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Skirmishing MDR strain of *Candida albicans* by effective antifungal CeO$_2$ nanostructures using *Aspergillus terreus* and *Talaromyces purpurogenus*

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Abstract

Emerging antibiotics resistance fungal infections is a major global health problem and new antifungal formulations are direly needed to fight drug resistant *Candida albicans strains*. This study is aimed to synthesize effective antifungal nanostructures of cerium oxide (CeO$_2$) using culture filtrates of two common fungal strains *Aspergillus terreus* and *Talaromyces purpurogenus*. The fungal strains used in the synthesis were identified by 18S rRNA gene sequencing and deposited to NCBI GenBank with the accession number of MN099077 and MN121629, respectively. The biofabricated CeO$_2$ NPs were characterized by X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). Pure CeO$_2$ nanoparticles (NPs) synthesized using *Aspergillus terreus* culture filtrate were depicted spherical morphology with average size of 28.5 nm. The CeO$_2$ NPs synthesized using *Talaromyces purpurogenus* revealed the presence of nanosponges with average size of 21.4 nm. Gas chromatography mass spectrometry of culture filtrates of respective strains indicated the presence of ethanol, 1-propanol and tri-chloromethane in culture filtrate of *Aspergillus terreus* and with addition of palmitic acid in *Talaromyces purpurogenus* culture filtrate which may have a function as bio reducers and capping agents. Dose dependent antifungal activity of CeO$_2$ NPs using various different concentrations (100, 200, 300, 600 μg ml$^{-1}$) synthesized by both fungal strains was observed by disc diffusion assay against *Candida albicans* evidenced by increase in size of zone of inhibitions with increasing concentration of CeO$_2$ NPs. Further *in-vitro* and *in-vivo* experiments are required to access the potential of CeO$_2$ NPs for controlling *Candida albicans* strains.

1. Introduction

The fungus *Candida albicans* is the most prevalent, opportunistic and nosocomial human pathogen which is responsible for 90%–100% mucosal infections such as skin, oral cavity and gastrointestinal tracts [1–4]. Candida is attributed to 35%–50% mortality rate in immune compromised patients [5]. Their significant biofilm activity makes a critical clinical challenge. The emergence of resistant strains has been increasingly reported in recent years. *Candida albicans* cells are highly resistant to antifungal agents such as fluconazole, nystatin, amphotericin B and chlorhexidine [6]. The cause of resistance could be extracellular enzymes such as proteinases, phospholipases, hemolysin and lipases because they facilitate adherence and penetration to the host [7]. Misuse of existing antifungal drugs led to the development of drug resistant in *Candida albicans*. Recently, microbial drug resistance is gained by nanomedicines based on nanoparticles as antimicrobial. CeO$_2$ NPs provide a new dimension to
biomedical field towards biomedicine via anti-bacterial effect, ROS suppression, tissue engineering, specific drug targeting and many others uses [8–11]. CeO$_2$ NPs can be used as potential antibiotic adjuvant to antibiotics to treat drug resistant pathogens to increase effectiveness of antimicrobials. The less toxicity of CeO$_2$ NPs makes them attractive candidate as antibiotic adjuvants against MDR pathogens [12]. In recent decades, myco-synthesis of nanoparticles is gaining attention in research since it does not require high energy inputs or the production of highly hazardous by-products. The enzymes, proteins and heterocyclic derivatives present in fungal extracellular membrane act as metabolites which work as reducing and capping agents, with high bio-catalytic activity [13–16]. There are many reports on transition metal nanoparticles. Among them, some recent reports are on the synthesis of CeO$_2$ NPs using Humicola sp and Aspergillus niger [17, 18].

In this study, we have used culture filtrate of two common fungal strains i.e. Aspergillus terreus isolated from soil sample and Talaromyces pupureogenus isolated from Buxus vahlii plant for mycological synthesis of CeO$_2$ NPs and the potential of synthesized nanoparticles against Candida albicans strain was also explored.

2. Materials and methods

2.1. Chemicals

Ce(NO$_3$)$_3$. (CAS number 10108-73-3) and other pure chemicals and biochemicals were procured from Sigma Aldrich and Merck. Potato dextrose broth (PDB) and Sabouraud dextrose agar (SDA) was obtained from Oxoid, United Kingdom.

2.2. Isolation of fungal strains

Agriculture soil samples were collected in container aseptically from microhabitat of International Islamic University Islamabad (IIUI) with the help of spatula. The samples were kept sterile and 4°C until processed for isolation of fungal strains. Soil was suspended in autoclaved deionized water (10 ml) and was shaken using a magnetic stirrer for 30 min to obtain homogeneous suspension. After serial dilution (up to $10^{-5}$) was made and 0.1 ml aliquot was inoculated on Sabouraud dextrose agar (SDA) plates and incubated at 28 ± 2 °C for 48–72 h. Single colonies were picked and subcultured on same media to get purified strains. A fast growing and morphologically different, purified strain IIUI-201 was selected for the synthesis of nanoparticles. To isolate other fungal species and given a special code 201. To isolate plant Buxus vahlii plant leaves were cut and washed (3 times), placed on SDA and incubated at 28 °C ± 2 °C for 48–72 h. After subculturing, a purified strain IIUI-236 was selected for mycomediated synthesis of cerium oxide nanoparticles.

2.3. Identification of the fungal strains

The isolated fungal strains were identified by morphological and microscopic observations followed by molecular identification based on sequencing of internal transcribed spacer (ITS). Spin kit (Qiagen, Hilden, Germany) was used to extract genomic DNA. The universal primers: ‘CTTGGTCATTAGAGGAAGTAA’ (ITS-1F primer) forward and ‘TCCTCCGCTTATTGATATGC’ (ITS-4 primer) reverse were used for amplification of ITS regions. Polymerase chain reactions (PCR) was performed on a Galaxy XP Thermal Cycler (BOER, PRG) in 20 μl reaction volume. To perform PCR, initial denaturation was done at 95 °C for 10 min followed by 32 cycles of denaturation (94 °C for 1 min), annealing (at 59 °C for 1 min). A final extension was done at 72 °C for 10 min. Amplified PCR was purified using QIA quick gel extraction kit (Qiagen) and was run on gel electrophoresis to confirm the size of amplicon. The PCR product was sequenced using Big Dye terminator (Applied Biosystems, USA) Sanger sequence technology using commercial services of Macrogen, Korea (http://www.macrogen.com/en/main/index.php). The resulting ITS sequence of the strains was analyzed using the BLAST (Basic Local Alignment Search Tool) of National Centre of Biological Information (NCBI) database to identify the strains. Bio Edit software was used for multiple sequence alignment and phylogeny was constructed by Neighbor joining method using MEGA 7 software [19–21]. Sequence of isolated strain (IIUI-201 and IIUI-236) were submitted to database of DNA Data bank of Japan (DDBJ) under following accession numbers MN099077 and MN121269, respectively.

2.4. GCMS analysis of fungal isolates

GC-MS analysis of the extracellular extract of fungal species was carried with fused silica 15 m × 0.2 mm ID × 1 μm of capillary column in Varian 4000 Ion trap GC/MS. Initial temperature of instrument was 110 °C and maintained it for 2 min. After this period, oven temperature was rose up at the rate of an increase of 5 °C min$^{-1}$ to 280 °C and maintained for 9 min. Temperature of injection port of ensured as 250 °C and rate of Helium flow as 1 ml min$^{-1}$. The ionization voltage was kept at 70 eV. The samples were injected as 10.1 in split mode. Mass spectral range was set at 45–450 (m/z). Using computer searches, spectrum obtained through GC-MS compounds present in the extracts were identified. The spectrum of unknown components was
compared with the spectrum of known components in NIST library. The name, molecular formula and molecular weight of samples were ascertained.

2.5. Mycogenic synthesis of CeO$_2$ NPs
Biomass of each fungal isolate was prepared by growing aerobically into 200 ml SDB media in Erlenmeyer flask with the help of wire loop and incubated on orbital shaker at 25 ± 2 °C and agitated at 150 rpm for 96 h. Biomass was harvested and washed with sterile distilled water. To prepare extract, 20 g of fungal biomass was transferred into 200 mL of autoclaved deionized water and again placed on orbital shaker for 96 h agitated at 150 rpm. After sieving this mixture through Whatman filter paper, 3.72 g of Cerium(III) Nitrate was treated with 100 mL of fungal extract. This solution was continuously stirred on hot plate at 80 °C with magnetic stirrer for 6 h. White precipitates were changed to bright yellow color by continuous stirring. Washing of nanoparticles was done with distilled water and ethanol via centrifugation at 2000 rpm. Nanoparticles were kept in furnace at 400 °C for 2 h.

2.6. Characterization of CeO$_2$NPs
Myco-mediated synthesized CeO$_2$ NPs were characterized by XRD, FTIR and SEM with EDX. The crystallinity of nanoparticles was investigated by XRD using GNR X-ray that uses CuK$_\alpha$ radiation ($\lambda = 1.54060$ Å) ranging with 2θ from 10° to 80°. For the analysis of functional groups variations, IRTRACER-100 Fourier Transform Infrared Spectrophotometer was used. KBr pellet technique was used. The wavelength range was 400–4000 cm$^{-1}$. Morphology of nanoparticles was examined by Hitachi UHR FE-SEM SU9000. This microscope was used to capture micrographs of synthesized nanoparticles. Hitachi UHR FE-SEM SU9000 was equipped with XMX1011 Energy-dispersive x-ray spectroscope that identified and quantified elemental compositions of nanoparticles under study.

2.7. Assessment of anti-fungal activity of CeO$_2$NPs
The antifungal activity of CeO$_2$ NPs was examined against Candida albicans using disc diffusion method. Candida albicans was grown in SDB at 37 °C until the suspension reached 1.5 × 10$^8$ CFU ml$^{-1}$ (0.5 Mac Farland standard solution). Molten SDA (20 mL) was added in petridishes and then these were cooled. This suspension was added over the medium. Then four different concentrations of 100, 200, 300 and 600 µg ml$^{-1}$ of CeO$_2$ NPs in DMSO were loaded in disks. The sterile forceps were used to place them over the plates. The incubation was done at 37 °C for 24 h. The zone of inhibition around each disk was measured. Each experiment was repeated three times and the data was presented as average zone of inhibition.

3. Results and discussion

3.1. Identification of fungal strains
The current study was intended to evaluate the fungi isolated from soil and plant sample for their capability for extracellular reduction of Cerium (Ce) ions to form Cerium nanoparticles (CeNPs). The potent fungi were tentatively identified based on the morphological observations such as colony morphology and cellular morphology without staining and Lactophenol Cotton Blue Staining (LPCBS) (figure 1) which were further confirmed by molecular characterization. IUUI-201 strain was identified as Aspergillus terreus whereas IUUI-236 strain was identified as Talaromyces pupurogenus. The nucleotide sequences obtained by 18S rRNA gene sequencing were deposited to NCBI GenBank. Aspergillus terreus accession number was MN099077 and Talaromyces pupurogenus accession number was MN121629. The closest homologues to the sequences were selected and the multiple sequence alignments were carried out. Phylogenetic tree was constructed based on the obtained 18S rRNA sequences using the neighbor-joining method with 1000 bootstrap replications in MEGA7. Figure 2 represents the phylogenetic relationship of fungi with other related strains.

3.2. GCMS analysis of fungal culture filtrates
GCMS analysis indicated the fungal isolates produced various bioactive compounds that are responsible for reduction of CeCl$_3$7H$_2$O salt to CeO$_2$ NPs. Main bioactive compounds found in extract of Aspergillus terreus were ethanol, 1-propanol and tri-chloromethane which indicate alcoholic compounds were the main source of reduction for nanoparticles synthesis. Main compounds found in Talaromyces pupurogenus extract were ethanol, 1-propanol, tri-chloro methane and palmitic acid which means palmitic acid in addition to alcoholic compounds played role in reduction of CeO$_2$ NPs. Many other compounds were found but they were in minor quantities. In the previous studies, it is reported that the proteins and reductases released in the fungal filtrates resulted in the reduction of ions to nanoparticles. Literature supporting the idea that the reduced form (NADH) and NADH dependent reductase enzyme are the possible reasons for the reduction of ions to nanoparticles [22].
3.3. Characterization of CeO₂NPs

3.3.1. XRD of myco-synthesized CeO₂NPs

Structural analysis of myco-mediated CeO₂ NPs was examined by XRD pattern. The presence of clear and intense peaks indicated the pure and cubic fluorite structured nanoparticles (figure 3). CeO₂ nanostructures by *Aspergillus terreus* had the highest intensity peaks seen at 2θ = 28.49° with (111) plane which is the lattice plane and other peaks were at 33.01°, 47.42°, 56.28°, 59.01°, 69.34°, 76.66°, and 79.17° indexed at 200, 220, 311, 222, 400, 331 and 420 planes, respectively. Plane values were closely agreed with JCPDS Card # 89-8436 standard diffraction data [12, 18]. The sizes of particles revealed by XRD were 28.5 nm. CeO₂ nanostructures by *Talaromyces pupureogenus* exhibited the same spectra and particle size was 21.5 nm. But the peaks were slightly broader which indicated the smaller size of particles. These peaks representing the purity and crystallinity of cerium oxide nanoparticles (CeO₂NPs). No extra peak is observed that confirms the purity of sample. These results are concordant to the previous literature [12, 18].

3.3.2. FTIR of myco-synthesized CeO₂NPs

FTIR spectra were analyzed and illustrated for functional groups analysis of CeO₂NPs (figure 4). CeO₂ nanostructures by *Aspergillus terreus* exhibited peaks at 468 cm⁻¹, 550 cm⁻¹ and 833.5 cm⁻¹ which shows the Ce–O stretch [23, 24]. The bands at 2432 and 3425 cm⁻¹ correspond to the vibrational bending of H–O–H and O–H bonds [25, 26]. CeO₂ nanostructures by *Talaromyces pupureogenus* exhibited peaks at 468 cm⁻¹ and 750 cm⁻¹ corresponds to Ce–O and Ce–C–O stretching bands, respectively. The absorption peak observed at 1340 cm⁻¹ confirms mycosynthesis of CeO₂ NPs. The peaks observed at 3425 cm⁻¹ shows presence of OH bond. These findings are well matched with the literature [26–29].

3.3.3. SEM of myco-synthesized CeO₂NPs

Morphological analysis of myco-mediated CeO₂ NPs with variable magnifications was examined by SEM images (figure 5). SEM of CeO₂ nanostructures by *Aspergillus terreus* exhibited spherical shape and average size was estimated to be 26–35 nm. CeO₂ NPs by *Talaromyces pupureogenus* were more homogenously distributed and exhibited a unique nano-sponges form of morphology. The average size was estimated to 20–25 nm. There was size and shape controlled reduction of CeO₂ NPs by *Talaromyces pupureogenus* due to the presence of acids in addition to alcohols as compared to CeO₂NPs by *Aspergillus terreus*. SEM showed the formation of Ce nanosponges with smaller size synthesized by *Talaromyces pupureogenus* as compared to spherical Ce nanoparticles with larger size synthesized by *Aspergillus terreus*. Similar spherical Ce NPs with average size of 24 nm was observed previously [30].

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**Figure 1.** Microscopic study of fungal species having potential to synthesize nanoparticles. Key: *Aspergillus terreus* (a) Colonial morphology on SDA (b) Cellular morphology without staining (c) Cellular morphology after staining with LPCBS image: *Talaromyces pupureogenus* (d) Colonial morphology (e) Cellular morphology without staining (f) Cellular morphology after staining with LPCBS image.
Figure 2. Phylogenetic analysis of both fungal species using MEGA7 software.

Figure 3. XRD patterns of CeO$_2$ nanostructures for both fungus.
3.3.4. EDX of myco-synthesized CeO$_2$NPs
Elemental composition of myco-mediated CeO$_2$ NPs was confirmed by EDX analysis (figure 6). EDX analysis for CeO$_2$ NPs by *Aspergillus terreus* showed the purity of sample by peaks of Cerium (Ce) and Oxygen (O) only. For CeO$_2$ NPs by *Talaromyces pupureogenus* EDX analysis, there were peaks of Ce and O elements. There was no any other element peak which confirmed the purity of nanostructures.

3.4. Assessment of anti-fungal activity of CeO$_2$NPs
In the current research, CeNPs are studied to discover their efficacy as potential anticandidal agents. The anticandidal activity of CeO$_2$NPs was performed against *Candida albicans* taken from human pathogens using
four different concentrations of 100, 200, 300 and 600 μg mL⁻¹ of CeO-1₂ NPs (Figure 7). The CeO₂ NPs by Aspergillus terreus did not show any inhibitory effect of zone formation at 100 μg mL⁻¹ concentration, 10 ± 0.33 mm and 13 ± 0.33 mm zone of clearance was observed with 200 μg mL⁻¹ and 300 μg mL⁻¹ concentration, respectively. Activity was stronger at 600 μg mL⁻¹ concentration as zone of clearance measured to 28 ± 0.33 mm. The inhibition zones with noteworthy effect was shown by CeO₂ NPs using Talaromyces pupureogenus. There was 7 ± 0.33 mm zone of clearance with 100 μg mL⁻¹, 12 ± 0.33 mm at 200 μg mL⁻¹, 16 ± 0.33 mm at 300 μg mL⁻¹ and 31 ± 0.33 mm at 600 μg mL⁻¹ concentrations. Anticandidal activity of Ce nanosponges was much better as compared to spherical Ce nanoparticles which may be due to easily entrapment of candidal cells into nanosponges due to its broader surface area (Figure 8). The nanostructures showed anticandidal activity due to their small sizes and higher surface area. Nanostructures might affect the cell by attacking the cell wall structures and permeabilize into the cell. They inhibit the growth or kill the cells by inhibition of certain pathways such as RNA or protein synthesis inhibition, metabolic pathways inhibition and synthesis of cell wall structures inhibition [23]. Another action of mechanism could be the production of reactive oxygen species (ROS) due to the electrostatic interaction between nanostructures and cell wall of Candida albicans [28] (figure 9). Production of OH radical that is present in the immediate vicinity of lipid membrane could be lethal. ROS generation results in the weakening of lipid membrane of candidal cell and denaturation of membrane permeability occur along the leakage of potassium ions that eventually results in cell apoptosis [30]. The present study explicates that mycosynthesized Ce nanosponges possess exceptional and mesmerizing properties that can be utilized as anticandidal agents against resistant strains.
4. Conclusions

The results of current study lead to the conclusion that two fungi were found to reduce Ce ions to cerium nanosponges and cerium nanoparticles. Myco-mediated synthesis of CeO₂ NPs is a cost effective and green route using *Aspergillus terreus* and *Talaromyces purpureogenus* culture filtrate. This study revealed the formation of CeO₂ NPs cubic fluorite structure with spherical morphology and unusual nano-sponges formed by *Talaromyces purpureogenus* due to presence of acids in extract for the reduction in addition to alcohols. Mycosynthesized Ce nanosponges exhibited potent anticandidal activity as compared to spherical Ce nanoparticles.

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