Cytotoxic effects of Gamma Irradiation on *Aspergillus occultus* MN259598 strain to augment biological copper nanoparticle synthesis and to corroborate antibacterial properties on food-borne pathogens

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Abstract

The current scientific study focuses on the effects of gamma irradiation on fungal isolate *Aspergillus occultus* MN259598 strain to monitor the efficacy of extracellular Copper nanoparticle (CuNP) synthesis using *Copper acetate* as the starting material through a biological route. This study is a first report of Gamma irradiation being employed in order to document the possible role of irradiational stress in nanoparticles synthesis in *Aspergillus occultus* MN259598. Gamma irradiation doses of 0.66 Gy, 0.99 Gy, 1.33 Gy, 1.66 Gy and 1.99 Gy were used for the induction of physiological changes. Macromorphological alterations of the mycelia were observed when grown in broth medium. The lethalities and fungal architectural distortions observed as a part of the study profile of micromorphological characteristics of the irradiated organism were documented. The irradiated fungal cell free extracts (CFE) were used for the production of biogenic CuNPs through optimization studies. Simultaneously, the CFE of the wild type strain was also used for the NP synthesis. The particles were characterized employing UV-Visible (UV-Vis) spectrophotometry, Dynamic Light Scattering (DLS), Fourier Transform InfraRed spectroscopy (FTIR), Scanning Electron Microscopy (SEM) and X-Ray Diffraction (XRD) analyses. Sizes of the NPs of treated (66.3 nm) and wild type strain (300 nm approx.) were found to differ enormously. The antimicrobial activity of CuNPs was determined using different food-borne pathogenic bacteria namely *Escherichia coli*, *Bacillus cereus* and *Listeria monocytogenes*.

1 Introduction

The revolution in the field of nanotechnology has opened many doorways in the world, medical science being the most significant one. Copper, since ancient times, has been used in households for its broad-spectrum antimicrobial activities against an array of human pathogens. With the advent of modern technology, it is now being employed in its *nano* form to engineer biosensors (for e.g., Glucose detector) due to a heightened catalytic activity. This application has served to be a reliable diagnostic measure for diabetics (Type II) who need constant monitoring of blood glucose level [1, 2]. They are used in cable wires due to their highly thermal and electrically conductive properties which make them a great choice of metal to be used. As compelling as it is, the biological aspect of Copper cannot possibly be avoided. In biological systems, Copper is an extremely important component of several enzymes, namely, Copper transporting adenosine triphosphates [3, 4], *cupro* proteins, which play significant roles as cofactors in enzymes such as tyrosine kinases, cytochrome-c oxidase, superoxide dismutase etc. They help aid in enzymatic activities and act as a trace metal in cell energy production through mitochondrial respiration [5, 6]. They also take part in metabolism of cell walls in plants. Copper plays an admirable role in biological systems as antioxidants by scavenging free radicals generated in cells to protect DNA and other macromolecules from being destroyed [7–9]. Copper shows high antibacterial activity against certain human pathogens [10], especially gram-negative bacteria. Surfaces of copper nanoparticles, just like any other nanoparticles, are highly charged due to their small size and show a high resonating property called Surface Plasmonic Resonance (SPR). Such phenomenon facilitates their interaction with the bacterial membranes and eventually kills them by cell lysis. The exact mechanism of how these

nanoparticles achieve this level of specificity is yet unknown to researchers but it seems that the released Cu^{2+} ions disrupt and penetrate the bacterial cell membranes. This causes an intervention of the many cellular biochemical pathways by inactivating enzymes and causing DNA damage [11–13]. Nanoparticles have drawn considerable worldwide attention due to their promising and unusual properties, namely- chemical, photoelectrochemical, optical [13], etc., to mention a few. As researchers have dug deep into the subject, some extravagant features like, enhanced structural integrity have come to light, which make them suitable to be used in an assemblage of applications such as- medicine (antimicrobial properties against an array of bacterial and fungal pathogens, target specific delivery of drugs and drug-nanoparticle conjugates, nanorobotics as a cutting edge tool for early diagnosis of diseases, microsurgery at tissue or genetic level), magnetic, catalysis, photo-electrochemical, electronics, optics [14], mechanics etc.[15, 16]. To achieve the nano range, a series of protocols have been used by the scientists. Most of these methods employ toxic chemicals which are otherwise hazardous for the environment as well as for human health. The other major drawbacks exhibited by the chemical synthesis are- intensified capital, high temperature required to carry out the experiments and objectionable features like by-products of the reactions released into nature as waste material and many more [17, 18]. Ever since chemical methods for nanoparticle synthesis have caused great toxicity to the environment, scientists have inquired to find alternatives that can substitute the production [18, 19]. As a solution to this problem, they have come up with an exciting mechanism which forms a bridge of interaction between the inorganic metal salts and bio-specimens like- bacteria, fungi and plants, leading to the mode of 'green synthesis' [20–22]. Biological entities like plants, fungi, actinomycetes and bacteria have successfully been used in the biosynthesis of these nanostructures. Biosynthesis using the aforementioned organisms has driven researchers to come up with simple, inexpensive, easy and viable alternatives to the more complex physical and chemical counterparts. The potential of these organisms to synthesize numerous kinds of nanomaterials [23] can be put into use for exploring new materials. The rationales behind using green methods for nanoparticle synthesis are that the resultant products are eco-friendly, non-toxic to the environment, easy downstream processing, cost effective nature and reliable, leading to an enhanced reduction in the usage of toxic chemicals [24].

Ionization by irradiation is a powerful yet weak form of energy that when comes in contact with atoms or molecules, causes them to ionize or excite to a higher energy state where uncharged molecules are produced like singlet oxygen moiety ($\text{O}^{\cdot-}$), singlet hydrogen moiety ($\text{H}^{\cdot-}$), hydroxide radicals ($\text{OH}^{\cdot-}$), peroxides from radiolysis of water present in and around the cells [25]. Water (H_2O) is usually present in and around the macromolecules like DNA, proteins etc., as a hydration layer. When these water molecules are broken down into their free radical form, the $\text{OH}^{\cdot-}$ radicals generated can alone cause 90% damage to the genetic material. This is an indirect mode of damage compared to a direct one where the high energy photons (of γ -rays) released from the atomic nuclei are directly deposited on the DNA [26]. As a consequence, two different types of DNA damage can occur: single strand and double strand breakages. These unusual appearances in the hereditary material sends signal to the cell to repair the damage. In case of single strand DNA break, as one of the complementary strands is un-affected, the damaged/ mutated portion of one strand is excised by the repair system and is again restored using the complementary strand as the

template. So, the structural uniformity of one strand is very important in single strand damage. Cells that are devoid of this repair system, are more prone to radiosensitivity [27, 28]. Double strand breaks are repaired through either homologous recombination or non-homologous end joining to avoid perilous after effects that can otherwise cause cell death [29]. A team of researchers showed in their study as to how these ionizing radiations can cause architectural and physiological alterations in fungal hyphae [30], a field that has been inadequately researched thus far. They further have shown how the effect of Gamma irradiation and other ionizing radiations lowered the rate of spore germination as a certain dose level was used. Although, metal tolerance in fungal species like *Aspergillus* has previously been documented with Cadmium (Cd), scientific reports on nanoparticle synthesis from bulk metal precursors by fungal species, subjected to irradiation is completely unavailable. Here, we present the use of *Aspergillus occultus* MN259598 in the production of CuNPs through a biological route, and the effect that gamma irradiation has on its synthesis potential, for the very first time.

2 Materials And Methods

2.1 Fungal isolate

The fungal isolate was a result of the isolation techniques involving soil samples that had been previously collected as a part of an ongoing research at the Fungal Genetics and Mycotoxicology Laboratory. The soil samples had been collected from the region of Madhya Pradesh, Central India, for studying the fungal flora associated with those soils. The fungal isolates were obtained and cultured in pure form by following standard protocols in order to study both macro-morphological and micro-morphological characteristics. *Aspergillus occultus* was revealed to be one of the soil samples' fungal inhabitants upon isolation. The same was identified up to the genus and species level using fungal keys and manuals [31–34] and authenticated using DNA-based Fungal barcoding.

2.2 Glycerol stock preparation

A storage vial (10 ml capacity) containing 5 ml of 50% glycerol stock was used for preserving the culture. The glycerol stock was prepared using appropriate amount of distilled water and glycerol ($C_3H_8O_3$). The mixture was sterilized by autoclaving at 121° C for 15 mins and at a pressure of 15 lbs/ inch². A five-day old culture of the fungal isolate *A. occultus* MN259598 previously grown in potato dextrose broth (PDB) (HiMedia®) and incubated at ambient laboratory conditions, was gently vortexed for 3–4 times. 1 ml of the spore suspension was transferred to the glycerol stock storage container. Later, the vial was gently shaken for proper mixing of the spore suspension and was preserved at 4° C for further studies.

2.3 Culture preservation

Culture plates (Borosil S-Line®) were used to prepare Mycological (Fungal) agar medium (Soya peptone- 10 gms/ litre, Dextrose- 10 gms/ litre, Agar- 15 gms/ litre; Himedia®). The petriplates were inoculated with *A. occultus* MN259598 using point inoculation technique. Also, culture tubes (Borosil®) containing fungal agar were prepared, inoculated and incubated at 28° C for five days. After the 5th day, the

petriplates and the culture tubes were sealed with parafilm® and were preserved in the refrigerator at 4° C in sterile storage pouches.

2.4 Culture revival of *A. occultus* MN259598

As and when the necessity for a fungal isolate for experimental methods arose, they were recovered from glycerol stocks by inoculating them onto Mycological agar plates. The cultures were inoculated by standard procedures and incubated at 28° C.

2.5 Phenotypic (morphological) characterization of *A. occultus* MN259598

In order to study the phenotypic characteristics of the fungal test isolate, namely, *A. occultus* MN259598, different mycological media were used for studying the growth characteristics, like, Potato Dextrose Agar (PDA), Dichloran Rose Bengal Chloramphenicol Agar (DRBC) and Czapek Dox Agar (CDA) media. An antibacterial antibiotic, namely, Chloramphenicol (Sigma Aldrich) was added at the rate of 20 mg/ 1000 ml in PDA and CDA. All media were prepared as per manufacturer's instructions, poured and were allowed to solidify. The test isolate was inoculated by point inoculation method and was incubated at 25° C for 3 to 11 days. Pictographs were taken at regular intervals to study growth patterns (Data not shown). Stereo-binocular and light microscopic studies were performed. Macro-morphological characters were studied by naked eye observation. Alongside, Stereo-binocular microscopic (Magnus MSZ-Bi, Model: 13M1009) studies were carried out predominantly by achieving magnifications up to 20X. The micro-morphological characters were studied by preparing Lactophenol Cotton Blue (LPCB) wet mounts and light microscopic (Olympus CH20i) observations. The identity of the fungal isolate was established up to the genus level by microscopic observations using fungal keys and manuals.

2.6 Genotypic characterization of *A. occultus* MN259598

Extraction of fungal genomic DNA

In order to obtain high quality fungal DNA of *A. occultus* MN259598, a rapid purification kit for genomic DNA isolation was used (GeneJET™, Thermo Fisher Scientific). As per manufacturer's instructions, at first, freshly grown mycelial mat of the fungus, preferably a four-day old culture was taken in mortar and pestle and was thoroughly ground using liquid nitrogen. Adequate grinding was performed to achieve mechanical shearing of the fungal mycelia. After that, 100 mg of crushed mycelia was transferred to a 1.5 ml microfuge tube along with 350 µL of Lysis Buffer A. The solution was vortexed for 10–20 mins for proper mixing. Then, 50 µL of Lysis Buffer B was added to ensure breakage of resistant mycelium. Additionally, 20 µL of RNase was added to the vial to deactivate any RNA released during the solution's preparation. After 10 mins of incubation at 65° C, 130 µL of precipitation solution was added and the tube was gently inverted 2–3 times for thorough mixing. This was followed by a brief incubation for 5 mins on ice. Next, centrifugation was carried out for 5 mins at 14,000 rpm. About 400 µL of supernatant was collected and was transferred to a fresh microfuge tube along with sequential addition of genomic DNA binding solution and ethanol (400 µL each). Eventually, 700 µL of the prepared mixture was

transferred to the spin column (provided with the kit) and the column was centrifuged at 8,000 rpm for 1 min. Following the removal of the flow-through solution, the left mixture was centrifuged for one minute using the same column. Thereafter, 500 µL of Wash Buffer I was added to the column and was centrifuged at 10,000 rpm for 1min. This was followed by centrifugation of the column at 14,000 rpm for 3 mins with 500 µL of Wash Buffer II, after discarding the flow-through of the previous step. Finally, a high yield of pure fungal genomic DNA (50 µL approx.) was achieved using two elution steps. In the 1st step, 30 µL of Elution Buffer was added to the centre of the column, incubated for 5 min at room temperature and then centrifuged at 10,000 rpm for 1 min. In the 2nd step, 20 µL of Elution Buffer was added and the solution was collected in a new tube. The pure fungal DNA, thus obtained, was then used for PCR amplification.

PCR amplification of the ITS region and the Beta-tubulin gene

Once the DNA extraction procedure was completed, the sample was subjected to a standard quantification process (ng/µl) using NanoDrop Spectrophotometer (Thermo Scientific 2000c™). The concentration and purity of DNA were checked and were used in PCR reactions. The reaction mixtures for the amplification of the ITS and the β-tubulin gene sequences were prepared using a commercial Kit namely Taq DNA polymerase 2x Master Mix RED, AMPLIQON [Tris-HCl, pH 8.5, (NH)₂SO₄, 3 mM MgCl₂, 0.2% Tween 20, 0.4 mM of each dNTP mix (dATP, dCTP, dGTP, dTTP), Ampliqon Taq DNA polymerase, inert red dye and stabilizer]. The PCR reactions were carried out in a 25 µL volume containing 12.5 µL (Taq DNA Polymerase 2x Master Mix RED, Ampliqon PCR Enzymes And Reagents), Forward primer (10 pmol) 2.0 µL, Reverse primer (10 pmol) 2.0 µL, Template DNA 2.0 µL, Molecular biology grade water (HiMedia®) 6.5 µL for ITS gene and 12.5 µL (Taq DNA Polymerase 2x Master Mix RED, Ampliqon PCR Enzymes and Reagents), Forward primer (10 µM) 1.0 µL; Reverse primer (10 µM) 1.0 µL; Template DNA 2.0 µL, Molecular biology grade water (HiMedia®) 8.5 µL for β-tubulin gene amplification. The 200 µL capacity PCR tubes were then placed in the PCR machine (Eppendorf Hamburg Mastercycler, Model: AG 22331). The cycling protocol consisted of an initial denaturation step of 94°C for 1 min 30 s, 32 cycles of denaturation at 95°C for 35 s, annealing for 55 s at 55°C for ITS and an initial denaturation step of 95°C for 3 min, 32 cycles of denaturation at 94°C for 30 s, annealing for 45 s at 65.5°C for β-tubulin and extension at 72°C for 1 min followed by a final extension of 10 min for ITS and 13 min for β-tubulin at 72°C. The PCR results were analyzed and documented using 1.5 percent agarose gel electrophoresis.

Primer sequences

The primer sequences of both ITS and β-tubulin sequences are as follows: ITS sequences (Synergy Scientific Services, Chennai, India)- ITS 1 (5′- TCCGTAGGTGAACCTGCGG-3′), ITS 4 (5′- TCCTCCGCTTATTGATATGC-3′) and β-tubulin gene (GENEi, Bangalore, India)- bT2a (5′- GGTAACCAAATCGGTGCTGCTTT-3′), bT2b (5′- ACCCTCAGTGTAGTGACCCTTGG-3′).

Sanger's sequencing of the ITS and β-tubulin sequences, Phylogenetic analysis and GenBank Submission

The PCR-amplified products of the ITS and β -tubulin genes were sent to Applied Biosystems (Biokart India Pvt. Ltd., Bangalore, India) for Sanger sequencing. The ITS gene sequences were used to characterize the fungal isolate and GenBank Accession No. MN259598 was obtained.

2.7 Scanning Electron Microscopy

Scanning electron microscopic studies were also conducted in order to obtain the three-dimensional structures of the fungal isolate (*A. occultus* MN259598). In the current study, four-day old cultures contained erect conidiophores on the cultivation medium used. A drop of double-distilled water was placed on micro-coverslips. Conidiophores were then separated from the agar surface using micro-tweezers and were laid on the coverslips. The coverslips were allowed to dry briefly, coated with carbon (Hitachi, Model: E-1010) over a duration of 20 mins and were subjected to analysis.

2.8 Irradiation studies

Petri plates were prepared in order to inoculate the test organism onto sterile solidified Fungal agar medium. Sterility was maintained during the execution of every single step of the experimental procedure. Five-day old cultures were subjected to irradiation using gamma irradiation chamber (BRIT, Model: GC-5000). Once the cultures were irradiated (with a dose rate of 1 kGy, 1.5kGy, 2.0 kGy, 2.5 kGy, 3.0 kGy/ hour respectively) for 40 mins, the culture plates were dismounted from the sample holder of the chamber and carefully shifted to the laboratory in sterile dark containers exclusively prepared for this purpose. Care was taken to incubate the irradiated cultures away from the exposition to light. The irradiation doses were given 4 days repeatedly. The rationale behind this approach was to shorten the time period for the organism to recover from the stressed condition followed by the isolate's ability to continue synthesis of nanoparticles in the desired nano size-range. Morphological and fungal architectural distortions were studied. The irradiated isolates were then subjected to nanoparticle synthesis on the 5th day. Appropriate controls were maintained.

2.9 Biogenic copper nanoparticle synthesis

In order to achieve Copper nanoparticle synthesis by biological methods, Copper acetate monohydrate [$\text{Cu}(\text{CH}_3\text{COO})_2$] and Sodium hydroxide (NaOH) extraction protocol was followed, wherein, a series of molar concentrations of the metal precursor solution was used with 0.06 M of sodium hydroxide initially. However, modifications were made to obtain optimum synthesis noticeable in terms of the efficiency of synthesis by visual observation (colour change from straw yellow to brick red and/ or muddy brown) [35].

2.9.1 Protocol optimization for synthesis of biogenic Copper oxide nanoparticle

A series of protocols were followed in order to control the size of the biogenic CuONPs, and to standardize the procedure. Extraction of the nanoparticles from media (PDB) alone was carried out, using both 0.01 M and 0.001 M metal precursor solutions of Copper acetate monohydrate of analytical grade. Sodium hydroxide (0.6% NaOH; Himedia, Molecular biology grade) was used as a reducing agent to

improve the rate of reaction. For the synthesis of nanoparticles from the wild type isolate and irradiated fungal isolates, the cell free extract (CFE) was separated from the mycelial mat by using Whatman filter paper No. 1. The pH of CFE, metal salt solution and NaOH were checked using digital pH meter. Then, with the addition of a magnetic bead, the conical flasks were placed on the magnetic stirrer (REMI 1MLH) at a speed of 370 rpm. An aliquot of Copper acetate solution (20 ml) was added to it under vigorous stirring condition while the pH was also being monitored. After stirring for about 2–3 mins, 20ml of 0.6M NaOH solution was added drop-wise and the pH was checked again. Following this, the solution was stirred for 10 mins. Colour changes that were observed throughout the process, were being documented by taking pictographs. After the completion of stirring, the flask was placed in water bath that was previously maintained between 78–80° C for 20 mins. As the colour changes became noticeable into dark red, blackish red or muddy brown, the flasks were drawn out of the water bath. The colour changes were indicative of the reduction of Copper, as stated in previous studies [35, 36]. An additional stirring was followed for 10 mins to complete the reaction. The flask was then left undisturbed to cool down to room temperature. They were later subjected to extraction of the nanoparticles. In the extraction process, aliquots of the solution were poured into respective sterile tubes (50 ml) and were centrifuged at 8,600 rpm for 20 mins. After the first spin, the supernatant was discarded and the pellet was washed with 70% ethanol, followed by a 100% ethanol wash at the same speed to dissolve the impurities in the solution from the pellet. Next, acetone was used 2–3 times, additionally and the tubes were spun under same conditions, to get a less crude/ unadulterated final product in terms of pure nanoparticle form. Later, the falcon tubes were covered adequately with tissue paper and kept for drying overnight. Following the drying step, the residual particles were retrieved from the tubes the next day with the help of a sterile spatula. The contents were transferred into a mortar, and ground into a fine powder texture which was stored in microcentrifuge tubes for further characterizations.

3. Characterization Of Copper/ Copper Oxide (Cu/ CuO) Nanoparticles

The working solutions of the NP suspension comprised aliquots of 1.5 ml each, dispensed in 2 ml microfuge tubes. The tubes were then subjected to sonication for 20 mins using an appropriate dispersant, namely, Poly Ethylene Glycol (PEG). The absorbance was recorded in the wavelength range of 200–800 nm (Shimadzu, Model- UV 3600 Plus). Nanoparticle suspensions (4 ml) from wild type and irradiated isolates were aliquoted, out of which 1.2 ml of the samples each were used to determine the particle size and density. The samples were prepared by using Polyethylene Glycol (20% PEG) and were sonicated for 20 mins for uniform particle distribution throughout. The technique was carried out using the particle size analyzer (Malvern Zetasizer, Model: ZEN1600). The 3-dimensional structure of nanoparticles were studied using scanning electron microscopy. The nanoparticles were dusted onto the sample holder and placed inside the coating equipment (Hitachi, Model: S-3400N). They were sputter coated with Carbon (Hitachi, Model: E-1010) over a duration of 20 mins and then examined. The composition of the nanoparticles was studied using Fourier transformed infra-red spectroscopy (FTIR) (Nicolet, Thermo scientific, Model: 6700) and X-ray diffraction (XRD) analysis (PANalytical X'Pert Pro™).

4 Antimicrobial Assays Using Biogenic Nanoparticles

4.1 Antibacterial effects of CuNPs from wild type strain using Well diffusion assay

Mueller-Hinton (HiMedia®) Agar plates were prepared by using the required medium contents. The plates were marked accordingly and were inoculated with subsequent bacterial cultures (*Bacillus cereus*, *Escherichia coli* and *Listeria monocytogenes*) by spread plate technique using appropriate lab accessories. Later, with the help of a sterile stainless steel cork borer, the agar surface was bored and wells were created, to place the nanoparticle suspensions. Then, 100 µl of different nanoparticle concentrations (20 µg/ mL, 40 µg / mL, 60 µg / mL, 80 µg/ mL) were added into four different wells while *Kanamycin* (100 µg/ mL) was used as a positive control. The fifth well contained sterile distilled water and served as negative control.

5. Results And Discussions

5.1 Morphological and Genetic characterization of *A. occultus* (Wild-Type and Irradiated isolates) by Microscopy and DNA Barcoding

An examination of the cultural characteristics of the test isolate *A. occultus* MN259598 was carried out alongside the main objective of this paper (complete data not shown here) to document and observe growth patterns of the organism, employing various mycological media. *Aspergillus occultus* MN259598 was grown on different media, namely- Czapek-Dox agar (CDA), Dichloran Rose Bengal Chloramphenicol (DRBC) agar, synthetic and semi-synthetic Potato Dextrose agar (PDA) and Fungal agar media. *A. occultus* exerted minor macro-morphological changes over a duration of six days of incubation. Many researchers in their work previously [34] have documented as to how nutrient availability and other extrinsic parameters could influence the fungal growth patterns as shown in (Fig. 1). As the organism grew into mature, interwoven hyphal filaments, sclerotia began to develop with age, which are known to reserve food material for utilization under uncongenial conditions and were detectable in PDA and Fungal agar media. Also, sclerotia formation, which generally are considered as a prerequisite to sexual development [32] were seen in all the cultures irradiated with different doses of Gamma irradiation (1 kGy, 1.5kGy, 2.0 kGy, 2.5 kGy, 3.0 kGy/ hr each)

The colonies ranged from 0.25-1cm in diameter in a highly crowded plate. Otherwise, the growth was profuse and spread as concentric waves when colonies fused with each other. The species started to appear as hyaline with conidial heads being white in the initial stages, transforming to a velvety outline of actively growing hyphae. The hyphae eventually changed through the color shades of cream, cream buff to light brownish, biscuit- brown, powdery colony upon maturation. Conidial heads were conspicuously circular when viewed with the naked eye and under the stereo-binocular microscope. They gradually spread layer by layer producing young conidiophores in concentric rings which were easily differentiable. Sometimes in old cultures they showed a coloration of snuff-brown. When viewed under stereo-binocular

microscope, a well distributed colony of conidiophores (Fig. 2) was spotted. When higher magnifications were used (20X), young conidiophores bearing the globose conidial heads were perceived. Long conidiophores (Fig. 3) were observed with a maximum of one to two septa. These exceptionally long conidiophores and cultural features are characteristic of yet another species namely *Aspergillus wentii*. In spite of meticulous inspections of the micromorphological characters, it led to an initial ambiguity in species identification and was overcome by using DNA barcoding of the Internal Transcribed Spacer (ITS) and β -tubulin gene sequences. Conidiophores were thick-walled, smooth, enlarged at tips to form vesicles varying in diameter; and were yellow to fade brown. Biseriate sterigma were observed circumferentially on the globose vesicle which in turn bore chains of conidia in basipetal succession. Spores were found to be brown, but sometimes young conidia took up the Lactophenol cotton blue (LPCB) stain and appeared blue. Conidial heads were large and radiate. Conidia appeared as pyriform to globose structures. Hülle cells, which are produced to indicate the isolate's sexual potential, however, were not observed during the current study.

While studying the 3-dimensional structures of the wild-type and of the irradiated isolates of *A. occultus* MN259598 using Scanning Electron Microscopy, it revealed a conidiophore length of 1360 μm (Fig. 4). In another instance (Fig. 5), the smooth-walled conidiophore was clearly visible with a length of 704 μm . This is a characteristic feature which was observed during both light microscopic studies and SEM analyses. In (Fig. 6), an enlarged and globose conidial head of the fungus with higher magnification is presented. The Scanning Electron Micrographic structure of irradiated *A. occultus* MN259598 species is being documented here for the first time, which showed severe architectural distortions of the fragmented vesicle and very poor formation of chains of conidia (Fig. 7) in comparison to the wild-type. Such ultra-structural studies have been rare, especially in context to nanoparticle production.

5.2 Genetic characterization of the test fungal isolate

In order to authenticate the identity of the fungal test isolate as *Aspergillus occultus*, DNA sequences which were generated by the PCR amplification of the ITS (Amplicon size: 594 bp) and β -tubulin genes (Amplicon size: 585 bp), were sequenced in the forward and reverse directions, compared with all known related sequences in the databanks and appropriate phylogenetic trees were constructed (Figs. 8 and 9). Phylogenetic trees were obtained by maximum parsimony (MP) and maximum likelihood (ML) methods, which yielded adequate results to examine the sensitivity of the resulting trees. Fungal phylogeny solely based on phenotypic characterization may not always elucidate the taxonomic status of the organisms in question [37]. Most certainly, the nuclear ribosomal DNA (rDNA) is the popular region in molecular studies because it is multi-copied and contains highly conserved genes [38]. Hence extensive morphological studies combined with nuclear-based molecular datasets provided valuable data in genus and species level identification of the fungal isolate under scrutiny. Due to many advantages like the ease of amplification and widespread use, ITS has been chosen as the official barcode for fungi by a consortium of mycologists [39, 40].

5.3 Synthesis Potentials of Wild-Type and Irradiated Fungal isolates of *A. occultus* MN259598

CuONPs were synthesized from the cell-free extracts of the wild type and irradiated fungal specimens of *A. occultus* MN259598 with two different concentrations of Copper Acetate (0.01M and 0.001 M) and Copper Nitrate (0.001M and 0.005 M) as metal precursors. Nanoparticle sizes of 559 nm and 295 nm were obtained when tested with 0.001M concentration of Copper acetate and Copper nitrate respectively. Upon irradiation of *A. occultus* MN259598 with a dose of 1.33 Gy (2 kGy/ hr), a desirable size of 66.3 nm was achieved with 0.01M concentration of Copper acetate, whereas, the wild type strain showed a particle size distribution in the range of 150–300 nm, where average particle size was found to be 362.82 nm. On the other hand, the size of the nanoparticles was found to be 78–90 nm with culture filtrates of irradiated fungal specimen (γ irradiation dose rate: 3 kGy/ hr) treated with 0.005M concentration of Copper nitrate. In any case, variation in colour changes of the solution throughout the biological synthesis process was similar for both wild-type and irradiated strains. The use of gamma irradiation to successfully inactivate fungi in stored seeds [41], paper, wood and soil [42] etc., have been carried out in past. Although, the effect of Gamma irradiation has not been a part of extensive research when it comes to studying the toxigenic profile of different fungi. A study led by a research team [43] found a vast difference in mycotoxin production between irradiated and wild type strains of *Aspergillus flavus* and *Aspergillus ochraceous*, where, the irradiated strains showed heightened levels of Aflatoxin B₁ and Ochratoxin, respectively. This can be explained as a stress response to stimulate excess toxin production. In another study [44], radiation doses of 2–10 kGy had been employed to inhibit Castor seed mycoflora. However, their application in bio-nanotechnology is being reported for the very first time in the current investigation. Irradiation adversely affected the membrane integrity and disrupted the fungal cell walls to a large extent resulting in the formation of malformed, stunted and thicker hyphae as compared to the slender mycelia observed with wild type specimens. Conidiophores showed severe fragmented appearance and were poorly developed. It is clear that the damage caused at the genetic level resulted in major mycelial distortions due to irradiation (because irradiated strains showed lower DNA concentration; data not shown here). Also, the extreme stress may have resulted in altered enzyme production leading to the formation of CuONPs in the size range of 66 nm and 78 to 90 nm as opposed to the approximate size range of 300 nm in case of wild-type specimens.

5.4 Characterization of the Nanoparticles

5.4.1 UV-Vis Spectroscopic studies

The formation of the metal nanoparticles namely CuONPs/ CuNPs was confirmed by UV-Vis Spectra as illustrated in (Fig. 10). Peaks were obtained with a maximum absorption at wavelengths of 245-295nm for wild type and 243nm, 295nm for irradiated fungal isolates. This is an indication of inter-band transition of electrons in the core Copper metal. The results obtained in the current study were in accordance with previous reports published on CuONPs/ CuNPs [35]. In a specific study by [45], the shape of the spectra and the wavelength at which maximum absorbance occurs are strongly related to particle

size and relative dimensions. It was also observed that there was a gradual decrease in the absorbance especially in the irradiated specimens. As and when the irradiation dose was increased, the frequency of the reduction of metal ions to metal nanoparticles showed a sequential diminishment. Although, the exact mechanism for reduction of Copper ions to Copper nanoparticles by test fungal isolate *Aspergillus occultus* MN259598 is unclear, irradiation may be used as a tool to achieve synthesis of nanoparticles in the nano-range (below 100nm or lesser). Further, irradiation served to selectively eliminate the formation of larger particles as seen in non-irradiated samples (Fig. 11a-b).

5.4.2 Dynamic Light Scattering analysis

DLS data were taken in order to determine the particle size and the distribution of the CuONPs in a measurable size range, produced by biological methods. Differences in particle sizes were observed when varying concentrations of metal precursors were used. The average sizes of the particles formed by the wild-type fungal isolate were found to be 362.82 nm and 615 nm when 0.01M and 0.001M concentrations were respectively used. On the other hand, irradiated fungal specimens produced nanoparticles in the size range of 120.5 to 201.9 nm when radiation doses ranged from 1–3 kGy with optimum dosage (2 kGy), producing nanoparticles in the size of 66.3 nm. Concentration of the metal precursor salt used, has a significant role in controlling the nanoparticle size along with other process parameters [46] like pH, medium used for extraction, temperature, duration of the reaction time, etc. Size control of nanoparticles synthesized using fungal specimens demand a control of several parameters like rotary conditions during the growth of the organisms, pH, etc. The whole focus in the current inquiry was the use of irradiation as one of the predominant parameters on studying the impact on the nanoparticle synthesis potential of the fungal isolate at a given set of parameters (Temperature – 30° C; pH 7)

5.4.3 SEM analysis

Several studies have reported the shape and size of Copper/ Copper oxide [47] and other metal nanoparticles [48, 49] using fungi. Surface morphology using Scanning Electron Microscopic (SEM) studies revealed polygonal particles with rounded ends and exhibited polydispersity, with diameters ranging in size from 60–200 nm (Fig. 12a-b), as against particle size ranging from 500–600 nm with non-irradiated samples, achieved with extracellular synthesis using the mycelium-free fungal extract of the isolate *A. occultus* MN259598. Both, individual particles and tiny aggregates were observed.

5.4.4 FTIR analysis

FTIR analysis was carried out for the biosynthesized CuNPs to deduce the presence or absence of specific functional groups present on them. The IR spectrum of the CuNPs showed peaks at 3421 cm^{-1} , 2925 cm^{-1} , 1618 cm^{-1} , 1398 cm^{-1} , 1050 cm^{-1} , 548 cm^{-1} and 457 cm^{-1} which correspond to H-bonded stretching in O-H group, C-H asymmetrical stretch vibration of alkanes, bending vibration of N-H bond, OH bending of tertiary alcohols, C-O stretch of primary alcohol, C-halogen stretching vibration and S-S stretch of aryl disulfides respectively [50, 51]. The broad band at 3421 cm^{-1} is in the range $3400 - 3200\text{ cm}^{-1}$, specifying the H-bonded stretching in O-H which is for the presence of hydroxyl groups in fungal culture extracts. The two peaks 2855 and 2925 are assigned to C-H stretching in $-\text{CH}_3$ and CH_2 groups

respectively. According to [52], the above two peaks 2855 and 2925 mainly show the presence of lipid molecules. The FTIR spectra containing peaks 1398 and 1050 cm^{-1} can be assigned to -OH bend of tertiary alcohols and C-O stretch of primary alcohols, which clearly confirm that the oxygen-containing groups such as hydroxyl, epoxy and carboxyl groups are successfully bound to the surface of nanoparticles due to oxidation. The bands 1618 cm^{-1} and 457 cm^{-1} are due to the appearance of bending vibrations of N-H bonds present in primary, secondary amines and S-S stretching vibration in disulphide bonds respectively [50, 51]. Based on the above FTIR data, it is clear that mainly lipids and proteins influence the synthesis and stabilization of nanoparticles. It confirms the presence of the proteins and lipids that have been secreted (Fig. 13) by the test isolate *Aspergillus occultus* MN259598 which could contribute to the formation of nanoparticles as well as their stabilization [53].

5.4.5 X-Ray Diffraction Studies

Powder X-ray Diffraction (XRD) of synthesized NPs was carried out which authenticated these structures to be of Cu NPs. The XRD spectrum showed formation of broader peak with less intensity. Such a pattern supported the fact that the NPs under study are composed of small crystals. The size of the NP plays a pivotal role in determining the crystalline structure of NPs. The peaks observed at 2θ (Bragg reflections) values of 43.08°, 50.23° and 73.91° (Table 1) correspond to (111), (200) and (220) sets of lattice planes of metallic Cu, which can be indexed to face-centered cubic (FCC) structure of copper [54, 55]. XRD data that was obtained in this experiment was in well accordance with the Joint Committee of Powder Diffraction Standards (JCPDS) data to identify the crystallographic phases. These peaks were in exact resemblance with the JCPDS file number- 04-0836, which ultimately proved the formation of nanocrystals of copper (Cu) in the biological nanoparticle sample (Fig. 14).

5.4.6 Antimicrobial assays against Food-borne pathogenic bacteria

The well diffusion method was used, in order to determine the effects of the CuNPs (from wild-type and irradiated fungal isolates) against three different genera namely *E. coli*, *B. cereus* and *L. monocytogenes*. The zones of inhibition (mm) obtained are presented in the (Fig. 15a-c). In the case of *E. coli* and *L. monocytogenes*, the nanoparticles produced from irradiated fungal isolate *Aspergillus occultus* MN259598 showed better inhibition rates. Such studies using wild-type and irradiated isolates, comparing their synthesis potentials and the effects on pathogenic bacteria is being reported for the very first time. This is also ascribed to the reduction in size of the nanoparticles which was achieved through irradiation. While size of the nanoparticle can have a significant bearing on its antimicrobial effect [56], extensive studies are required to explain this phenomenon in adequate detail. One of the rationales is the distinction between the Gram-negative and Gram-positive bacteria, with respect to their cell wall structure and thickness, making the former more vulnerable to nanoparticle exposure and inhibitory effect. Although, the Gram-positive bacteria lack an outer membrane, they are surrounded by a thicker peptidoglycan layer [57, 58]. Few studies have reported the antimicrobial properties of CuNPs [59], and may have similar mechanism of action as that of silver. However, the precise mechanism regarding how CuNPs cause an inhibitory activity is unclear. It is thought that Copper acts by combining with the

Sulfhydryl groups (-SH) of key microbial enzymes [60]. According to previous reports, studies on antibacterial effects of CuNPs are limited when compared to AgNPs or ZnONPs and higher concentrations of the NPs are required to achieve similar results. Some researchers [61] opine that copper being less expensive than other nanometals, can be utilized as nanocomposites for increasing antibiotic efficiency.

6 Conclusions

The synthesis of nanoparticles by biological routes is not only exciting for a researcher but also extremely challenging. The current era has seen a surge in nanotechnological studies pertaining to the use of especially fungi in nanoparticle synthesis and has emerged as a branch of science called *Myconanotechnology*. The use of *Aspergillus occultus* MN259598 in the current exploration was novel, because, scientific reports involving this organism with special reference to Gamma (☒) irradiation, being used to determine nanoparticle synthesis potentials are being reported for the very first time. Irradiation of the fungal test isolate was one of the foci, in order to study the excruciating effect on the phenotype of the fungal organism and the role of Gamma (☒) irradiation on the NP synthesis potentials of the same. The study revealed that irradiation of the fungal isolate *Aspergillus occultus* MN259598 caused a drastic reduction in the size of the nanoparticles. This study also provided specific insights to reveal that *Aspergilli*, such as the fungal isolate used, could survive radiations up to 3 KGy/ hour. Although, severe phenotypic malformations were observed in their structural/ hyphal architecture (as evident by microscopic studies), nanoparticle synthesis was still achievable. Characterization studies of nanoparticles were especially helpful in order to determine features like type, purity, size distribution, dispersive nature, etc. Nanoparticles produced by such methods could be used in the production or design of drugs, in food-preservation techniques and development of biopesticides for agriculture. Further, evidence-based studies to deduce the pathways involving the enzymatic reduction of metal ions to metal nanoparticles, are the need of the hour to understand the complex mechanisms of nanoparticle synthesis in fungi.

Declarations

Ethics approval and consent to participate

This manuscript contains no animal research, no human studies, and no potentially identifiable human photos or data.

Consent for publication

Each author confirms that the publisher has obtained the author's consent to publish research findings.

Availability of data and materials

Internationally recognized scientific procedures were employed, and all data supporting the findings in the article should be widely available following publication. The datasets analyzed in this study are publicly available.

Competing interests

The authors state that no commercial or financial ties that might be considered as a potential conflict of interest existed during the conduct of the research.

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Authors' contributions

RM and RD were involved in planning and designing the research study. RM designed and conducted the experiments, analyzed the data, and drafted the manuscript. RD created the results, drafted the article, corrected errors, and aided in the manuscript's preparation. PM carried out the phylogenetic analysis, whilst MG carried out the experiments and drafted the article. The paper was written by all authors and their contributions were approved before to submission.

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Tables

Table 1 is not available with this version.

Figures

Figure 1

Macromorphological observation of *Aspergillus occultus* MN259598 on (a) synthetic PDA, (b) DRBC and (c) Fungal agar medium with naked eye observation on the top panel. (d), (e), (f), Bottom panel shows the corresponding stereomicroscopic observation at 10X (Flow of images: Top to bottom)

Figure 2

Stereo binocular microscopic obverse view of a colony of *A. occultus* MN259598 showing conidial heads (20X)

Figure 3

Light microscopic image of a young and mature, unusually long conidial head of *A. occultus* MN259598 enlarged (left). The right-hand side image is showing the sterigmata at 40X magnification.

Figure 4

Scanning Electron Microscopic image showing a tuft of conidiophores and length measurement of the longest conidiophore (1360 µm) of *A. occultus* MN259598.

Figure 5

Conidiophore of *A. occultus* MN259598 scanned by SEM at 704 nm resolution.

Figure 6

SEM image of an enlarged, globose conidial head of *A. occultus* MN259598 at higher magnification.

Figure 7

Following irradiation, light microscopic pictures showed architectural (conidiophore) deformities in *A. occultus* MN259598. The light microscopic images demonstrate inadequate conidial chain formation, fragmented vesicles, and spore dispersion throughout the field (shown by a black triangle-shaped indication).

Figure 8

A phylogenetic tree (taxon branching diagram) which presents the genetic analysis of the *Aspergillus occultus* RSDR2 clade belonging to the Section *Circumdati*, exhibiting a maximum parsimony analysis of partial ITS1 and ITS4 concatenated sequences, acquired.

Figure 9

A phylogenetic tree (taxon branching diagram) which presents the genetic analysis of the *Aspergillus occultus* RSDR2 clade belonging to the Section *Circumdati*, exhibiting a maximum parsimony analysis of partial β -tubulin concatenated sequences, acquired.

Figure 10

UV-Vis spectra showing the absorption maximum of the CuNPs of both wild type and irradiated samples. Wavelength range of CuONPs synthesized from wild type *Aspergillus sp.* SR2927 indicated by yellow line showing peaks in the range of 200-400. CuONPs synthesized from gamma irradiated strain showed peaks at 243 and 295 nm indicated by green line. (Violet color line represents data from another experiment).

a

b

Figure 11

Graph reflecting the dynamic light scattering (DLS) properties of the formed CuNPs from (a) PDB in a size range of 559 nm. (CuA- 0.01 M). Histogram showing analysis of particle distribution by DLS of the (b) biologically synthesized CuNPs (0.01 M) from the wild type *A. occultus* strain MN259598. Polydispersity of particles were observed in the range of 150- 300 nm approximately.

a

b

Figure 12

(a), (b) Scanning Electron Micrographs of Copper nanoparticles

Figure 13

FTIR Spectrum of Copper nanoparticles.

Figure 14

The X-Ray diffraction pattern of the copper nanoparticles.

a

b

c

Figure 15

Graphical presentation of the effect of copper nanoparticles synthesized from wild type and irradiated strains of *A. occultus* MN259598 on food borne bacterial pathogens (a) *Escherichia coli* (b) *Bacillus cereus* (c) *Listeria monocytogenes*. In all three scenarios, the particles synthesized from irradiated samples showed maximum inhibition compared to the wild type.