistry. 2009;44(8):939-943.
24. Cenis J, Rapid extraction of fungal DNA for PCR amplification*.* Nucleic Acids Res. 1992;20(9):2380.
25. Patle TK, et al, Phytochemical screening and determination of phenolics and flavonoids in Dillenia pentagyna using UV–vis and FTIR spectroscopy. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 2020;242: 118717.
26. Agarwal M, et al, Preparation of chitosan nanoparticles and their in-vitro characterization*.* International Journal of Life-Sciences Scientific Research. 2018; 4(2):1713-1720.
27. Weiss JN, Dynamic Light Scattering (DLS) Spectroscopy, in dynamic light scattering spectroscopy of the human eye. Springer. 2022;13-17.
28. Walbrück K, et al. Synthesis and characterization of PVP-stabilized palladium nanoparticles by XRD, SAXS, SP-ICP-MS, and SEM. Journal of Nanomaterials. 2019;1-7.
29. Bhuyar P, et al. Synthesis of silver nanoparticles using marine macroalgae Padina sp. and its antibacterial activity towards pathogenic bacteria. Beni-Suef University Journal of Basic and Applied Sciences, 2020; 9:1-15.
30. Keshari AK, et al, Antioxidant and antibacterial activity of silver nanoparticles synthesized by Cestrum nocturnum*.* Journal of Ayurveda and integrative medicine. 2020;11(1):37-44.
31. Parvekar P, et al., The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of silver nanoparticles against Staphylococcus aureus. Biomaterial investigations in dentistry. 2020;7(1):105-109.
32. Sreelekha E, et al, A comparative study on the synthesis, characterization, and antioxidant activity of green and chemically synthesized silver nanoparticles*.* BioNanoScience. 2021;11: 489-496.
33. Adebayo EA, et al., Fungi as veritable tool in current advances in nanobiotechnology. Heliyon. 2021; 7(11).
34. Priyadarshini E. et al. Metal-Fungus interaction: Review on cellular processes underlying heavy metal detoxification and synthesis of metal nanoparticles. Chemosphere. 2021;274: 129976.

Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle5.com/review-history/106457