MYCOBIOSYNTHESIS OF METAL NANOPARTICLES

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ABSTRACT

The extensive applications of metal nanoparticles in the fields of nanomedicine, electronics, cosmetic and food industries along with other areas have intensified the search for eco friendly pathways for their production. Chemical and physical methods have various limitations in terms of synthesis procedures which may involve use of drastic experimental conditions coupled with release of toxic byproducts, thus damaging the environment. Polydispersity and instability of particles add to the disadvantages. An alternative approach is their production by the process of mycosynthesis, using different species of fungi which act as nanobiofactories, since they produce and secrete enzymes which help in reduction of metal ions to nanoparticles. They are further easy to culture, maintain and due to considerable biomass favor the large scale production of a wide variety of metal nanoparticles including those of silver, gold, iron, cadmium, selenium and platinum. Although the mechanistic pathways are yet to be elucidated due to the complexity of the process, the synthesis procedures have been well standardized and in some cases optimised for maximum production. Particles have also been thoroughly characterized by different advanced instrumental techniques. Subsequently, they have been found to possess monodispersity, stability and other favorable properties which have lead to their wide usage and applications in different industries.

Keywords: Fungi, nanobiofactories, nanoparticles, synthesis mechanisms, characterization.

INTRODUCTION

Advances in the field of nanotechnology have resulted in the production of different metal nanoparticles which have found innovative applications in various fields. These particles are ultra small in size, biocompatible, have high surface area to mass ratio, and show considerable surface activity along with plasmon resonance bands.

Metal nanoparticles are currently being used in nanomedicine for precise diagnosis and advanced therapy. An important impact is seen in the field of molecular imaging and therapeutics with respect to cardiovascular diseases encompassing restenosis, atherosclerosis and myocardial infarction. *In vivo* and *ex vivo* detection techniques employing nanoparticles facilitate early detection of these conditions for advisory and intervention programs[1]. A similar perspective applied to cancer nanotechnology is expected to change the very basis of malignant tumor detection and treatment. They are an excellent choice for transporting chemotherapeutics to targeted sites. Increased efficacy of these drugs is a natural consequence as reported in case of pancreatic cancer [2].

Targeted drug delivery techniques adopting nanoparticles are effective in the pharmacotherapy of neurodegenerative disorders like Alzheimers' disease where this extremely successful strategy aids therapeutic moieties to penetrate the CNS very effectively [3]. Antibacterial activity of some metallic nanoparticles reduces the need for antibiotics, to which microbes are gradually getting resistant. It has been reported that silver nanoparticles interact with *E.coli* causing complete cell death, with higher biocidal activity being observed with triangular particles compared to rod shaped and spherical ones [4].

Beyond nanomedicine, the applications of nanoparticles extend to water purification technology where noble metal particles in the nanoscale range are employed successfully for removal of heavy metal particles, micro organisms and pesticides including halogenated organics [5]. Metal oxide nanoparticles like those of molybdenum have found use as renewable source of energy [6]. Some of the period four metal nanoparticles also have antifouling potential[7]. Cosmetic industry extensively uses nano titanium and zinc oxides in its products[8]. Food packaging and preservation techniques currently employ different nanosized materials for extending the shelf life of products and to prevent spoilage of

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edible items[9].The wide spectrum of existing as well as ever growing new applications for engineered nanoparticles warrants their extensive synthesis by various techniques.

Metal nanoparticles have been produced by different chemical methods and some include the use of chemical reductants like hydrazine hydrate[10] and sometimes montmorillonite (MMT) as a solid support, for their preparation at room temperature. Physical methods of synthesis involve chemical vapour deposition, pulsed laser ablation, spark discharge, thermal plasma and flame synthesis, ion sputtering, etc [11].

However biological methods, which make use of fungi, bacteria and plants, are more ecofriendly and are being explored as alternatives to conventional techniques which invariably involve hazardous waste generation. Adopting green nanotechnology for this purpose will be a positive step towards reduction of global warming and also lead to sustainable development. Further, biological routes are easy, usually involve a single step with enhanced energy efficiency and are economical too. The environmental impact is minimal, with the process also being conducive for scaling up for synthesis of large amounts of products, in a controlled manner, to get the particles of uniform shape, size and dispersity based on the desired applications.

Plants such as *Desmodium triflorum* and *Sesuvium portulacastrum L* have been successfully employed for the synthesis of silver nanoparticles which show antimicrobial activity against clinical isolates of bacteria and fungi [12,13]. Green synthesis using ribosomes and also microorganisms including bacteria, fungi, and various yeasts species have been reported [14,15].

Among the biological sources, filamentous fungi produce stable nanoparticles which do not aggregate even upon prolonged storage and are thus characterized by longevity. Also, the enhanced tolerance of fungi towards a higher concentration of metal nanoparticles, which are also are well dispersed in the medium offer additional advantages. Fungi also secrete a number of enzymes and are easy to grow and maintain. There are no requirements for special equipments and the biomass concentration remains much higher than bacteria. *Verticillium* sp, *Cladosporium cladosporioides*, *Trichoderma asperellum*, *Neurospora crassa* and some species of *Aspergillus*, *Penicillium, Fusarium,* etc. have all been exploited for this purpose [16,17,18,]. Synthesis of metal nanoparticles using immobilized fungus remains a cost effective, safe process without requirement of any specific instruments [19]. On the other hand, nanoparticles from *Pseudomonas stutzer* is also capable of synthesizing triangular, hexagonal silver nanoparticles, but extracting and purifying them is more difficult compared to fungi, especially for large scale production [20].

MECHANISTIC BASIS FOR METAL NANOPARTICLE SYNTHESIS BY FUNGI

Fungi can reduce metal ions to nano sized particles by two different mechanisms. The extracellular synthesis method may possibly involve

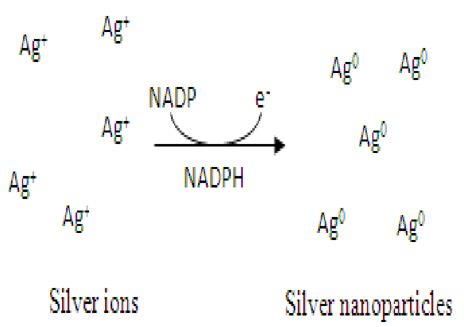

Fig 1: Hypothetical mechanism for extracellular synthesis of silver nanoparticles [22]

the NADPH dependent nitrate reductase enzyme which is secreted by the fungi into the reaction medium. The process of reduction of metal ions to the nano level is accompanied simultaneous conversion of NADPH to $NADP^+$**(Figure 1).** This was observed in case on *Fusarium oxysporum* where during the synthesis of nano metal particles, strong absorption bands corresponding to proteins(260nm), NADPH and hydroxyquinoline are observed along with bands which are obtained for nanoparticles (413nm) [21] . It is hypothesized that the hydroxyquinoline shuttles the electrons generated during the enzymatic reaction, involving the conversion of nitrate to nitrite, to the Ag^+ ions, converting them to Ag^0. Absence of enzyme from the reaction medium leads to conspicuous disappearance of all the bands thus validating the active enzymatic role in the whole process.

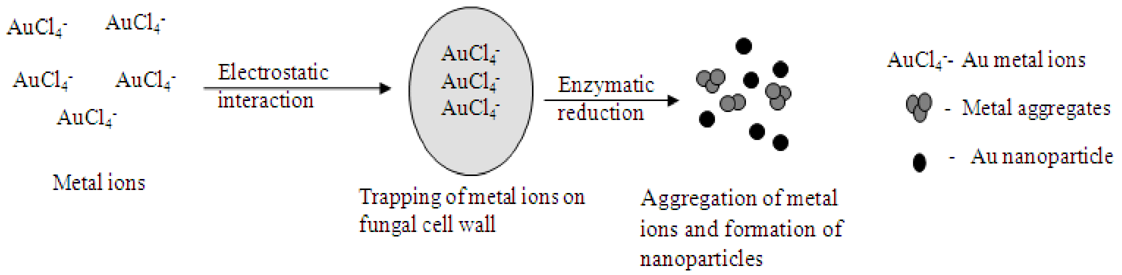

Figure 2: Hypothetical mechanism for intracellular synthesis of silver nanoparticles[22]

The other mechanism involves the intracellular process **(Figure 2)** wherein the fungal cell wall along with sugars present in them play a very important role. The cell wall composition varies during the fungal life cycle and its inner side is associated with a microfibrillar component which is embedded in an amorphous matrix material. The latter usually is made up of water soluble polysaccharides whereas the cell contains chitin and beta linked glucans[23]. The positively charged groups of cell wall enzymes may be responsible for absorbing metals ions from the medium and subsequently these get reduced to nanometals by enzymes in the cell wall. Microscopic studies show aggregates of these particles in the cell wall as well as in the cytoplasmic membrane and cytoplasm. This probably leads to the conclusion that some particles may diffuse through the cell wall and enter the cytoplasm where the reduction takes place with the help of cytoplasmic enzymes also.

CHARACTERISATION TECHNIQUES FOR METAL NANOPARTICLES

The availability of advanced instrumentation techniques for the characterization of metal nanoparticles has proved to be very useful for gaining an insight into various morphological and structural features with respect to their size, shape, crystal structure and degree of aggregation. The stability of the particles can also be ascertained by these techniques. One of the earliest nanoparticles to be synthesized was that of gold from the *Verticillium* sp.and their formation was confirmed through UV-Visible spectroscopy. The pale yellow colour of fungal extract was found to change into vivid purple over a time period of 24 hours[24]. The absorption peak was seen at 540nm which is characteristic of gold nanoparticles. UV-Vis spectroscopy is probably the most widely used technique for detecting the formation and presence of nanoparticles in the fungal medium. The simplicity and reliability with which this technique can be applied is the reason for its wide applicability **(Figure 3)**.

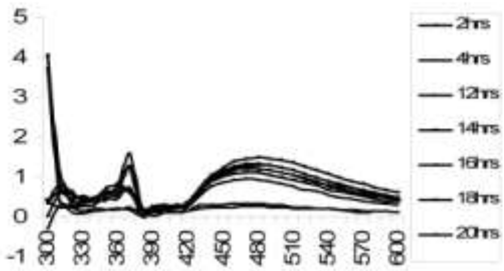

Fig 3: UV-Vis spectra of silver nanoparticles from *Aspergillus* niger

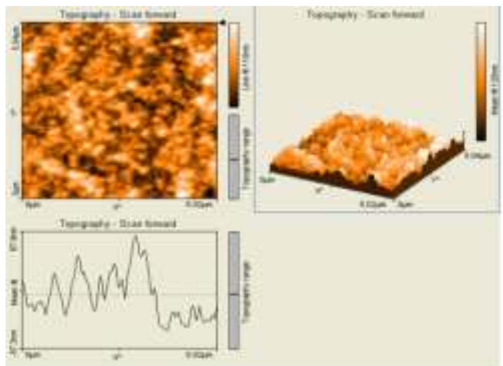

Fig 4: Atomic Force Microscopic image of silver nanoparticles from *Aspergillus* niger

Photo biosynthesised silver nanoparticles from *Fusarium oxysporum* have been observed with a Plasmon peak at 440nm and this corresponds to large silver nanoparticles[25]. Elemental spectroscopy imaging has been adopted as an interesting technique to detect the presence of protein shells around the nanoparticles as these are largely responsible for stabilizing them˙ Microscopic techniques are employed to study the surface morphology and topology of the particles. Atomic Force Microscopy has been used to study the shape and size and also shed light on a 3D topographic view **(Figure 4).**

Transmission Electron Microscopic studies also decipher their size and distinguish between shapes as triangular, spherical, hexagonal, etc. ESEM has been used to study morphology of silver nanoparticles.

Silver nanoparticles synthesized using fungus *F. semitactum* was found to have diffraction signals (111, 200, 220 & 311). This pattern corresponds to the face centred cubic structure of silver and the results were arrived at using X ray diffraction analysis which is another useful technique for deciphering the crystal structures of particles[26] **(Figure 5)**. The mean diameter of particles can be calculated using Scherrer's equation.

rouxii based on sequence analysis, effectively synthesized nanoparticles in its water extract and their average size was confirmed to be 20nm by Atomic Fluorescence Microscopy [29]. Availability of toxicity data for these particles would probably enhance their usage in medical aids.

Mycelia free media of *Bipolaris nodulosa,* a phytopathogenic fungus of nodular group can produce anisotropic silver nanoparticles which show efficient antimicrobial activity against representative organisms of public concern[30].These particles, when conjugated with proteins, can probably catalyze chemical reactions in aqueous solutions and be utilised as biolabelling

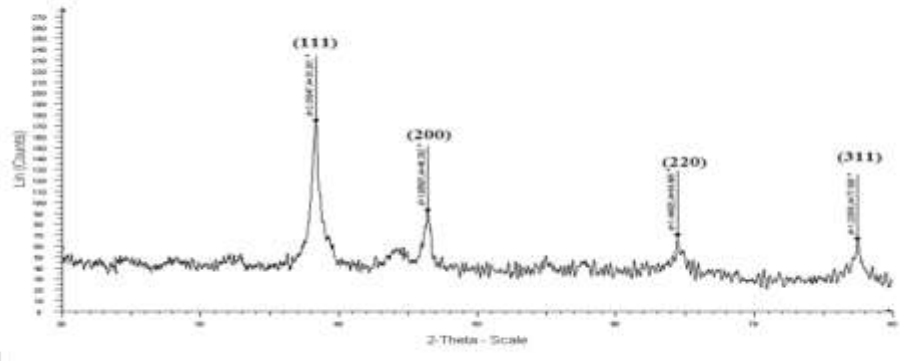

Fig 5: Powder-XRD data of silver nanoparticles in the filtrate of *Aspergillus niger*

FTIR analyses have also been extensively done on nanoparticles to detect presence of stretching and bending vibrations in them and also study the functional groups present in various associated proteins along with their bonds.

SILVER NANOPARTICLES FROM FUNGI

Nano silver is a bludgeoning molecule and has extensive therapeutic applications. Its green synthesis would hence be very useful for biomedical purposes [27]. *Aspergillus fumigatus* has shown a great potential for extracellularly synthesizing silver nanoparticles in the size range of 5-25nm.The formation of fairly monodispered particles is very rapid which makes it suitable for downstream processing [28]. But an understanding of the biochemical and molecular mechanisms involved in this process would be beneficial for achieving better control on the particle size. Spherically shaped monodispersed silver nanoparticles, synthesized from *Amylomyces rouxii* fungal strain KSU-09, showed activity against *Shigella dysenteriae* type I*, Citrobacter* sp., *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans* and *Fusarium oxysporum*. The fungus, which is isolated from date palm root and confirmed as *Amylomyces*

agents. Particles of 10-100nm have been biosynthesized from *Cladosporium cladosporiales* and characterized extensively. In this fungi, it is probably the proteins, which are secreted into the medium, cap the nanoparticles through their free amino groups and prevent them from agglomeration[31]. Particles from *Fusarium semitectum* may also be stabilized by the same process[26]. Fungi from this genus have been thoroughly exploited.

Several strains of *Fusarium oxysporum* can carry out the extracellular production of silver nanoparticles with the help of nitrate dependent reductase enzyme and a shuttle quinone extracellular process[25, 32]. Other *Fusarium* sp. include *Fusarium solani,* a phytopathogenic fungus[33] and *Fusarium acuminatum*[34] isolated from infected ginger. The latter acts against many multidrug resistant and highly pathogenic bacteria especially MRSA. This property may be very beneficial for treatment against this deadly organism.

A previously unexplored fungus *Hormoconis resinae* produces silver nanoparticles within an hour upon treatment with silver nitrate solution. The shape however is not uniform and varies from triangle to spherical and the size from 20-80nm[35]. Better control of synthesis conditions may be needed to achieve more uniformity in shape and size. Non pathogenic filamentous fungus *Neurospora crassa* produces silver nanoparticles intracellularly and extracellularly. This fungus shows a fast growth rate, with the ability to reduce metallic ions rapidly to uniformly sized nanoparticles[15]. Incubating mycelium of a white rot fungus *Phaenerochaete chrysosporium* with silver nitrate solution produces silver nanoparticles extracellularly. Pyramidal shape of the nanoparticles is observed under TEM. The fungus remains non-pathogenic and its easy handling and mass productions gives it an advantage [36].

A marine fungus *Penicillium fellutanum* isolated from sediments of coastal mangrove is able to synthesize nanoparticles under controlled parameters of pH, temperature, silver ion concentration and time of exposure [37]. This fungi is also able to facilitate quick synthesis within few minutes of substrate being added to the culture filtrate. High polydispersity is obtained in compactin producing fungus *Penicilliun brevicompactum* and nanoparticles in size 23-105 nm is obtained as observed under TEM. Protein induced stabilization is observed in this case also [17].

Particles synthesized from *Rhizopus stolonifer* is observed by TEM to be very small and in the size range of 3 to 20nm which may prove advantages for specific applications. This extracellular synthesis method produces nanoparticles in 24 hours. The combined action of these silver nanoparticles and antibiotics shows efficient activity as antibacterials against ESBL strains [38]. A pathogenic fungus of potato plant *Phytopthora infestans,* an edible mushroom *Pleurotus florida,* and *Pleurotus sajor caju* fungi are all capable of producing nano sized particles showing activity against pathogenic organisms [39,40,41].

Various species of *Trichoderma* produces large amounts of extracellular metabolites and enzymes which can catalytically reduce toxic silver ions to non toxic nanoparticles when the mycelium is challenged with silver nitrate solution, for its own survival. This fungus shows higher growth rate in both industrial and laboratory scale[16]. *Trichoderma viride* is reported to behave similarly[42]. Nanoparticles synthesized from non-pathogenic bio control agent *Trichoderma asperellum* is found to be stable even after 6 months of storage. Capping of these particles is probably responsible for their stabilization as confirmed by FTIR and Raman spectroscopy studies[43].

Particles from an endophytic fungus *Aspergillus clavatus* shows spherical and hexagonal shapes with ease of downsteam processing and good antibacterial activity against against *Candida albicans*, *Pseudomonas fluorescens* and *Escherichia coli*. The average minimum inhibitory concentration is reported to be 5.83 µg ml^{-1} with minimum fungicidal concentration of 9.7 µg ml^{-}against *C. albicans*[44].

FUNGAL SYNTHESIS OF GOLD NANOPARTICLES

Gold nanoparticles have many applications in tumor hyperthermia, in scanning tunnelling microscopes as conductive tips and as optical coatings. These particles are produced by both live and dead cells of *Aspergillus oryzae,* which is economically important in the food industry. Trivalent aurum could be reduced to gold nanoparticles which is visually seen by the purple colour formation in the reaction medium[45]. However high polydispersity is observed by TEM studies with their sizes ranging from 10-400nm.

Synthesizing gold nanoparticles from *Candida albicans* were also found to be economical, easy and eco friendly with their amount being inversely proportional to the cytosolic extract of the fungus[46]. The 60-80nm sized antibody conjugated particles can differentiate between normal and cancerous cells. This could be extremely useful in cancer therapy and treatment. Gold nanoparticles from *Cylindrocladium floridanum* were able to catalyse the degradation of 4 nitrophenol, a toxic organic pollutant, to 4 amino phenol.. Recycling and recovery of these particles is also thus made possible [47].

In the process of waste treatment, large amount of biomass of non conventional yeast *Yarrowia lipolytica* is generated and this has been utilized for the generation of gold nanoparticles, as biological properties, required for interactions with metal interactions, has been shown by this fungus[48]. It is interesting to note that synthesis takes place in a pH dependent manner in both sea water and fresh water. The mechanism underlying the process is not clearly defined but presence of inherent proteases and reductases may probably be involved.

Extremely stable gold nanoparticles, synthesized from *Trichoderma viride,* when bound with vancomycin is reported to have antibacterial activity against vancomycin resistant and vancomycin sensitive *Staphylococcus aureus*[49]. The results prove that binding of vancomycin to the nanoparticles probably destroys the pathogenic bacteria by an alternative mechanism than the one adopted by vancomycin alone. The gold nanoparticles produced by fungal culture filtrate of *Alterneria alternate* average only a size of 12 nm and are very stable [50]. An excellent control of the synthesis parameters would probably result in such small particles which may find many uses in nanomedicine for targeted delivery of drugs.

In another interesting study laccase, purified from *Paraconiothyrium variabile* is used to reduce $HAuCl_4$[51]. Formation of particles increased with decreasing activity of laccase inferring that exposure to reducing functional groups may be responsible for particle formation. Gold nanotriangles can be extensively used in optics, biomedicine and electronics and these have been synthesised from endophytic fungus *Aspergillus clavatus* intracellularly when reacted with chloroaurate ions which may find have potential applications in these areas [52].

The presence of additional proteins in the system probably favours nanoparticle synthesis as seen in the case of extracts from naturally occurring edible mushroom *Volvariella volvacea* which synthesizes gold nanoparticles along with silver and gold-silver ones of 20-150 nm. The productivity enhancement is a positive factor and using mushrooms is more economical too[53].

Size controlled nanoparticles can also be produced from *Penicillium* sp. where the separation of nanoparticles could be easily achieved from the fungal cell lysate by using the ultrasonication and centrifugation techniques[54]. A similar synthesis is also seen for *Aspergillus niger* where the interaction of gold ions with the supernatant of the fungus lead to the production of gold nanoparticles [55]. The extracellular enzyme responsible for the reduction process may also cap the particles and prevent them from aggregation.

MYCO PRODUCTION OF NANO CADMIUM

The green route has been adopted for the synthesis of Cadmium nanoparticles also. Cadmium sulphide particles are of considerable interest due to their potential applications in solar cells, photoelectric devices and diodes. *Coriolus versicolor*, immobilized on a ceramic bead column has been shown to reduce cadmium to nanoparticles which were also quite stable without the addition of any stabilizers. SH containing proteins, released by the fungus, may probably be involved in the production of these particles[56]. Two different types of yeasts *Schizosaccharomyces pombe* and *Candida glabrata* were able to accumulate cadmium metal when cultivated in a metal rich batch fed process [57]. As the metal is toxic to cells, detoxification by an intracellular mechanism is adopted by them during which the metal is reduced to CdS nanoparticles of 35kDa. This could probably be adopted as a bioremediation strategy.

Q-state cadmium sulphide nanoparticle production has been reported from *Fusarium oxysporum.* The enzyme sulfate reductase from the fungus is shown to mediate the synthesis of nanoparticles and this enzymatic pathway can be utilized for the synthesis over a wide range of nanoparticles [58].

FUNGAL PRODUCTION OF IRON NANOPARTICLES

Microbial synthesis of iron based nanomaterials has been reviewed[59]. Fungal mediated biosynthesis of iron nanoparticles by *Pleurotus* sp. is possible where the presence of siderophores account for transfer of iron particles into the cell from the medium to facilitate the intracellular reduction of iron to nano levels[60]. Magnetite nanoparticles, produced by a combination of ferrous and ferric salts, using *Fusarium oxysporum and Verticillium* sp, is also a possibility where the extracellular synthesis leads to the formation of crystalline magnetite particles[61]. Iron phosphate nanoparticles are reported to be obtained from yeast cells. Precipitation method, when applied to them, provided nucleation sites where nanoparticle formation is regulated. This method remains cost effective and a rapid way nanoparticle production [62].

PLATINUM, ZIRCONIA AND SELENIUM NANOPARTICLES FROM FUNGI

A mechanism for the bioreduction of platinic acid and platinum chloride by *Fusarium oxysporum* has been proposed for the synthesis of platinum nanoparticles[63]. Two steps of two electron transfer leads to the production of these particles, where in the first step, platonic acid is converted to platinum chloride and then reduced to nano platinum in the second step. Nano platinum finds extensive use in the field of cancer diagnostics and therapy.

Challenging *Fusarium oxysporum* with zirconium fluoride anions leads to protein mediated hydrolysis of these anions and the subsequent synthesis of nanocrystalline zirconia [64]. It is probable that certain cationic proteins may be responsible for this process. Nano alpha selenium particles in the size range of 30-150nm can be produced from the culture filtrate of *Alternaria alternate* by the bioreduction of sodium selenate[65]. The presence of protein shell surrounding them is found to stabilise the particles.

CONCLUSION

Various microbial strains have been evaluated for their ability to produce metal nanoparticles. Silver, gold, cadmium, platinum, iron and zirconium particles have been synthesized by utilizing them, which provides a green pathway for their production. There is no involvement of toxic chemicals, and drastic experimental conditions are also avoided. The process is further eco friendly as there is no release of toxic byproducts into the environment. The mechanism of synthesis has however not been clearly established but the probability of microbial secretary enzymes, *viz.,* NADPH reductase being involved, is highlighted in several reports. The variations in the metabolic pathway of different fungal species further impede the unraveling of the synthesis routes. Hence identification of the active species involved in the process of nucleation and growth of these nanoparticles becomes a complex activity and requires further research. But there is no doubt in the fact that the different metal nanoparticles synthesized using green chemistry find extensive applications in various fields.

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CONFLICT OF INTEREST

Nil

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