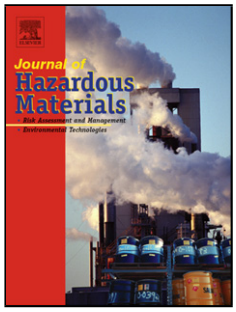

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Toxicity of fungal-generated silver nanoparticles to soil-inhabiting *Pseudomonas putida* KT2440, a rhizospheric bacterium responsible for plant protection and bioremediation

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Highlights

1. This study incorporates the mycosynthesis of AgNPs and their characterisation by various methods.

2**. A first attempt demonstrating the toxicity assessment of AgNPs on beneficial soil microbe**.

3. **Use of Biosensor in *Pseudomonas putida* KT2440, gave accurate antimicrobial results.**

Abstract

Silver nanoparticles have attracted considerable attention due to their beneficial properties. But toxicity issues associated with them are also rising. The reports in the past suggested health hazards of silver nanoparticles at the cellular, molecular, or whole organismal level in eukaryotes. Whereas, there is also need to examine the exposure effects of silver nanoparticle to the microbes, which are beneficial to humans as well as environment. The available literature suggests the harmful effects of physically and chemically synthesised silver nanoparticles. The toxicity of biogenically synthesized nanoparticles have been less studied than physically and chemically synthesised nanoparticles. Hence, there is a greater need to study the toxic effects of biologically synthesised silver nanoparticles in general and mycosynthesized nanoparticles in particular. In the present study, attempts have been made to assess the risk associated with the exposure of mycosynthesized silver nanoparticles on a beneficial soil microbe *Pseudomonas putida.* KT2440. The study demonstrates mycosynthesis of silver nanoparticles and their characterisation by UV-Vis spectrophotometry, FTIR, X-ray diffraction, Nanosight LM20- a particle size distribution analyser and TEM. Silver nanoparticles obtained herein were found to exert the hazardous effect at the concentration of 0.4 µg/ml, which warrants further detailed investigations concerning toxicity.

Keywords: Silver nanoparticles, Mycosynthesis, Toxicity, *Pseudomonas putida* biosensor.

1. Introduction

Nanotechnology offers new applications in science and technology [1]. Synthesis of various nanomaterials is achieved by physical, chemical and biological methods. However, biological synthesis is a green approach, and therefore, has been a topic of attraction.

Out of the vast array of nanomaterials, silver nanoparticles (AgNPs) play a pivotal role particularly in biomedical applications. The use of silver ions as a bactericide was common upto 1940 [2-5]. The silver ions provide protection against a variety of microbial pathogens [6]. Colloidal silver solutions (CSSs) are effective in pharmacology, human and veterinary medicine, food industry, and water purification [7]. The development of nano-silver as antimicrobial compounds is an active area of research [8, 9] because it has higher antimicrobial activity than its bulk counterpart [10].

Microbes and plants are remarkably effective in reducing metal ions to generate nanoparticles [11-18]. Among all microbes, the biosynthesis of nanoparticles by using fungi is preferred because the culture is cost- effective and requires simple growth conditions. The fungal mycelia when starved release nitrate reductase into the surrounding medium. This enzyme reduces metal ions to nanoparticles [11, 19].

AgNPs synthesized by fungi possess significant antimicrobial activity [11, 12, 20-27]. Efficacy of AgNPs produced by *Fusarium acuminatum* [19] was demonstrated against multidrug-resistant *Staphylococcus aureus, S. epidermidis, Salmonella typhi,* and *Escherichia coli.*

The production and application of nanomaterials increase the likelihood of environmental threat to microbes essential for ecosystem functions including biogeochemical cycles, degradation of

pollutants, and the basis of food webs and soil health. *Pseudomonas putida* strain KT2440 is an example of the ubiquitous pseudomonads that thrive in the rhizosphere [28], providing plant protection [29], and participating in bioremediation of xenobiotic compounds [30]. *P. putida* KT2440 is a plasmid-free derivative of the original *P. putida* mt-2 isolate [31]. The isolate typifies many uses in biocatalysis [32]. Gajjar *et al*. [10] demonstrated dose dependent changes in energy status and culturability in a strain of *P. putida* KT 2440 engineered to act as an energy biosensor when treated with physically prepared AgNPs.

The aim of the present study was to evaluate the activity of mycosynthesized AgNPs against *P. putida* KT 2440. We have synthesized nanoparticles by filtrates of *F. culmorum*, *F. moniliforme*, *F. tricinctum* and *Phoma glomerata*. In this study we used biosensor to test the sensitivity of *P. putida* KT 2440 to mycosynthesized AgNPs. .

2. Experimental

2.1. Fungi used for the synthesis of AgNPs

The fungal isolates *F. tricinctum (*MTCC-1978*), F. culmorum (*MTCC-1893*) F. moniliforme (*MTCC- 7375*),* and *P. glomerata (*MTCC-2710*)* were used for the synthesis of AgNPs. These isolates were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India. The cultures of the test fungi were maintained aerobically on potato dextrose agar (PDA) at 28 ± 2 °C. Aliquots of the cultures were stored in refrigerator at 4 °C on PDA slants.

2.2. Mycosynthesis of AgNPs

For the synthesis of AgNPs, flasks containing potato dextrose broth (200 g/l potato infusion and 20 g/l dextrose) were inoculated and incubated with shaking at 120 rpm at 28 ± 2 °C for 48-72 h. Biomass was harvested by filtration through Whatman No. 1 filter paper and the mycelia were rinsed with sterile distilled water four times to remove the medium components. Approximately 20g of the wet mycelia were transferred to 100 ml sterile double distilled water for 48 h at 28 ± 2°C with shaking at 120 rpm. The suspension was filtered with Whatman filter paper No. 1 and the filtrate was amended with 1 mM silver nitrate in darkness for 14 h. The mixture was filtered through a 0.22 µm filter paper and the unreacted silver ions were removed by centrifugation of the cell filtrate three times at 10000g each for 15 min [22] to pellet the nanoparticles. From each

aliquot of 20 g fungal biomass, a yield of 10 mg AgNPs was obtained. The NPs were stored in water suspensions at room temperature shielded from light.

2.3. Detection and Characterisation of AgNPs

2.3.1. Visual observation and UV-Vis Spectrophotometry

To demonstrate production of AgNPs, aliquots of the purified suspensions were scanned for absorbance using a Perkin –Elmer (Lambda-25) spectrophotometer. The plasmon resonance bands for AgNPs characteristically have a peak of absorbance near 420 nm [19].

2.3.2. Fourier Transform Infrared Spectroscopic (FTIR) Analysis

FTIR analysis was performed to check whether the NPs were capped with organic materials. The suspensions of NPs in water were treated with acetone in 1:5 ratio (NP suspension: acetone) and immediately centrifuged at 10000 g [22] for 15 min. This step was repeated twice. After centrifugation, the supernatant was discarded and 1-2 ml acetone was added to the pellets. The pellets were air dried and examined by FTIR (Perkin- Elmer FTIR-1600, USA) in the range of 500-4000 cm^{-1} at a resolution of 4 cm^{-1}.

2.3.3. XRD Analysis

The AgNPs were dried in oven at 60 $^{\circ}$C and the powders were subjected to X-ray diffraction (XRD) analyses using a PAN analytical X-PRT PRO, D-8, 130 Advanced Brucker instrument, (Netherlands). The diffraction patterns were recorded for the range from 20° to 80°.

2.3.4. Size and its Distribution Analysis

For size and morphology of the nanoparticles, each sample was examined by transmission electron microscopy (TEM) (Phillips, CM12) operating at 120KV. At least three images per sample were viewed. Aliquot of 5 µl each of the samples was placed on carbon coated copper grids (400 meshes, Plano Gmbh, Germany) and dried under infrared light for 30 minutes before inspection. The size range of the NPs was also examined using the Nanosight (LM-20, UK) instrument. The AgNPs were suspended in nuclease-free water by sonication for this analysis.

2.4. Evaluation of Antibacterial Activity

The biosensor constructed in strain *P. putida* KT2440 was used as described by Gajjar *et al*. [10]. In this construct toxicity from commercial AgNPs and Ag ions was demonstrated by a loss in light output [10]. The biosensor cells were grown overnight at 25 $^{\circ}$C and regrown to early log phase in a basal salts medium devoid of chloride. The cells were pelleted by centrifugation and resuspended to 10^8 cells/ml in water before being treated with known dilutions of the fungally-prepared AgNPs. Water was used as the matrix to eliminate possible chelation of metal ions by materials often present in culture medium [10]. The treated cultures were shaken for aeration during the assay period and light output from the biosensor cells was measured at 10 min intervals for one hour. At the end of the treatment the cultures were serially diluted and samples were transferred to LB medium (no salt) plates to assess culturability. Colonies were counted after 48 hours.

3. Results and Discussion

3.1. Synthesis of AgNPs

AgNPs were synthesized by different isolates of fungi including *F. tricinctum*, *F. culmorum*, *F. moniliforme* and *P. glomerata*, designated as A, B, C, and D respectively. The development of a brown colour from the initially colourless reaction mixture was indicative of the formation of AgNPs by ion reduction. Each preparation showed a sharp absorbance peak near 420 nm (Fig 1), characteristic of the presence of AgNPs [19]. The intensity was greatest for the products from the *Phoma* isolate. This absorbance peak of 420 nm was absent when fungal filtrates without addition of Ag ions were scanned.

3.2. FTIR spectroscopy

FTIR spectroscopy revealed vibration spectra with defined peaks between 1011 to 1041 (i.e. 1022, 1025, 1027) indicating the presence of stretch vibrations typical of –C-O-C- bond [33]. These findings suggested the possible presence of proteins capping the AgNPs as discussed in previous literature [34-38]. During sample preparation, AgNPs after acetone treatment gets precipitated. Which, supports the possibility of protein capping. This is due to the fact that AgNPs would get precipitated by acetone only when they are capped with protein. Whereas, the presence of amide II linkage and ether linkage due to the possibility of protein glycosylation,

indicates the protein as capping agent. The FTIR analysis of sample A from *F. tricinctum* filtrates showed peaks at 2350, 1527, and 1025 indicating the presence of a –COO group, an amide II and aliphatic amines respectively. Sample B from *F. culmorum* filtrates had peaks at 3243, 1556, 1394 and 1022 revealing a –C-C stretch, an asymmetric and a symmetric stretch of COO^- and aliphatic methyl group respectively. Sample C produced with *F. moniliforme* filtrates generated peaks at 3295, 1567, 1371 and 1046 confirming presence of hydroxyl group, carboxylate and N-O bond stretch. Sample D from *P. glomerata* filtrate demonstrated peaks at 2345, 1544, 1380 and 1027 indicating the presence of COO^-, amide and methyl binding and C-O bonds respectively. These spectra were consistent with the possible presence of proteins on the AgNPs.

3.3. Size analysis

Figure 3 displays the particle populations by size and intensity as determined by Nanosight analyses. The analyses showed that each preparation had a range of particle size. The preparations from the *Fusarium* filtrates were generally smaller and less diverse in size than those from the *Phoma* filtrate. Peaks from the preparations were at 56 nm, for *F. tricinctum*, 47 nm from *F. culmorum*, 46 nm from *F. moniliforme* and 71 nm from *P. glomerata* with size of about 30 nm in each of the preparations. TEM imaging (Fig 4) showed the spherical particles, ranging from 5 to 12 nm in all samples. However, the aggregations were also evident in each sample.

3.4. Crystallization

X-Ray diffraction peaks were observed at positions 38°, 44°, 66° and 78° of 2Θ, (Fig 5), showing the presence of (111), (200), (220) and (311) facets of faced centred cubic (fcc) crystalline structures. These values are in agreement with the previous report (Joint Committee on powder diffraction, standard file no. 04-0783) for Ag crystallization. The observed optical absorption band peak in the range of 3 to 4 keV was distinctive for the absorption of metallic silver.

3.5. Toxicity to *P. putida* biosensor

Treatment of the biosensor cells with the mycosynthesized AgNPs caused dose- dependent loss in light output. The gradual decline seen in the control cells was due to a decline in energy status

of the cells during their incubation in the water suspension. Using doses of 0.8 µg/ml complete inhibition was observed after 10 minutes with preparations from filtrates of *F. culmorum* (B), and *F. moniliforme* (C), (Fig 6 a and b). Loss in light output corresponded with loss in culturability of the biosensor cells. At the 0.4 µg/ml dose, only preparation C caused complete loss of light output and reduction in cell culturability.

It is known that cytotoxicity is dependent upon the size of the nanoparticles. Smaller the nanoparticles, more will be its cytotoxicity at lower doses. This is because the smaller nanoparticle dissolves/ redistributes fastly in the suspension media and can interact more efficiently with the organism present in its vicinity. These factors might be playing a role in showing the toxicity of samples synthesized herein at 0.4µg/ml. Additionally, by reviewing the literature published in recent past it can be said that Gram negative bacteria are more prone to the harmful effect of AgNPs as compared to Gram positive bacteria as the former has thinner peptidoglycan layer than the later one [39]. Therefore, to evaluate the adverse effect of soil environment the present study made use of *P. putida*, a Gram negative bacterium recovered from rhizospheric soil.

Our results indicate that bacteria died within 10 minutes after exposure to AgNPs at the concentration between 0.4 to 0.8 µg/ml. These observations might be driven by an increased bioavailability of the AgNPs. However, as the nanoparticles from all samples have nearly similar size range distribution, we also found similar toxicity to *P. putida*. This signifies the phenomenon that similar sized AgNPs with similar characteristics, demonstrate similar toxicity regardless of the origin from where they have been synthesised.

As described earlier, TEM images of all AgNPs samples with size range in between 5-12 nm showed toxicity. It is because of the well-known notion that any nanoparticle, at lower concentration, in nanosize possess an extremely large surface area to volume ratio. The high surface area to volume ratio promotes interaction of nanoparticles with the bacterial surface at much faster pace thereby interacting actively with their cell membrane, increasing cell permeability and ultimately leading to the their cell death. The bacterial cell death may also result due to the nanoparticle driven disturbance of DNA replication, ribosomal subunit and ATP synthesis [40-42].

In addition to the size and shape, the capping of nanoparticle also contributes to its property. This is because capping by different molecules demonstrate different surface charges to the nanoparticle to which it is binding [43]. Therefore, they interact differently with any of the living system in fungal mediated synthesis of nanoparticle, the capping use to be protein in nature. These capping agents by coating on nanoparticles avoid their agglomeration to provide stability to them [44]. Furthermore, for the application like pharmacology, the protein capping is beneficial for nanoparticle, as it can play an important role in anchoring and carrying the drug inside human system. Fungi use to produce high amount of protein as compared to bacteria leading to the formation of higher amount of nanoparticles with protein cap [38, 45-46) .The occurrence of protein cap also intensifies uptake and retention of nanoparticles inside the human cells [47].

4. Conclusion

Fungal extracellular materials from isolates of *Fusarium* and *Phoma* reduced Ag ions to AgNPs as characterized by absorption spectra with a peak at 420 nm and XRD analyses. Thus, reduction of ions to AgNPs was not restricted to a specific fungal isolate. The size of the spherical nanoparticles varied and some agglomerations were present. The FTIR analysis showed that the synthesized nanoparticles were capped with proteins.

Each of the preparations of AgNPs was toxic to the soil bacterial biosensor generated in *P. putida* KT2440. As reported previously [10] depletion of Lux activity was more sensitive than detection of the change in bacterial culturability. The preparation from *F. moniliforme* was more toxic than the products from *F. culmorum*. The product from *P. glomerata* was more effective than the product from *F. tricinctum.* The findings supported the toxicity studies reported previously with commercial AgNPs [10]. Nanoparticles at concentrations of 0.4 µg/ml were hazardous to *P. putida.* The present study and the data published till date, shows that bacteria are model organisms to find potential harmful effects. Therefore, more studies are required to explore the possible hazardous effects of AgNPs to the environment through proper model organisms in order to set standards for safe use of nano-silver based technology for the betterment of mankind.

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Figures captions:

Fig 1. UV–Vis spectra of suspensions of AgNPs synthesised by filtrates of *F. tricinctum* (A), *F. culmorum* (B), *F. moniliforme* (C) and *P. glomerata* (D). The subscript E denotes spectra from the filtrate with Ag ions and C filtrates without added ions. The peak absorbance wavelengths (nm) for the preparations are shown in insert.

Fig 2: FT-IR spectra for AgNPs prepared from filtrates from: (A) *F. tricinctum*, (B) *F. culmorum,* (C) *F. moniliforme,* and (D) *P. glomerata*.

Fig. 3 Size distribution determined using NanoSight LM-20 for Ag NPs generated from filtrates of: (A) *F. tricinctum*, (B) *F. culmorum*, (C) *F. moniliforme*, and (D) *P. glomerata*. The vertical red lines show the size distribution of the nanoparticles: a white line encloses these data. The height of each bar represents the number of nanoparticles of particular size. The sigmoidal white line shows percent of nanoparticles below a defined size.

Fig 4: Transmission electron micrograph (TEM) of aggregated and protein-capped Ag NPs generated from extracellular produced from filtrates of (A) *F. tricinctum*, (B) *F. culmorum*, (C) *F. moniliforme,* and (D) *P. glomerata*. The red lines in image D show how the particles were measured to obtain their diameters.

Fig 5. Spectra obtained by powder XRD-spectroscopy of AgNPs synthesised using filtrates of: *F. tricinctum* (A), *F. culmorum* (B) and *F. moniliforme* (C) and *P. glomerata* (D).

Fig 6. Response of the *P putida* KT2440 biosensor to Ag NPs at A, 1: 500 [0.8 mg/L] and B, 1:1000 dilutions [0.4 µg/ml]) and 1.0 mg/L ions. Changes in Lux output in relative light units (RLU) and cell culturability (colony forming units Cfu) are shown. Data are from one study typical of at least three generated under the same conditions. Means and standard errors are shown. The labels Sample A, B, C and D are assigned to the AgNPs synthesised using filtrates of *F. tricinctum, F. culmorum, F. moniliforme* and *P. glomerata* respectively.

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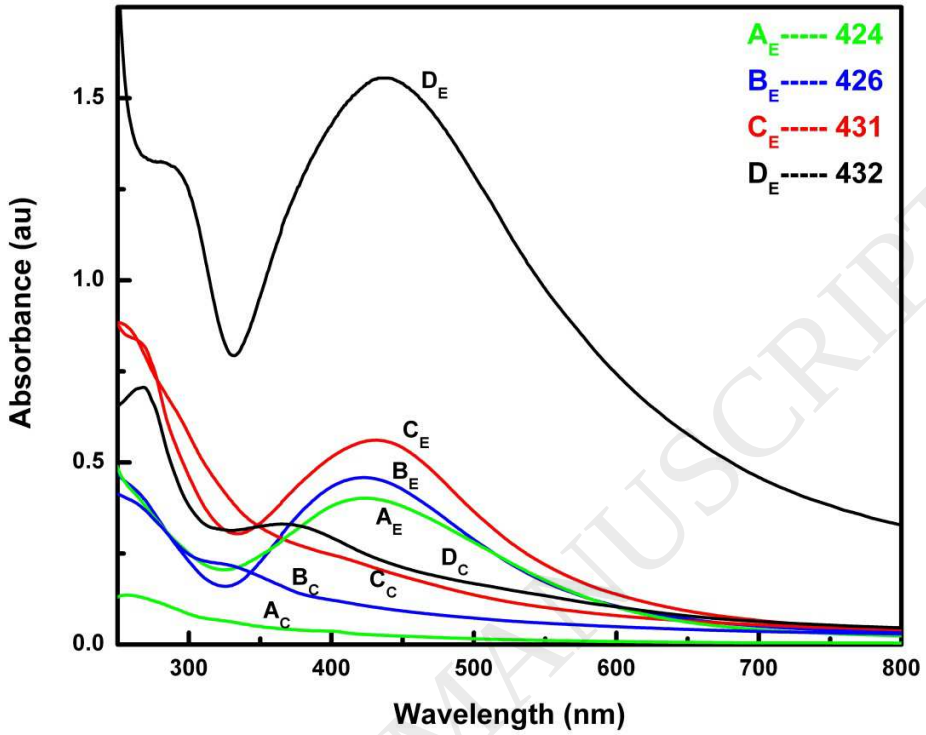

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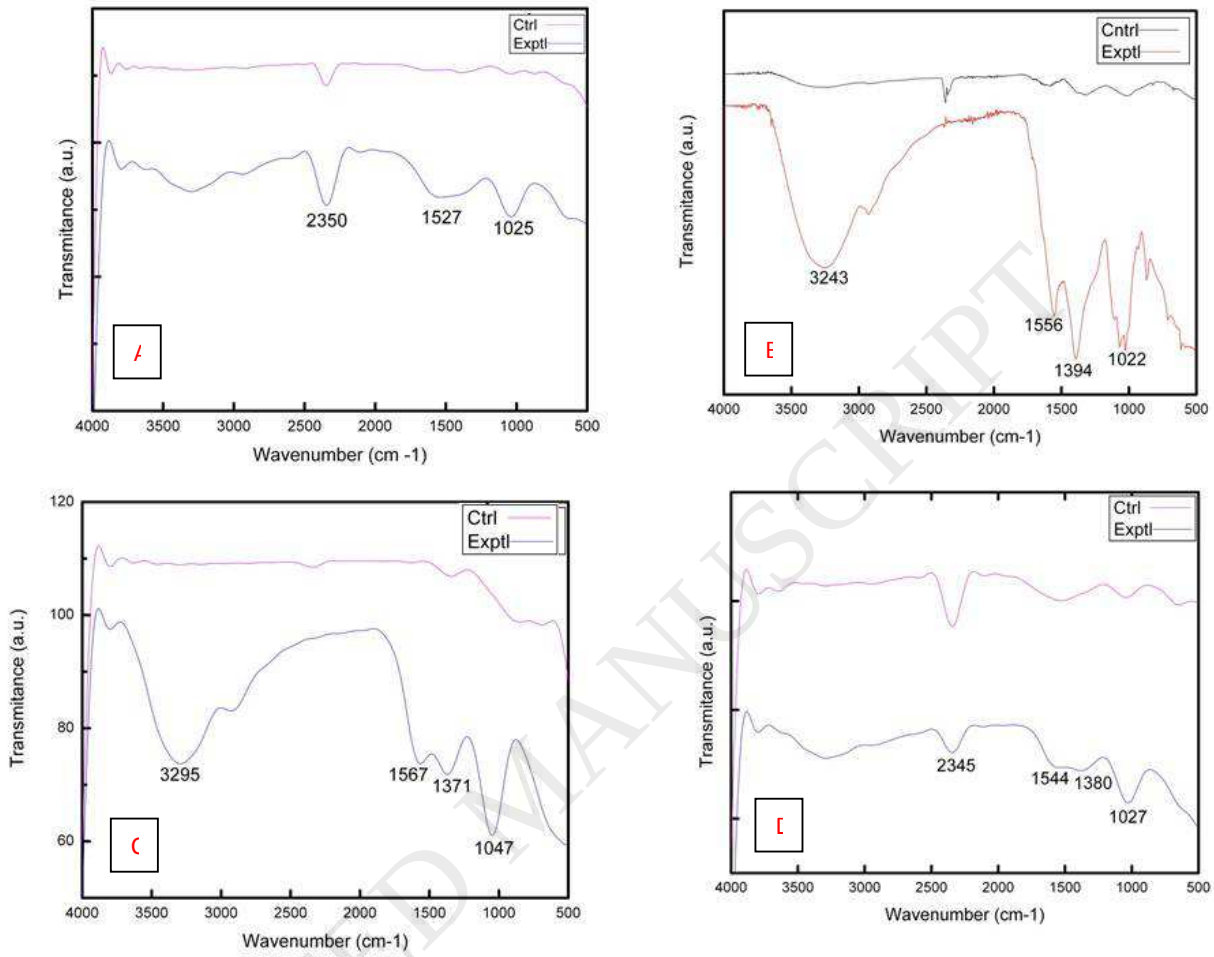

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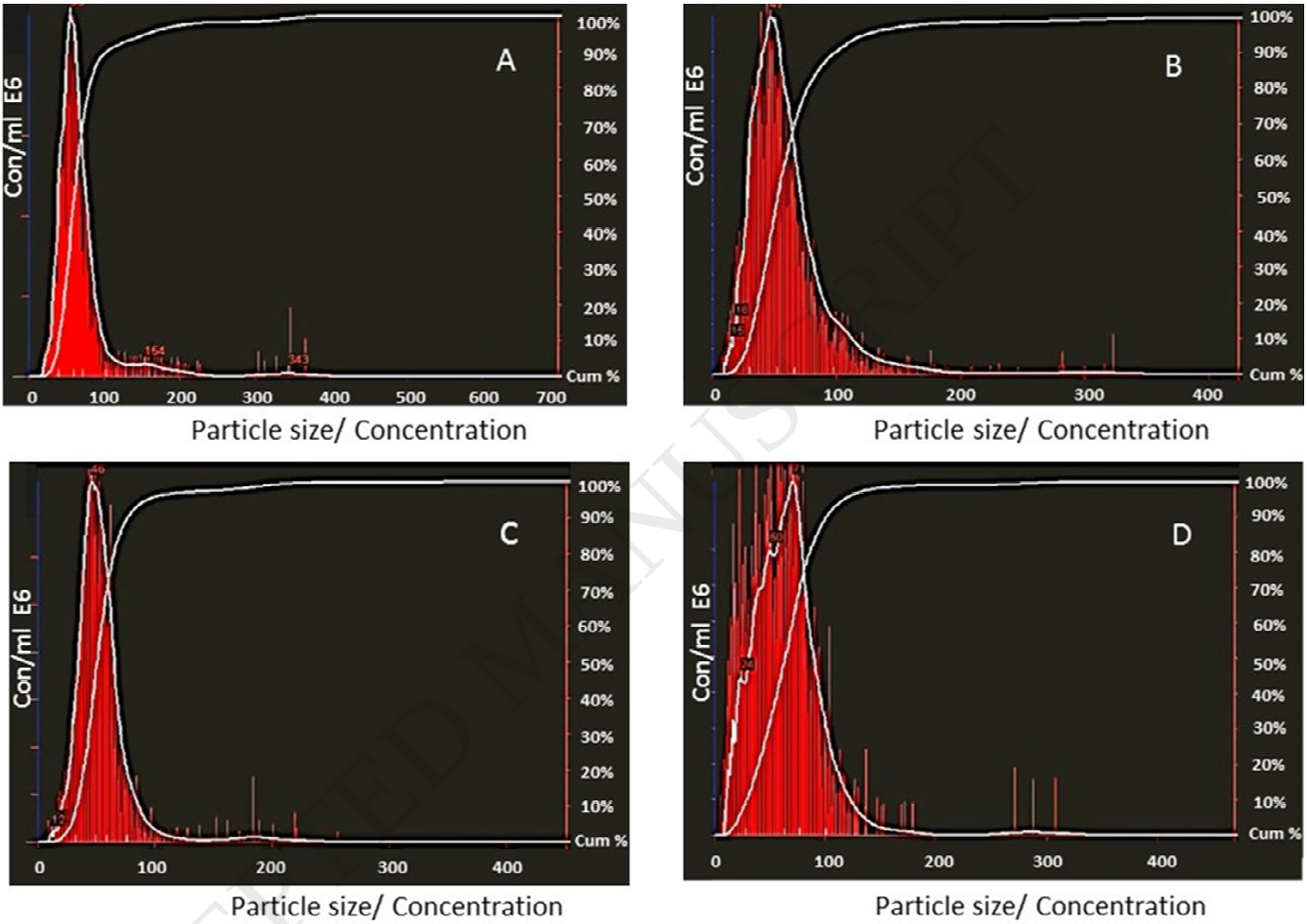

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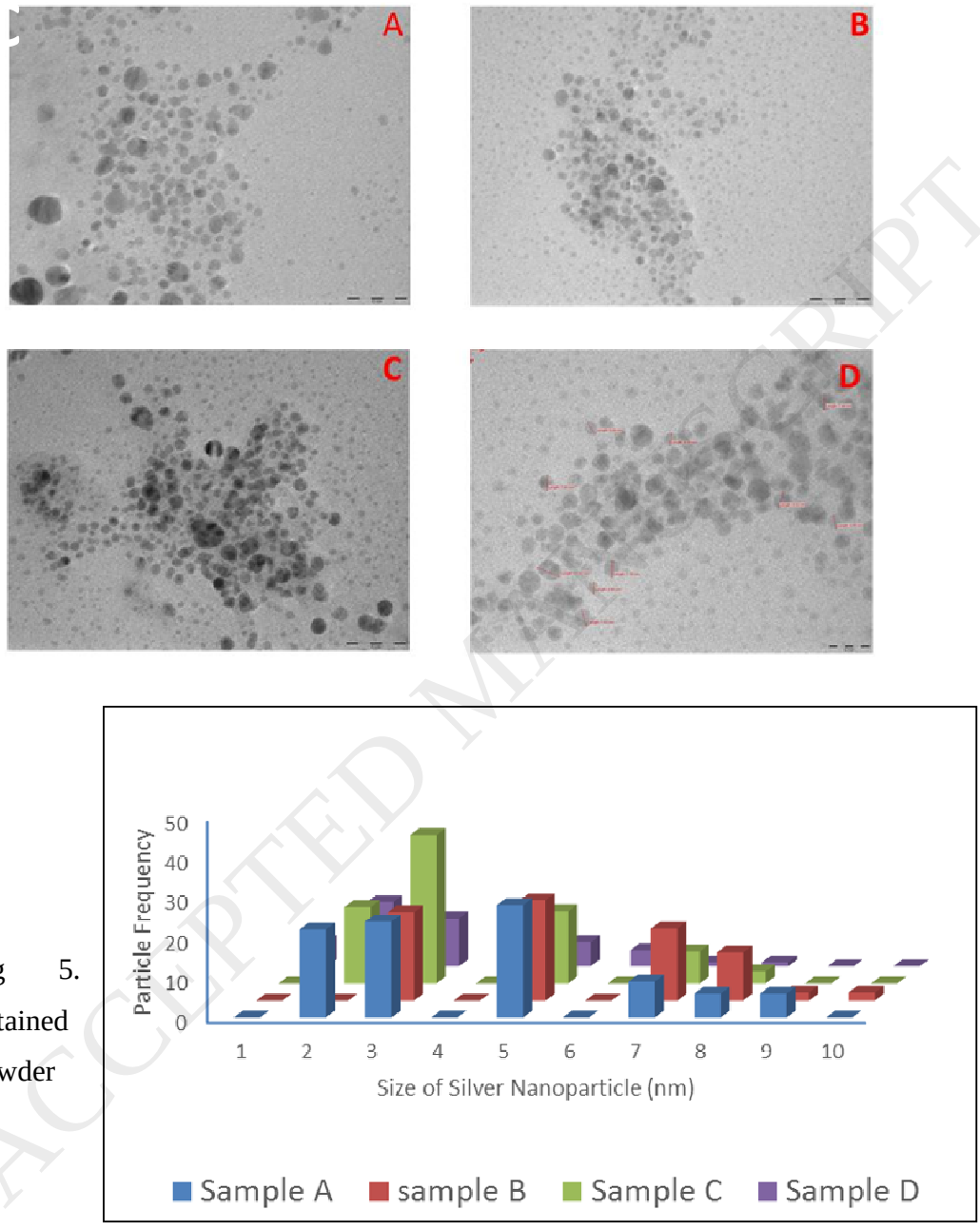

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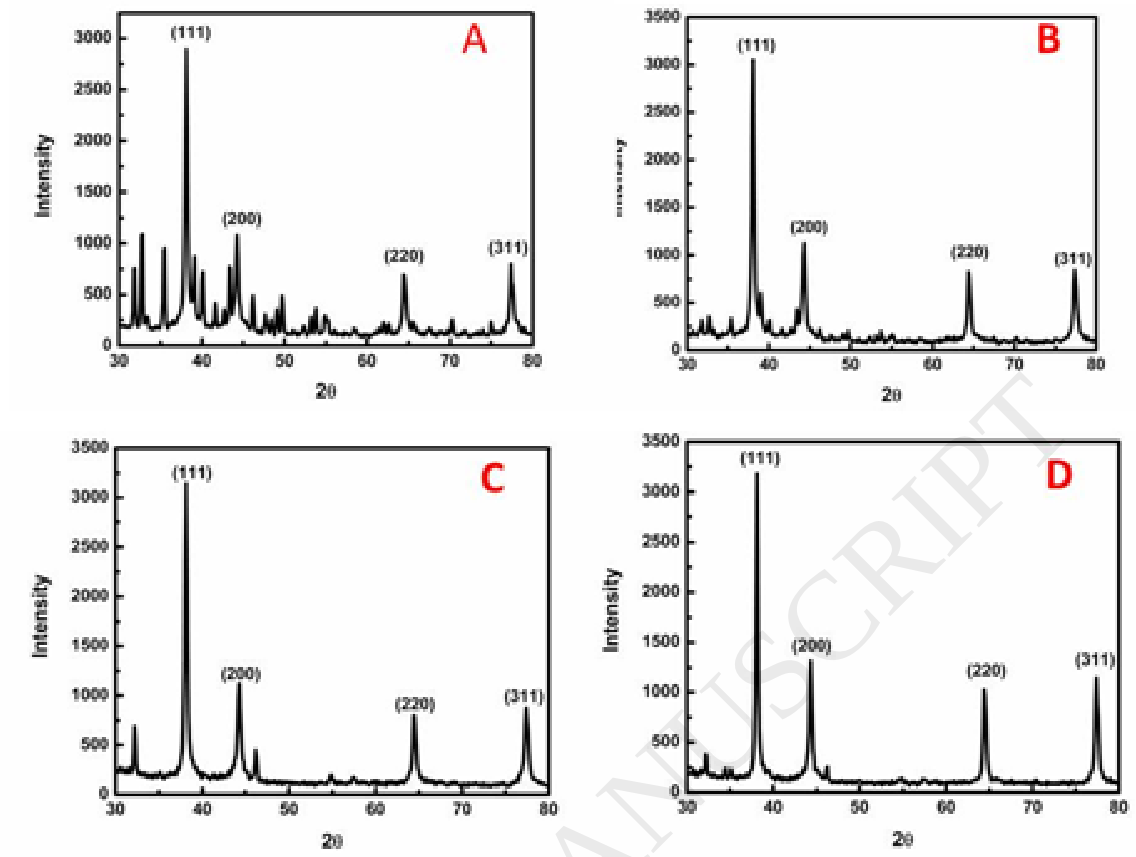

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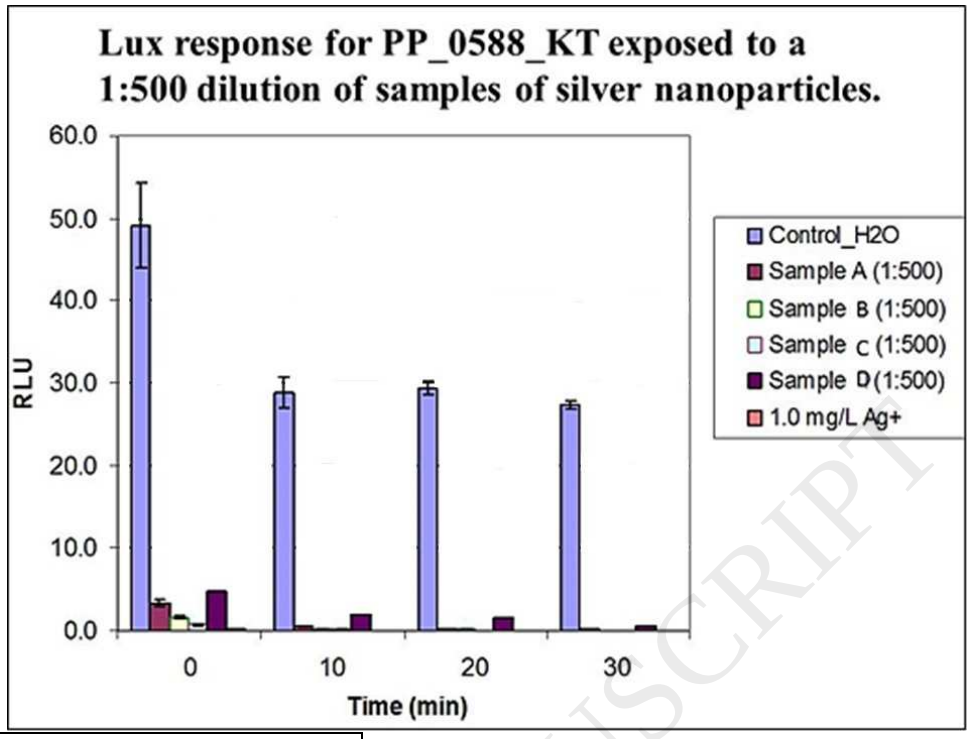
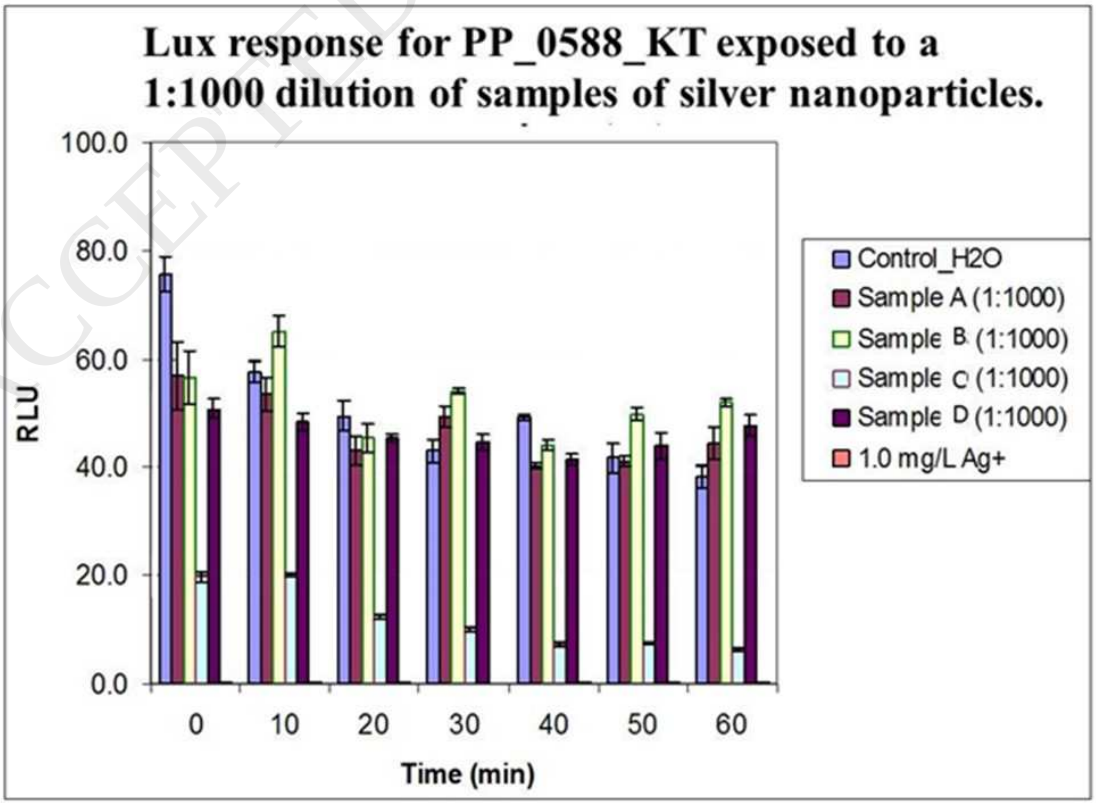
A)



Culturability (cfu/ml)	
Control	1.00E+07
Sample A (1:500)	3.00E+04
Sample B(1s:500)	0.00E+00
Sample C (1:500)	0.00E+00
Sample D (1:500)	4.00E+04

B)



Culturability (cfu/ml)	
Control	1.00E+08
Sample A (1:1000)	1.00E+08
Sample B (1:1000)	5.00E+07
Sample C (1:1000)	3.50E+05
Sample D (1:1000)	6.50E+07

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