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Decolorization of Methylene Blue using Silver Nanoparticles Synthesized from Endophytic Fungus

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Silver nanoparticles (AgNPs) have been synthesized and characterized in the current study, and evaluated in terms of their use for the decolorization of methylene blue dye. AgNPs were successfully prepared using a green chemistry process from isolated endophytic fungus. Nine fungal endophytes were isolated from the leaves of *Ocimum balilicum*L. The four major isolates of *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., and *Alternaria* sp. were tested for the extracellular synthesis of AgNPs. The formation of AgNPs was initially observed as a change in color and was subsequently confirmed by Ultraviolet-visible spectroscopy, which showed a characteristic absorption peak for silver at 420 nm. *Aspergillus* sp. was determined to be the most potent producer of AgNPs and was subjected to further characterization. Transmission electron microscopy studies showed that the AgNPs were 4–15 nm in size. X-ray diffraction analysis revealed the crystalline pattern of the AgNPs. Based on the sequences of the ribosomal DNA, internal transcribed spacer regions, the major endophytic species was identified as *Aspergillus niger* and the sequence data were submitted to the GenBank [GenBank: LC009511.1]. Further analysis showed that AgNPs efficiently decolorized methylene blue dye up to 96% within 72 h of incubation. AgNPs could therefore be used as highly economical agents for the rapid removal of dye-based pollutants from the environment and could also be used for the control of other reducible contaminants.

Key words: Endopytic fungi; Silver nanoparticles; Biosynthesis; Dye decolorization.

The field of nanotechnology is one of the most active research areas in modern materials science. Nanoparticles exhibit new or improved properties based on specific characteristics such as size, distribution, and morphology. There have been numerous impressive developments in the field of nanotechnology during the course of the last decade, and several new methods have been developed for the synthesis of nanoparticles with specific size and shape characteristics depending on their requirements. Furthermore, the number of potential new applications for nanoparticles and nanomaterials continues to increasing rapidly. Nanoparticles have a high surface area, with a high fraction of surface atoms[1] and serve as the fundamental building blocks of nanotechnology[2] because of their extensive range of applications across numerous areas of research[3], including optics, electronics[4], bioimaging, sensors, and diagnostics, as well as the development of novel therapeutic agents in biomedical research[5]. Metallic silver and silver nanoparticles (AgNPs) have been used as antimicrobial agents in various products, including cosmetics[6], animal feed[7], the coating used for catheters[8], wound dressings[9], anti-dimorphic materials[10], and water purification systems[11] because they pose a minimal risk of toxicity to humans. The use of biological systems

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for the synthesis and assembly of nanoscale materials has grown in popularity during the course of the last decade as a viable alternative to conventional physical and chemical processes. Green chemistry approaches for the synthesis of nanoparticles using biological entities have several significant advantages over conventional approaches because they are environmental benign, safe, cost effective, and less toxic and time consuming. Furthermore, reactions of this type can be conducted as single-step processes[12] and the reducing agents and stabilizers typically involved in the synthesis of AgNPs can be replaced by molecules produced by organisms such as bacteria, fungi, yeasts, algae, and plants[13,14]. Fungi are useful for the synthesis of metal nanoparticles, and the use of fungi in this regard is potentially exciting because they secrete large amounts of enzymes and their biomass is easy to manage[15]. Most of the nanoparticles generated in this way can be used in chemical reactions for the degradation of organic compounds[16,17,18,19]. The degradation of effluents containing dyes represents a significant problem for several industries[16]. Continuous exposure to dyes can have an adverse influence on anaerobic biomass and cause irritation to the respiratory and gastrointestinal tract. With this in mind, the development of physical and chemical treatment processes for the removal of dyes from waste water systems is very important[20]. Dyes are used in a variety of different industries, including the pigmentation of cosmetics, textiles, paper, ceramics, leather, inks, and food-processing products, where most of the materials are derived from azo dyes. About 15% of the dyes used in these industries are discharged in the natural water sources, such as streams and rivers, and this waste represents a significant hazard to humans and the environment because of the toxic nature of these dyes[21]. Numerous techniques have been developed for the removal of dyes from water, including adsorption[22], nanofiltration[23], and biological treatment processes[24]. Among the many different types of dye, methyl green is particularly toxic[25]. Methyl green is a heterocyclic aromatic compound that is also cationic in nature[26], and the triphenylmethylene functional group of this dye represents a significant pollution hazard to natural water sources[20]. Research towards the use of heterogeneous catalysts involving metals and metal nanoparticles for the treatment of waste water has yielded several successful results[27,28]. The aim of the current study was to synthesize AgNPs using endophytic fungi and evaluate these nanoparticles in terms of their dye degrading abilities.

MATERIALS AND METHODS

Isolation of endophytic fungi

Endophytic fungi were isolated according to the method described by Petrini *et al*. (1986)[29]. Random samples of the leaves were taken from the *Ocimum balilicum* L plant. Prior to surface sterilization, the leaves were gently rinsed in running water to remove any dust particles. After being washed, the leaves were cut into small pieces (0.5 × 0.5 mm) using a flame-sterilized cork borer both with and without the midrib under aseptic conditions. Surface sterilization was achieved using a 10% sodium hypochlorite (NaOCl) solution. The plant material was initially immersed in 70% ethanol for 3-5 min followed sequentially by immersion in 10% sodium hypochlorite and 70% ethanol for 30 s each. The segments were then rinsed three times with sterile distilled water and blotted on sterile blotting paper. The efficiency of the surface sterilization procedure was evaluated for all of the tissue segments according to the imprint method described by Schulz *et al*. (1993)[30]. Five segments were placed on potato dextrose agar supplemented with 50 mg l^{-1} ampicillin and the dishes were sealed with parafilm and incubated at 28±2 °C for 3 weeks in the absence of light. The fungi growing out of the plant segments were purified and identified according to their macro and microscopic structures[31,32,33]. Endophytic fungi displaying the highest level of AgNP production were identified based on the analysis of the nucleotide sequences of the internal transcribed spacer (ITS) regions of their rDNA. The methods and reagents used for DNA extraction and the polymerase chain reaction (PCR) amplification of the ITS regions have been described by Sambrook and Russell (2001)[34]. PCR amplification was conducted using ITS1 and ITS4 primers, and the resulting sequence was used as a query sequence to search for similar sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). These sequence data were also submitted to the GenBank.

Biosynthesis and characterization of AgNPs

The fungal biomasses used for the biosynthetic experiments were grown aerobically in a liquid growth medium containing 5.0 g L^{-1} malt extract and 10.0 g L^{-1} glucose[35]. Flasks were inoculated with culture and incubated in an orbital shaker (100 rpm) for 72 h at 25 °C. The resulting fungal biomass was harvested using plastic sieves and then washed extensively with distilled water to remove any residual medium. The fresh clean biomasses were then weighed and incubated in 200 mL of MilliQ-purified water on a shaker (100 rpm) for 72 h at 25 °C. The resulting biomass was then filtered (Whatmanfilter paper No. 1) and the cell-free filtrate was collected to be used for further experiments. Fifty milliliters of the cell-free filtrate was placed in a 250-mL conical flask followed by 8.4 mg of silver nitrate ($AgNO_3$) to give a 1 mM solution, and the resulting mixture was agitated in a shaker (150 rpm) at 25 °C in the absence of light. A control experiment was also conducted at the same time under the same conditions but without $AgNO_3$ (i.e., just the cell-free filtrate). A negative control experiment using only $AgNO_3$ in water under the same conditions as those described above was also conducted. The formation of nanoparticles in these experiments was confirmed by Ultraviolet-visible (UV-Vis) analysis using a UV-Vis spectrophotometer (Optizen 2120 UV; Mecasys, Korea). All of the spectra were recorded at wavelengths in the range of 300–700 nm.

X-ray diffraction (XRD) analyses of the freeze-dried AgNP powders were conducted at 25Øß values in the range of 20° to 80° (Philips PW 1830) using Cu K±radiation (» = 0.15408 nm) at 30 mA and 45 kV. Samples for analysis by transmission electron microscopy (TEM) were prepared as follows. The samples were sonicated (Vibronics VS 80) for 5 min. AgNPs were loaded onto carbon-coated copper grids and the solvent was allowed to evaporate under infrared light for 30 min. TEM images were recorded on a Phillips model CM 20 instrument, which was operated at an accelerating voltage of 200 kV, and the shapes and sizes of the AgNPs were fully characterized.

Dye decolorization properties of silver nanoparticles derived from endophytic fungi

In a typical experiment, 10 mg of methylene blue dye was added to 1000 mL of double distilled water, and the resulting mixture was used as a stock solution. Different concentrations of the biosynthesized AgNPs (i.e., 25, 50, and 100 µg) were then added to 100 mL of the methylene blue dye stock solution, and the resulting mixtures were incubated at 30 °C for 72 h. A blank experiment was also conducted under the same conditions except no AgNPs were added to the stock solution. The concentration of dye during the degradation was calculated by the absorbance value of the solution at 660 nm. The percentage of dye degradation in each experiment was estimated by the following formula:

$$\text{Decolorization (\%)} = (C_0 - C)/C_0 \times 100,$$

where C_0 is the initial concentration of the dye solution and C is the concentration of the dye solution after degradation.

RESULTS AND DISCUSSION

Isolation of endophytic fungal strain

Nine endophytic fungal isolates were obtained in the current study from *Ocimum balilicum* (Table 1) and characterized according to their culture characteristics, including their colony growth and conidia morphology. The endophytic fungi were subsequently evaluated in terms of their AgNPs production properties and the best fungi were identified using molecular techniques.

The results of these experiments revealed *Aspergillus* sp., *Penicillium* sp. *Cladosporium* sp., and *Alternaria* sp. were the four dominant isolates, and these fungi were subsequently evaluated as cell factories for the production of AgNPs.

Biosynthesis and characterization of AgNPs

Fungi are extremely good candidates for the synthesis of metal nanoparticles. In this study, AgNPs were synthesized via the reduction of aqueous Ag^+ ions within the cell-free extracts of four endophytic fungal isolates at 25 °C. When $AgNO_3$ was incubated in the cell-free endophytic fungal isolate, the mixture became dark brown in color, whereas the negative control flasks containing only water and $AgNO_3$ remain unchanged. UV-Vis spectroscopy has been widely used as an analytical technique for the structural characterization of AgNPs[18, 36]. The absorption spectra of the pale yellow-brown silver colloids generated following the incubation of $AgNO_3$ in

the cell-free endophytic fungal isolates for 72 h revealed that the nanoparticles exhibited surface plasmon resonance (SPR)[37,38,39]. It has been reported that SPR shifts to longer wavelengths as the particle size increases[40]. The UV-Vis spectrum of the particles generated in the presence of the cell-free extract of *Aspergillus* sp. contained a strong SPR peak at about 420 nm, which is characteristic of colloidal silver. Taken together, these results revealed that *Aspergillus* sp. was the best producer for AgNPs after 72 h of incubation, whereas *Alternaria* sp. gave the poorest results of the four isolates tested (Figure 1). These results were comparable to those reported by Huang *et al*. (2007)[41], Verma *et al*. (2010)[42], Yehia and Al-Sheikh (2014)[10], who reported SPR peaks around 450 nm in the UV-Vis spectra of AgNPs prepared from *Pleurotus ostreatus*. The role of nitrate reductases derived from microorganisms in the biosynthesis of AgNPs has been discussed previously elsewhere[44,45,10]. In this study, *Aspergillus* sp. was identified as a potent producer of AgNPs, and the AgNPs produced by this fungus were therefore selected for further experiments.

TEM analysis was conducted to provide further insight into the size and shape of the AgNPs generated by *Aspergillus* sp. A TEM image of the AgNPs placed on a carbon -coated copper TEM grid is shown in Figure 2. AgNPs consisting of polydispersed nanospheres with a spherical shape could be used as highly active catalysts in a range of different applications. Most of the nanoparticles shown in the micrograph of the AgNPs generated by *Aspergillus* sp. were 4–15 nm in diameter.

The AgNPs produced by *Aspergillus* sp. were subjected to XRD analysis to determine their crystalline size (Figure 3). The results of this analysis revealed that the AgNP crystals were face centered cubic (FCC) in structure, which was consistent with previous results from the literature[46,47]. A number of Bragg reflections with 25Øß values of 32.6, 38.8, and 55.06° were observed, which were assigned to the (1 1 1), (2 0 0), and (2 2 0) sets of the lattice planes of the FCC structures of silver. These XRD patterns therefore confirmed that the AgNPs synthesized by *Aspergillus* sp. were crystalline in nature[48].

The *Aspergillus* sp. responsible for the efficient production of AgNPs was identified based on its morphological characteristics with the help of an identification key, and classified as a species of *Aspergillus niger*. Furthermore, 18 S rRNA gene sequencing was used to confirm the identity of the fungus. Sequence data for the nucleotides were obtained by DNA sequencing experiments and submitted to the GenBank [GenBank: LC009511.1]. After BLAST analysis, specific amplicons showed 99% identity with *Aspergillus niger* [GenBank:EF661058.1], *Aspergillus niger* [GenBank: AM270260.1], and *Aspergillus niger* [GenBank: EF661059.1].

Decolorization studies

The activity of the AgNPs synthesized by *Aspergillus* sp towards the degradation of dye was evaluated using methylene blue dye. The degradation of methylene blue was carried out in the presence of different concentrations of the AgNPs over different periods of time. The percentage decolorization efficiency of the AgNPs was determined to be 96% at 72 h (Table 2). Notably, the decolorization efficiency increased as the concentration of the AgNPs increased. These

Table 1. Fungal endophytes isolated from the leaves of *Ocimum balilicum*

Species	Isolates
Aspergillus sp.	34
Cladosporium sp.	29
Alternaria sp.	22
Penicillium sp.	24
Fusarium sp.	16
Epicoccum sp.	2
Arthrinium sp.	1
Curvularia sp.	1
Acremonium sp.	1

Table 2. Percentage decolorization of methylene blue by different concentrations of AgNPs at different time intervals

AgNPs conc.(µM)	% of methylene blue decolorization		
	24 h	48 h	72 h
25	15±0.15	29±0.47	33±0.19
50	30±0.25	38±0.50	48±0.10
100	58±0.27	82±0.18	96±0.30

±Standard Error.

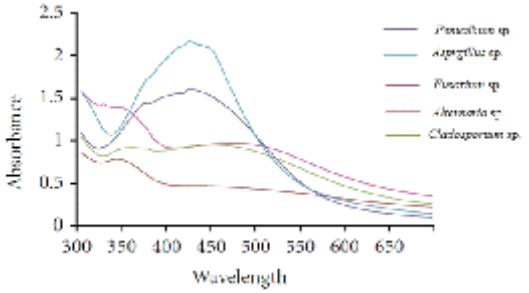

Fig. 1. UV-Vis absorption spectra of the AgNPs produced by different endophytic fungi after 72 h

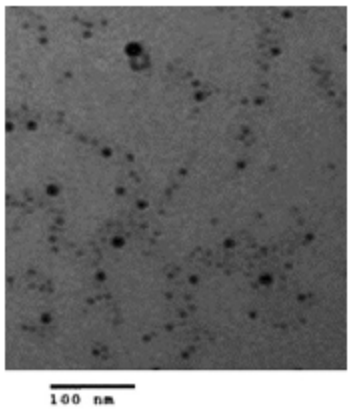

Fig. 2. TEM micrograph showing silver nanoparticles of different sizes

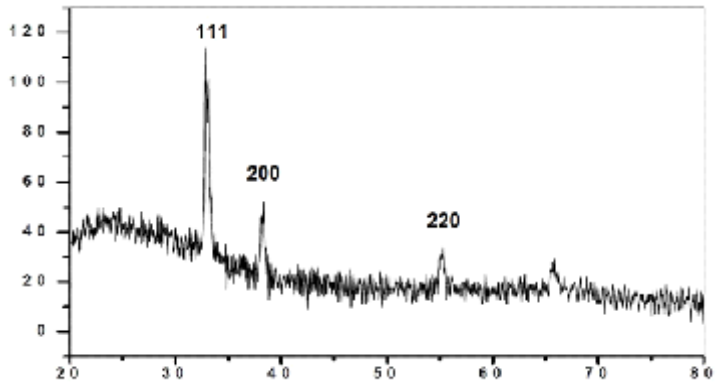

Fig. 3. XRD pattern of AgNPs synthesized by *Aspergillus* sp

results therefore suggest that the AgNPs synthesized in the current study could be used as efficient catalysts for the degradation of methylene blue dye. Furthermore, this study provides an economical solution for the removal of dyes from polluted water systems that could also be used to remove several other pollutants.

CONCLUSION

The biosynthesis of AgNPs using endophytic fungi is an eco-friendly, low cost, and efficient process for the production of AgNPs. In this study, AgNPs were synthesized using *Aspergillus niger* and subsequently characterized by UV-Vis spectrometry, TEM, and XRD analyses. The AgNPs were also evaluated in terms of activity towards the degradation of methylene blue dye. The results of this study show that the AgNPs synthesized from *A. niger* exhibited excellent activity towards the degradation of methylene blue dye molecules and could therefore be used in water purification systems and for the removal of dyes during effluent treatment processes.

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