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Mycogenesis of silver nanoparticles by different *Aspergillus species*

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Abstract. The bio-synthesis of nanomaterials is emerging as an innovative methodology, which is comparatively eco-friendly and inexpensive. Among different microbes, the role of fungi has been proved considerably promising in the in-vitro synthesis of nanomaterials. In this study, the comparative efficacy of four different species of *Aspergillus (A. fumigatus, A. niger, A. flavus* and *A. terreus)* was investigated for the synthesis of silver nanoparticles (AgNPs). Initially, the synthesis was monitored through changes in coloration (yellow to dark brown) of the reaction solution containing $AgNO_3$ reacted with the fungal biomass, of each fungi for 96 hours (hr) at 28°C. The UV-visible spectra of the reaction mixture taken at different times showed a gradual change in absorbance between 400-420 nm, corresponding to changes in the surface plasmon resonance of the Ag metal. Comparatively, *A. fumigatus* showed a higher rate of nanoparticle synthesis than the other fungi. X-Ray Diffraction (XRD) spectra showed peaks of various intensities, with respect to the angle of diffraction (2θ), thus, revealing the crystalline nature of AgNPs. Nanoparticles fabricated through *A. fumigatus* (5-18 nm) and *A. flavus* (13-26 nm) exhibited more drift towards monodispersity, which was relatively higher (6-70 nm) than in the other two fungi. Transmission Electron Microscopy (TEM) further confirmed the configuration of AgNPs in the range of 3-80 nm.

1. Introduction

The growing area of nanoscience has been viewed with great interest owing to its implications in disciplines of electronics, biomedical science, energy, transportation and the construction industry [1], and investigations in this area have been noticeably increasing globally in recent years. Metallic nanoparticles exhibit distinctive and considerably unusual physico-chemical and biological properties compared to their macro scaled counterparts. These properties of nanoparticles have been specifically associated with their high surface-to-volume ratio [2]. Metallic nanoparticles can be used for the development of biosensors (e.g., DNA labels), and in the precise transmission of drugs/medicine to target organs in an organism. Currently, the synthesis and application of nanoparticles has been the subject of research in scientific circles [1,2]. Advancements in the manufacturing of new materials with nanometer dimensions, like nanorods, nanowires, nanoparticles, and nanotubes, etc., are the prime areas of focus [1,3].

Generally, production of metal nanoparticles has been achieved through different methods. Among these, chemical synthesis routes are the most common. However, these protocols cannot avoid the use of certain chemicals that have become considerably more toxic to workers and associated aquatic and terrestrial environments [3]. Presently, there is a growing need to develop safe processes of nanoparticle synthesis that avoid any hazardous implications to

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the corresponding environments. A striking opportunity for green nanotechnology is to utilize microbial resources for the synthesis of nanoparticles [4]. The utilization of microbial technology in the bioremoval of perilous metals by reduction is not so uncommon, but nanomaterial synthesis using microorganisms, especially fungi, has recently been focused upon [5].

With growing interest in the aforesaid discipline, scientists have explored the abilities of certain microbes, including cyanobacteria, actinomycetes, bacteria, yeast and fungi, that help in the reduction of certain metal ions towards the formation of metallic nanoparticles [4-11]. However, the detailed mechanisms of nanoparticle biosynthesis are not fully understood [5]. Comparatively, bacteria and fungi have gained more interest compared to other microbes, due to their already established physiology and metabolism, and a marginal understanding related to the synthesis of nanoparticles [7-9]. Various reports have mentioned that the types of microbe and bioprocess used significantly affect nanoparticle synthesis [2,9,10]. Filamentous fungi are found to be an excellent candidate for the synthesis of metal nanoparticles of elements like Au, Ag, Pt and Pd [4,5,7,8,9,12]. They can be easily handled, cultivated at laboratory scale and are relatively less prone to metal toxicity [12]. The methodology involved in the extracellular synthesis of metal nanoparticles by microbes, especially fungi, has proved to be enormously low cost with reduced time consumption, hence, offering a superior option in the future [1,2].

Compared to other metal nanoparticles, AgNPs have gained much importance as sensors, antimicrobial agents, bio-labels and in bioremediation [1,3]. Preliminary results (data not shown) indicated *Aspergillus species* as a promising candidate for this purpose. Therefore, the current work highlights and compares the relative efficacy of different *Aspergillus* species, i.e., *A. fumigatus, A. niger, A. flavus* and *A. terreus*, in the extracellular biosynthesis of AgNPs. The study reveals the detailed kinetics and characterization of AgNP synthesis using techniques like UV-visible spectroscopy, X-ray diffraction and Transmission Electron Microscopy.

2. Materials and methods

2.1. Fungi used in the study

Four *Aspergillus sp.*, including *Aspergillus fumigatus, A. niger, A. flavus* and *A. terreus*, were obtained from Microbiology Research Laboratory (MRL), at Quaid-i-Azam University, Islamabad, and maintained on Potato Dextrose Agar slants. Stock cultures of the fungi were maintained by sub culturing them twice a month in Sabouraud Dextrose Broth.

2.2. Experimental setup for the synthesis of silver nanoparticles using different Aspergillus species

2.2.1. Media composition

The fungal biomass was grown aerobically in a liquid media (MGYP medium) containing (g/100 ml) malt extract, 0.3; glucose, 1.0; yeast extract, 0.3 and peptone 0.5 (pH: 5.8).

2.2.2. Cultivation of fungal biomass

The flasks were inoculated and then incubated on an orbital shaker at 28°C and agitated at 150 rpm. The biomass was collected after 6-7 days of growth by filtering through a filter paper, followed by extensive washing with distilled water to remove any medium component from the biomass.

2.2.3. Assay for the synthesis of silver nanoparticles

Specifically, 20 g of biomass (fresh weight) came into contact with 200 ml of precursor salt solution, i.e. $AgNO_3$, 0.1 M final concentration in a 500-ml Erlenmeyer flask and agitated at 28°C in dark for 96 hr. The reaction was carried out with Milli-Q water. Positive control (only fungal biomass in deionized water, without the silver ion) and negative control (only 0.1 M $AgNO_3$ solution) were also run along with the experimental flasks.

2.3. Analytical tools

Spectroscopy. About 1 ml of the sample was continuously withdrawn at different time intervals and the absorbance was measured between 200-800 nm wavelengths using a UV-visible spectrophotometer (Agilent 8453). After 96 hr of incubation, the filtrate was obtained by passing the reaction mixture through Whatman filter paper no. 1.

Colloidal suspension containing silver (filtrate) was first concentrated by time and, again, centrifugation using a high speed centrifuge (Kokusan Model H-251, Japan) at 12,000 rpm for 20 minutes. After concentrating, the silver colloidal suspension was extensively washed out three times using sterile deionized water, then ethanol, and centrifuged using a microcentrifuge (Beckman coulter^{TM} Microcentrifuge 18 centrifuge, Germany).

XRD. The silver colloidal suspension was dried and sent for X-Ray Diffraction (XRD) analysis. A drop-coated film of dried sample containing nanoparticles on silica were subjected to XRD analysis using PANalytical X'pert PRO XRD, Netherlands, operating in transmission mode at 30 kV, 20 mA, with Cu Kα radiation. The diffracted intensities were recorded from $10'$ to $80'$ of 2 θ angles.

Scherrer method. From this study, the average particle size was estimated using the Debye-Scherrer

formula:

$$D = 0.9\lambda/\beta\cos\theta,$$

where λ is the wave length of X-Ray (0.1541 nm), β is FWHM (Full Width at Half Maximum), θ is the diffraction angle, and D is particle diameter (size) (Table 1) [13].

TEM. Finally, the dried silver nanopowder was also sent for morphological analysis using Transmission Electron Microscopy (TEM). The AgNPs film was formed on carbon-coated copper TEM grids and analyzed by transmission electron microscopy (JEOL JEM-1010, Japan) at an accelerating voltage of 80 kV.

3. Results

The extracellular biosynthesis of AgNPs was carried out by exposing Ag salt solution to six-day old biomasses of four *Aspergillus* species, including *A. fumigatus*, *A. niger*, *A. terreus* and *A. flavus* for 96 hr at 28°C under agitated conditions. During incubation, the Ag salt solution showed a steady change in color intensity from yellow to dark brown. However, it varied with different fungi and was comparatively higher with *A. fumigatus*. The Ag solution in each flask remained as hydrosol and no precipitation occurred even after 96 hr of incubation. Controls (positive control; fungal biomass solution in sterile de-ionized water without Ag salt solution, and negative control; sterile de-ionized

Table 1. Diffraction angle, d spacing, FWHM, range and mean sizes of AgNP corresponding to different fungi.

Organisms	Diffraction angle (2θ)	d-spacing (nm)	Delta (FWHM) (radians)	Range of particle size (nm)	Mean particle size of NPs (nm)
A. fumigatus	38.1947	0.235635	0.001717	5-18	10 ± 6
	44.4224	0.20394	0.006869		
	64.6052	0.144265	0.005496		
	77.522	0.123037	0.010052		
A. niger	16.9062	0.524448	0.004122	9-70	31 ± 15.4
	26.1657	0.3058	0.006182		
	28.5336	0.312832	0.003606		
	29.4731	0.303072	0.003091		
	32.0445	0.279314	0.002061		
	36.2137	0.248057	0.008243		
	38.5022	0.233824	0.003091		
	39.1915	0.229868	0.004122		
	41.2037	0.219096	0.016485		
	44.681	0.202819	0.006182		
	46.6416	0.194741	0.012365		
	51.2706	0.178193	0.005152		
	53.0531	0.172619	0.006182		
	59.6641	0.154847	0.010052		
A. flavus	38.3144	0.234926	0.005667	13-26	12 ± 5.6
	44.5224	0.203505	0.011333		
	64.6885	0.144099	0.007213		
	77.5709	0.122971	0.007539		
A. terreus	28.1077	0.317475	0.024729	6-47	21 ± 13
	32.5924	0.274743	0.012365		
	38.423	0.23428	0.003091		
	44.7145	0.202675	0.012365		
	46.5794	0.194986	0.012365		
	64.7685	0.14394	0.004122		
	77.7275	0.122763	0.012565		

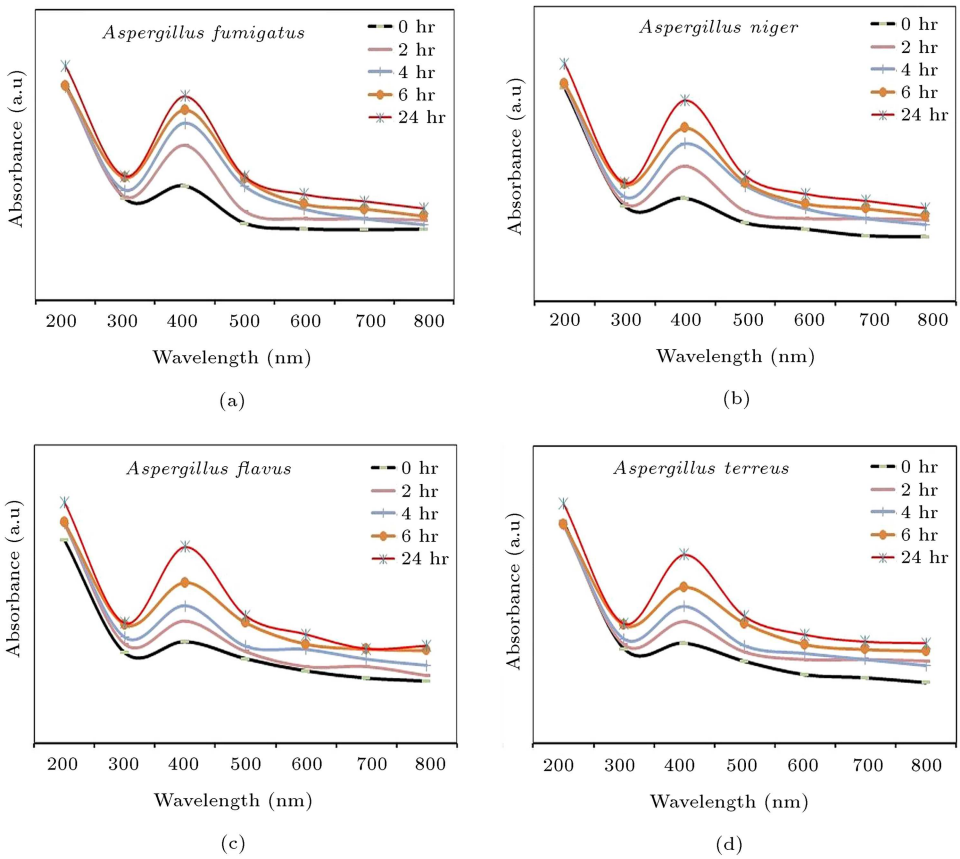

Figure 1. UV-visible spectra of aqueous medium containing reaction mixtures of silver salt (0.1 M) and a) *A. fumigatus*, b) *A. niger*, c) *A. flavus*, and d) *A. terreus*.

water with only Ag salt) showed no change in color in the Ag salt solution when incubated under the same experimental conditions. At the second stage, the biosynthesis of AgNPs was monitored through UV-Vis spectroscopy by scanning the reaction solutions in the range 200-800 nm. The reaction mixtures showed spectra of increasing intensity in the range of 350-500 nm (Figure 1), where major peaks were centered around 400-470 nm. The UV-visible spectra recorded at different time intervals showed increased absorbance with an increasing time of incubation, critically around 420 nm. While both control filtrates do not show any peaks in this 400-470 nm range, the positive and negative controls exhibited peaks around 230 nm and 300 nm, respectively.

X-ray diffraction analysis using the Debye-Scherrer equation confirmed the crystalline nature of nanoparticles with varying sizes (Figure 2(a)-(d)). Along with other peaks, the XRD diffractograms showed four intense peaks around 2θ angles at 38°, 44°, 64° and 77° and were indexed as (111), (200), (220) and (311), respectively. The presence of distinct peaks corresponds to elemental Ag. Overall, the crystallites estimated though the XRD technique revealed that their size was within nanometer range. Comparatively, higher peaks in the analysis indicated the active Ag composition and were mentioned by indexing. This was owing to the unit cell of the face centered cubic (fcc) structure. The sizes of the Ag nano crystallites, as estimated from the FWHM of the peaks using the Scherrer formulae, demonstrated significant variation corresponding to different fungi. The maximum mean size (nm) of the nanoparticles was 31 ± 15.4 for *A. niger*, followed by 21 ± 5.6 for *A. flavus*, 21 ± 13 for *A. terreus*, and 10 ± 6 for *A. fumigatus* (Table 1). The representative TEM micrographs of AgNPs synthesized by different fungi were obtained after 96 hr (Figure 3(a)-(d)). The micrographs showed considerable variability in the shapes of the AgNPs; though they appeared to be mostly spherical in shape. The overall sizes of these myco-genized AgNPs varied from 3 to 80 nm. The majority were scattered in the micrographs with only some places showing large size aggregates of varying size. Different fungi showed different sizes (nm) of nanoparticle, i.e., *Aspergillus fumigatus* 3-80, *A. niger* 3-75, *A. flavus* 5-75, and *A. terreus* 5-60 nm (Table 2).

4. Discussion

Currently, biological ways of synthesizing metal nanoparticles have been considered environmentally

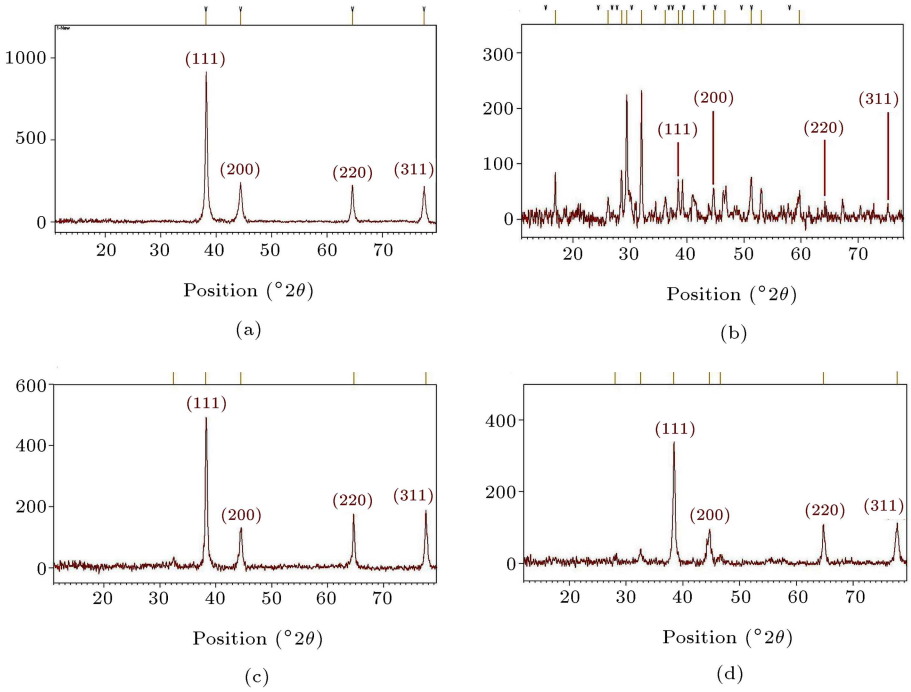

Figure 2. XRD patterns of nanoparticle film on Cu surface obtained from reaction mixtures of a) *A. fumigatus*, b) *A. niger*, c) *A. flavus*, and d) *A. terreus*.

Table 2. The complete experimental setup revealing conditions used to synthesize NPs by different *Aspergillus* sp.

Sample ID	Fungal strain	Experimental conditions	Range of NPs through XRD	Range of NPs through TEM	Average size of NPs
1.	*Aspergillus fumigatus*	pH 5.8, temp 28°C, RPM 150, biomass growth time 6 days, Reaction time 96 hr	5-18 nm	3-80 nm	10 nm
2.	*A. niger*	-do-	9-70 nm	3-75 nm	31 nm
3.	*A. flavus*	-do-	13-26 nm	5-75 nm	21 nm
4.	*A. terreus*	-do-	6-47 nm	5-60 nm	21 nm

safe [1]. In this reference, the synthesis of metals like Ag, Au & Pt nanoparticles has been reported utilizing different fungal genera [4,5,7-9,14-18]. In this study, an attempt was made to compare the potential of four different species of *Aspergillus sp.* to synthesize AgNPs. When the reaction mixtures containing $AgNO_3$ salt and fungal biomasses of *Aspergillus* sp. were incubated under agitated condition (28°C), the color of the reaction solutions changed from light yellow to dark brown, indicating synthesis of AgNPs. This color change has been previously associated with structural configuration and with surface plasmon excitation of AgNPs [4,8,14]. Similarly, the UV-Vis spectra of the Ag salt solution that reacted with the biomasses of different fungi showed a strong surface plasmon response (absorption spectrum) at $\lambda_{max} = 400-500$ [19]. Excitation of the localized SPR causes strong light scattering by an electric field at the wavelength where resonance occurs. This phenomenon results in the appearance of strong SPR bands which increase in intensity with reaction time. This is due to changes in the electronic properties and surface modification of the metal particles after the formation of nanoparticles, and can be easily investigated through simple spectrophotometric methods [20]. These observations indicated the release of certain catalytic proteins into the solution by the fungi that helped reduction of the metal ions [4]. It was confirmed through formation of the absorption spectrum at 400 nm due to the formation of AgNPs [15] and was absent when non reacted Ag salt solution was used for UV-Vis spectroscopy [8].

Various research groups have provided evidence of the extracellular generation of AgNPs by the XRD method. XRD data indicated particle sizes ranging

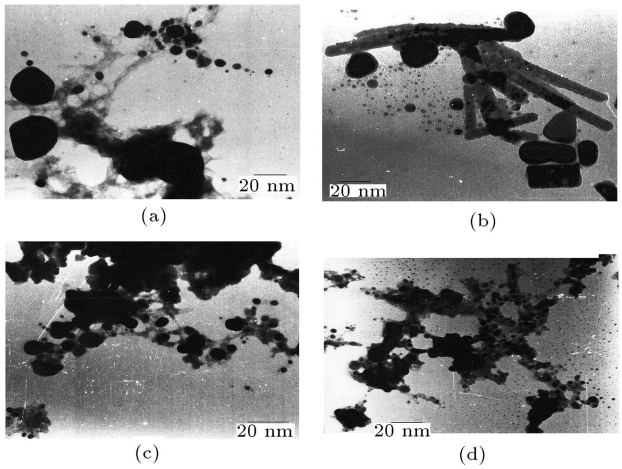

Figure 3. TEM micrographs of AgNPs produced by reaction mixtures of a) *A. fumigatus*, b) *A. niger*, c) *A. flavus*, and d) *A. terreus*.

from 5-70 nm. Results were more or less the same as the previous findings, where nanoparticle sizes varied from 5-100 nm [8,16-18]. Bhainsa and D'Souza in 2006 reported AgNPs synthesis ranging from 5-25 nm by *A. fumigatus* [8], whereas they were more homogeneous in size (5-18 nm) in the present study. In the case of *A. niger*, the size of nanoparticles obtained in this study was around 31 nm, while it was reported to be 5-35 nm previously [9]. Recently, the size of AgNPs synthesized by *A. flavus* was found to be 17 nm [18], which is in-line with the present findings (i.e., 21 nm). Similarly, AgNP formed by *A. terreus* was around 21 nm and was reported to be 6 nm in another report [7]. A few other obvious peaks at different 2θ angles, like 29°, 32°, 42°, 46°, 52°, 54° etc., can be attributed to silver chloride and other ions used in the preparation of the cultural medium and also to the biomass residue (Figure 2(b)) [7]. In addition to size, shape and crystallinity are important factors in determining the physicochemical and electronic properties of nanoparticles [11].

TEM micrographs showed that most of the nanoparticles were spherical in shape and ranged in sizes from 3-80 nm with different fungi (Figure 3(a)-(d)). Nanoparticle sizes showed more polydispersity compared to observations made through XRD, which was specifically noticed in the cases of *A. fumigatus* and *A. flavus*. In the TEM micrographs, some aggregates were also observed (it was mentioned previously that they formed when reaction time was increased beyond certain limits [21]). Since polydispersity has been a major issue in the biological synthesis of nanoparticles, it is important to optimize the conditions viz. pH, temperature, growth medium, type of organisms, substrate concentration and exposure time [2,22]. Nonetheless intracellular production of NP proved to resolve this issue [11].

5. Conclusions

Different genera of *Aspergillus* have exhibited significant potential (though varying) for extracellular synthesis of AgNPs in the range of 5-80 nm. However, the synthesis of nanoparticles can be further improved (towards monodispersity) and enhanced if reaction conditions are optimized.

Acknowledgments

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Biographies

Syed Zeeshan Haider Naqvi obtained his M.Sc. degree in 2006 from the Department of Biological Sciences and a MPhil degree in 2008 from the Department of Microbiology at Quaid-i-Azam University, Islamabad, Pakistan, where, since 2009, he has been studying for his PhD degree. He is working on a project entitled, "Synthesis of nanomaterials by microorganisms; their characterization and applications", sponsored by the Higher Education Commission (HEC), Government of Pakistan. He has also visited HST, Harvard-MIT, USA, for partial fulfillment of his research work.

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Abdul Hameed is a renowned Microbiologist in Pakistan. Presently, he is serving as Professor in the Department of Microbiology, and Director of the Office of Research and Innnovation at Quaid-i-Azam University (QAU), Islamabad, Pakistan. He paved the way in establishing the Department of Microbiology and in launching the Department of Pharmacy and Biotechnology. During his 28 years of university career, he has worked on multi-dimensional aspects of Microbiology, varying from basic to applied levels. Currently, he is pursuing the development of linkages between universities and industries in order to translate innovative research ideas and transform the role of a university from a "knowledge house" to a "knowledge hub", in order to uplift the local industrial sector. In recognition of his services in teaching and research, the President of Pakistan has conferred upon him the award of Izaz-e-Fazeelat in 2003 and Sitar-e-Imtiaz in 2011, while the Higher Education Commission (HEC) awarded him the Best Teacher award in 2000.

Safia Ahmed obtained her PhD degree in the field of Biotechnology from Imperial College, London, UK, in 1992. She was also a Post-Doc fellow in the

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Naeem Ali completed his PhD degree in Microbiology, in 2007, from Quaid-i-Azam University (QAU), Islamabad, Pakistan, where he is currently Assistant Professor. After serving a period of one year in an International Islamic University, in 2012, he undertook one year post-doctoral study in the School of Environment and Natural Resources, Ohio State University, USA, funded by the Higher Education Commission of Pakistan. Dr. Naeem Ali started his career in 1996 as a biology lecturer, serving in a number of colleges and universities, and is currently working on a number of projects focusing on applied aspects of Microbiology. In this reference, he is specifically pursuing a project related to biosynthesis and application of nanoparticles. He has published a number of research articles in internationally recognized journals.