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# Optimization and spectroscopic characterization of the biosynthesized Silver/Chitosan Nanocomposite from *Aspergillus deflectus* and *Penicillium pinophilum*

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**Abstract:** Twenty seven of fungal species were isolated and screened from Egyptian soil samples during a previous study. Among all isolates, two species were selected for the biosynthesis of silver nanoparticles (AgNPs). *Aspergillus deflectus* filtrate and *Penicillium pinopholium* mycelia isolated from Abu Qir and Helwan sites respectively showed the best surface plasmon resonance (SPR) and stability. In the present study the SPR of silver AgNPs synthesized by the two fungal *sp*., were optimized via pH and salt concentration. Shape, size, molecular structure of the capping molecule and crystalinity have been investigated by UV/Vis, HRTEM, and FTIR. The effect of exposure to ionizing radiation during synthesis on the physical properties of the AgNPs has been considered.

**Keywords:** *Aspergillus deflectus, Penicillium pinophilum, pH, silver nitrate concentration, FTIR, HRTEM*

## INTRODUCTION

Nanotechnology is an emerging field with its application in science and technology for the purpose of synthesis and development of nanomaterials at the nanoscale level[1]. Large number of research were focusing to control the size and shape, which is crucial in tuning their physical, chemical, and optical properties[2,3,4]. The green synthesis of nanoparticles with the help of biological sources like plant and microorganisms is carried out because they are less toxic to human and environment[5]. Environmentally benign methods were developed for the synthesis of these nanoparticles which eliminate the use of toxic chemicals during their synthesis process[6]**.** Different types of metal nanoparticles are produced such as copper, zinc, titanium[7], magnesium, gold[8], alginate[9], and silver. Silver is medically considered as one of the most powerful elements due to its activity against mammalian tissues where it acts as an antiseptic agent[10]**.** Silver, exhibits cytotoxicity against several microorganisms and hence is used as an anti-microbial agent[11]. Some microorganisms are also able to accumulate silver by adsorption[6]**.** Silver nanoparticles have been exploited for their unique properties and their vast applications in biomedicine.

Due to vast emerging applications of AgNPs in distinct fields, there is an increase in demand for AgNPs and to fulfill the demand, there is a pressing need to increase in their yield, for which optimization of the process is very important step. Few contributions were made regarding the effect of cultural and physical conditions on the biosynthesis of SNPs. The synthesis of AgNPs at nano-range is still a challenge. In order to increase the yield and the shelf-life (stability) of AgNPs with minimum investment, it is necessary to optimize the cultural conditions and various physical parameters like pH, light intensity and temperature[12]**.**

Therefore, in the present study, *Penicillium pinophilum* and *Aspergillus deflectus* were investigated for their intracellular and extracellular biosynthesis of silver nanoparticles, its optimization, and characterization studies; to obtain mono-dispersed silver nanoparticles.

## MATERIAL AND METHODS

**Isolation of fungal isolates:** *Penicillium pinophilum* and *Aspergillus deflectus* were selected for the present investigation. They were isolated from local Egyptian soil, Helwan sites and Abu Qir respectively. The two fungal isolates were growing and maintained on potato dextrose agar media (PDA) and stored at 4 ºC until use.

**Biosynthesis of silver nanoparticles**: In sterilized 250 ml Erlenmeyer flasks. Fifty ml aliquots of the media were dispensed. After sterilization flasks containing potato dextrose broth were inoculated with 1cm disc of tested fungal isolates obtained from 7 days old plate culture, at 25ºC, under shaking condition (180 rpm).

**Extracellular biosynthesis of silver nanoparticles:** Synthesis of nanoparticles outside the cell, extracellular, has many applications as it avoids unnecessary adjoining cellular components from the cell. Mostly, fungi are regarded as the organisms that produce nanoparticles extracellularly because of their enormous secretory components, which is involved in the reduction and capping of nanoparticles[13].

The culture was filtered (Whitman filter paper No.1) at the end of incubation period to separate the biomass from filtrate. Detection of silver nanoparticles was carried in filtrate in case of

*Aspergillus deflectus*. Fifty ml of silver nitrate (1mM $AgNO_3$) were added to fifty ml of the culture of filtrate and the flasks were incubated in shaking incubator for 72 h.

**Intracellular biosynthesis of silver nanoparticles:** The nanoparticles formed inside the organism could be smaller compared with the size of extracellularly reduced nanoparticles. The size limit could have been related to the particles nucleating inside the organisms[13]**.** Fungal biomass was extensively washed with distilled water to remove any component. Five grams of biomass of *penicillium pinophilum* were mixed with 50 ml of aqueous solution of 1mM $AgNO_3$.The flasks were incubated under shaking condition for 72 h. Aliquots of the isolates which showed color change from yellow to brown were subjected to UV visible absorption spectroscopic investigation.

***Optimization studies for silver nanoparticles production***

There is always a continuous interaction between organism and the environment in which they live. The environmental conditions exert an influence on growth and development of organism. The enzyme production by fungi is influenced by the condition at which the organisms are cultivated[14,15] **.** Therefore, optimization studies will not only support good growth but also enhance product yield.

**Effect of $AgNO_3$ Concentrations**: The production of nanoparticles depends on substrate concentration. The concentrations of $AgNO_3$ from **(0.25, 0.5, 1, 1.5, 2, 2.5 mM)** were investigated. The optimum concentration for the synthesis of nano-silver is confirmed by UV-visible absorption spectroscopy.

**Effect of pH on biosynthesis of nanosilver:** Hydrogen ion concentration (pH) has a strong influence on growth and secondary metabolites production which is required for the biosynthesis of AgNPs. Investigation of nano-silver production either intracellular or extracellular was carried under various pH values (**5.5, 6.5, 7.5, 8.5 and 9)** by using 0.1M sodium chloride, 0.1M hydrochloride. All adjustments were carried out by using pH-meter (3310 Jenway).

**Effect of irradiation:** Production of nanosilver of *P. pinophilum* and *A. deflectus* was carried out by adding silver nitrate to mycelia and filtrate under optimized conditions and exposure to gamma irradiation was carried out using a gamma $^{60}Co$ source (1.17–1.33 MeV) of dose rate 2.2 kGy/h with doses 25 kGy. The facility used was $^{60}Co$-Gamma chamber 4000-A-India, available in the National Center for Radiation Research and Technology (NCRRT), Egyptian Atomic Energy Authority (EAEA).

**Characterization of AgNPs:** The formation of AgNPs was preliminarily confirmed by visual observation of color change from pale white to reddish brown, further by UV-visible spectra using UV/Visible spectrophotometer ( JASCO V-630), a resolution of 4 nm was used for the screening and detection of the best sample for the biosynthesis of silver nanoparticles. Liquid samples were diluted 1:4 and scanned in the range 200 to 1000 nm. Sharp peak given by UV/visible spectrum confirms silver nanoparticles at the absorption range between 400 and 450 nm.

The morphology and structure of samples was determined using High Resolution Transmission electron microscope (JEM-2100HR, Japan) at 200 keV. Samples of the aqueous suspension of

silver nanoparticles were prepared by placing a drop of the suspension on carbon-coated copper grids and allowing the water to evaporate. To study the molecular groups responsible for the reduction of silver ion and capping of the produced silver nanoparticles, we performed Fourier transforms infrared spectroscopy. The vacuum dried silver nanoparticles were mixed with Potassium Bromide (Sigma Aldrich) at a ratio of 1:100 and the spectra were recorded with a Vertex 70 Bruker Fourier Transform Infrared Spectrophotometer at resolution $1cm^{-1}$. The scanning data were obtained in the range between 4000 to 400 $cm^{-1}$. Base line of all samples has been corrected at multi-points.

## RESULTS AND DISCUSSION

**Effect of substrate concentration:** One of the factors making the reaction more economical and efficient is finding the maximum concentration of substrate which could be converted to the final product. Therefore, we investigated different concentrations of silver nitrate in the reaction mixture in order to evaluate the maximum concentration of the substrate for nanoparticle production (figure (3) a and b), by gradual increase in concentration of $AgNO_3$ to 2 mM, resulted in an increase in the intensity of AgNPs SPR until it reaches 2 mM for the two studied species. The effect of substrate concentration (silver nitrate) is a key parameter that affects the process of nanoparticle synthesis. Very few reports are available about the study of different substrate concentration, and their effect on mycofabrication process of AgNPs. The synthesis of AgNPs by using fungal system was reported by several researchers[16,17,18,19,20,21,22]**.** They have reported the synthesis of AgNPs by using 1mM silver nitrate concentration, but there is no explanation why only 1mM silver nitrate is used and not less than 1 mM. These results are in disagreement with the authors who reported that maximum production of AgNPs from *Penicillium sp* and *Fusarium oxysporium* were at concentration 1.5 mM[6,12].

**Effect of pH on biosynthesis of nano-silver:** Hydrogen ion concentration (pH) has a strong influence on the biosynthesis of AgNPs. Different pH values from 5.5 to 9.0 were used with the difference of 1.0 to study the influence of pH on AgNPs production from fungi, *Aspergillus deflectus* and *Penicillium pinophilum*. The alkaline pH 9 showed the maximum synthesis of nanoparticles while in acidic pH 5.5 aggregates were observed.

AgNPs SPR peak for *Aspergillus deflectus* and *Penicillium pinophilum* was at 422 nm and 419 nm, respectively, showed the blue shift in spectra with increasing pH (figure (4) spectra A and B). hydroxyl ($OH^-$) ions are nucleophiles which play crucial role in maintaining the stability of AgNPs by adsorbing on it and in synthesis of smaller size AgNPs by providing electrons for reduction of silver ions.More nucleation centers are formed due to the availability of $OH^-$ions. Increasing the rate of nucleation center formation help in turn, preventing the aggregatation by adsorbing on nanocrystals and therefore, maintains the smaller size of AgNPs[23]. Similarity with authors findings that at alkaline pH AgNPs are stable and aggregates are formed at lower pH[24]. It indicates that, by controlling the pH of AgNPs synthesis, controlling AgNPs size becomes easier. The proton concentration affects conformational changes in reducing agent present in the fungal filtrate, which may change the morphology and size of the AgNPs[25]. It has been stated that when the condition of the AgNPs myco-fabrication is alkaline, the synthesis will be faster than in acidic conditions. In other words, synthesis enhances as the pH increases towards alkaline region[26]. In alkaline conditions there were no need of agitation of the mixture for the formation of AgNPs and

all the silver ions supplied were converted to AgNPs within 10 min.The proteins involved in the synthesis may bind with silver at thiol regions (–SH) forming an S–Ag bond, a clear indication of which aids the conversion of $Ag^{+}$ to $Ag^{0}$. In addition, the alkaline ion ($OH^{-}$) is very much required for the reduction of metal ions. Moreover, under alkaline conditions, the ability of the 243 enzymes responsible (not only nitrate reductase) for the synthesis of AgNPs increases[27]. The present findings corroborate with worker who proposed the mechanism for the synthesis of AgNPs by using lactic acid bacteria[28]. The authors reported that whenever pH increases, more competition occurs between protons and metal ions for negatively charged binding sites; therefore, a better synthesis at alkaline as compared acidic pH were recorded. These results were in agreement with S. Sonal Birla et.al. [11] and disagreement with K.M. Narayanan *et.al*. [12] who reported that maximum production of $AgNO_3$ from *Penicillium sp* at pH 6.0 and other authors reported that maximim production from *Rhizopuss stolonifer* at pH 7 [29]**.**

The extracellular and intracellular synthesis of AgNPs using *Aspergillus deflectus & Penicillium pinophilum* respectively involves the bioreduction of silver ions in the biomass and filtrate as shown in fig (1&2). Reaction solution was monitored by periodic sampling of the reaction mixture at regular time intervals by using UV-visible spectroscopy. Synthesized AgNPs showed maximum absorbance peak at 410 nm for *Penicillium pinophilum* and 420 nm for *Aspergillus deflectus* figure 3. The AgNPs formed were highly stable up to 72 h after the reaction. The AgNPs were characterized and confirmed by HTEM analysis. Similar results were observed by[30] authors who revealed plasma resonance of AgNps between 380 and 450 nm, and other worker[31] revealed absorption peak at 400 nm and 423 nm by endophytic fungi isolated from leaf samples of *Garcinia Xanthochymus* and *Aravaelanata*.

Irradiation with 25 KGy gamma radiation was carried out after addition of $AgNO_3$ under the optimized condition (pH 9 and $AgNO_3$ concentration 2 mM) to reveal the effect of irradiation on the rate of synthesis and the physical properties of biosynthesized nanoparticles. The data showed that the AgNPs SPR intensity duplicated in response to irradiation for the two fungal species. The peak position of AgNPs from *Aspergillus deflectus* exhibit significant blue shift indicating a possible particle size reduction which will be confirmed by HRTEM, where, the AgNP SPR peak show no significant changes. Also there is only one SPR peak for each sample indicating that all the particles were spheres in shape without contribution from other shapes.

**High Transmission Electron Microscopy (HTEM):** HR-TEM measurements were carried out to determine the morphology and shape of AgNPs. Figure 7 revealed that the particles are almost spherical and well dispersed without agglomeration. The particle size of AgNPs synthesized by *Penicillium pinophilum* and *Aspergillus deflectus* ranges from 6.57 to 23.7 nm. Various reports provided evidence of extracellular synthesis of AgNPs by TEM images. The authors reported well distributed spherical shaped AgNPs in the range of 5–30 nm by Penicillium diversum[32] and others revealed spherical and poly-dispersive AgNPs ranging from 10 to 40 nm by endophytic fungus Pestalotia sp[33]. isolated from leaves of Syzygiumcumini. Other reports showed that spherical shaped AgNPs with the size ranging between 3 and 20 nm by Rhizopus stolonifer[29]. The HRTEM demonstrate that the lattice spacing is ~ 0.08, 0.06 ,0.1 and 0.1 nm for *Penicillium pinophilum* before and after irradiation and *Aspergillus deflectus* before and after irradiation respectively.

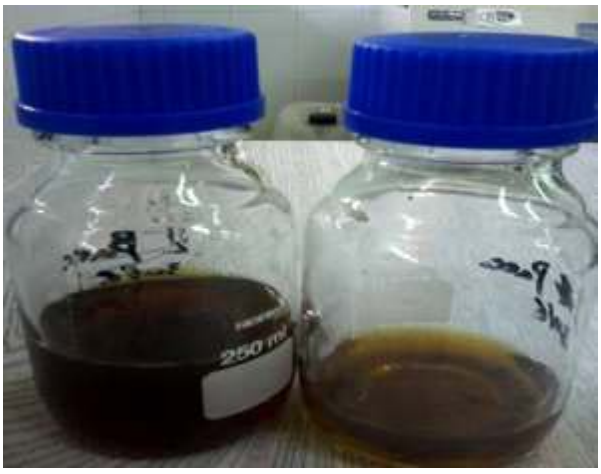
**Fourier Transform Infrared Spectroscopy (FTIR):** Fourier Transform Infrared Spectroscopy measurement of the powdered sample was carried out to identify the possible interaction between silver and bioactive molecules, which may be responsible for synthesis and stabilization of AgNPs. FTIR spectrum was performed for the AgNPs biosynthesized from *Aspergillus deflectus* and *Penicillium pinophilum* at pH9 and 2Mm $AgNO_3$ before and after irradiation. As this condition has been chosen to be the optimum, it revealed the presence of nine bands as shown in table 1 and figure 5. The bands at 1026 and 1034 $cm^{-1}$aromatic C–N stretching for *Aspergillus deflectus* and *Penicillium pinophilum* respectively. The band at 1249 $cm^{-1}$ is attributed to the C-O deformation. The bands at 1639, 1633$cm^{-1}$ correspond to the bending vibrations of C=O amide I, 1459 and 1485 $cm^{-1}$are attributed to N–H of amide II, whereas, their corresponding stretching vibration could be at 2932 and 2855 $cm^{-1}$in the same order. The band assignments of the *Aspergillus deflectus* and *Penicillium pinophilum* were similar to the characteristic bands of chitosan (chitin is one of the constituents of fungus cell wall). The vibrational bands shifts of amide I and amide II revealed that the C=O and N-H are involved in the reduction and stabilization[34.] The split of amide bands after irradiation is due to the interaction of radiation energy with C=O band resulting of free C-H bonds. The Molecular structure of the AgNPs reveals that chitosan (the deacetylated form of chitin) was involved in the reduction $Ag^{+}$, stabilization and capping of AgNPs and confirming the formation of Ag/Cs nano-composites.

**Table (1):** FTIR of optimized culture and irradiation condition of *Aspergillus deflectus* and *Penicillium pinophilum*

| **Main bands** | ***P. pinophilum*** **Non- irradiated** | ***P. pinophilum*** **irradiated** | ***A. deflectus*** **Non- irradiated** | ***A. deflectus*** **irradiated** |
|---|---|---|---|---|
| 1 | 3679 | 3696 | 3671 | 3671 |
| 2 | 2922 | 2922 | 2922 | 2922 |
| 3 | 2854 | 2854 | 2854 | 2854 |
| 4 | 1637 | 1645 | 1637 | 1645 |
| 5 | 1551 | 1583 two peaks | 1551 | 1577 two peaks |
| 6 | 1464 | 1455 | 1464 | 1455 |
| 7 | 1249 | 1266 | 1244 | 1266 |
| 8 | 1033 | 1033 | 1033 | 1033 |

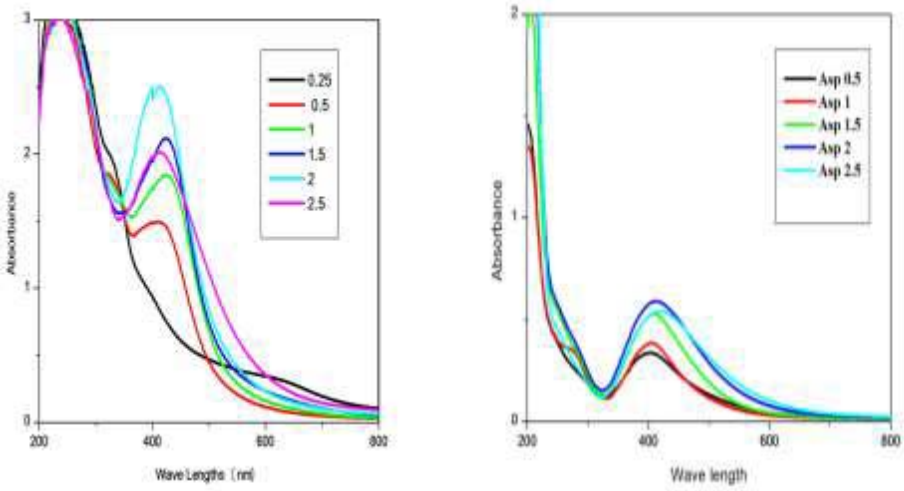
**(a) After** **(b) Before**

**Figure (1):** Silver nanoparticles production before and after optimizing the condition of *Aspergillus deflectus.*



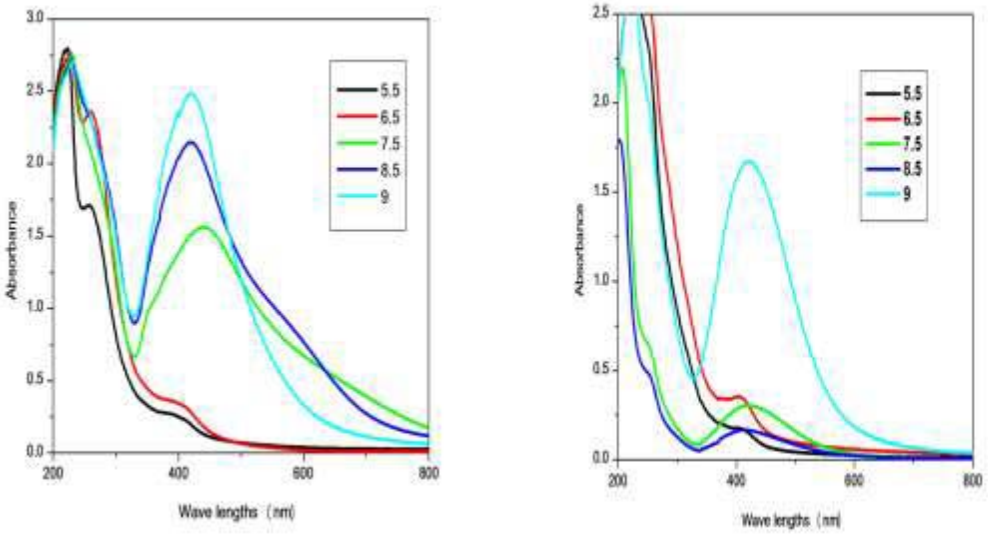
**(a) After** **(b) Before**

**Figure (1):** Silver nanoparticles production before and after optimizing the condition of *Penicillium pinophilum.*




(a) (b)

**Figure (3):** UV-Vis spectroscopy of the effect of different concentration on synthesis of SNPs of (a) *Penicillium pinophilum* and (b) *Aspergillus deflectus.*




(a) (b)

**Figure (4)** a: UV-Vis spectroscopy of the effect of different pH on synthesis of SNPs of (a) *Penicillium pinophilum* and (b) *Aspergillus deflectus.*

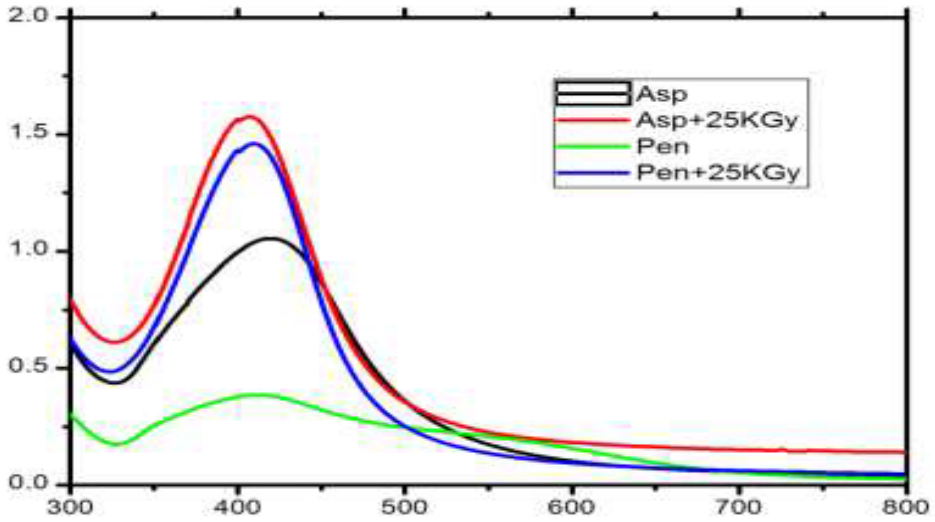



**Figure (5):** SPR of optimized culture and irradiation condition of *Penicillium pinophilum* and *Aspergillus deflectus*.

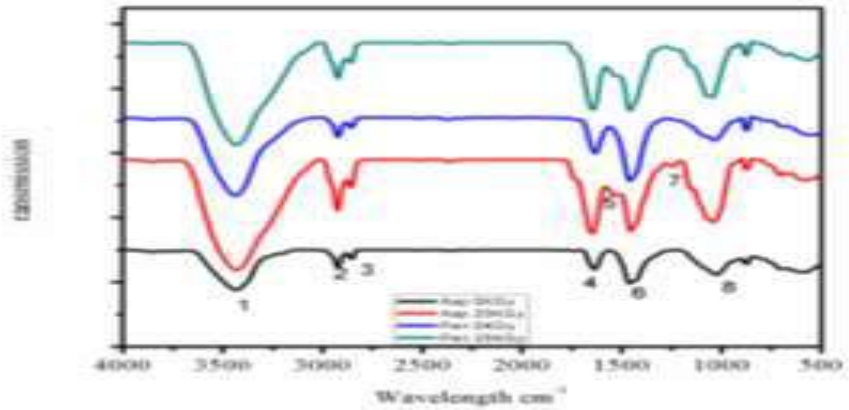



**Figure (6):** FTIR of optimized culture and irradiation condition of *Aspergillus deflectus* and *Penicillium pinophilum*

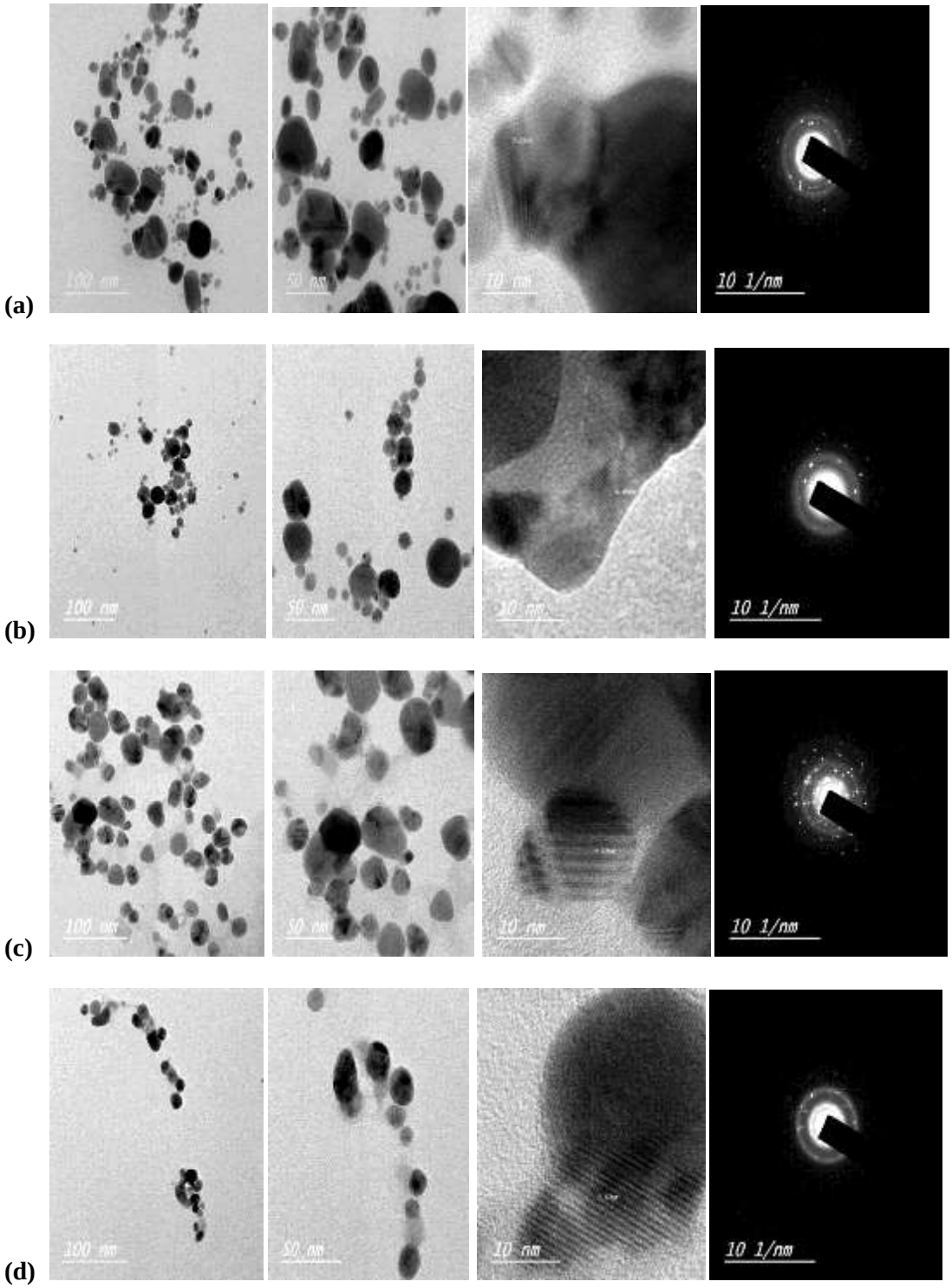



**Figure (7):** HRTEM of optimized culture and irradiation condition (a & b) of *Penicillium pinophilum* and (c & d) of *Aspergillus deflectus* respectively.

## CONCLUSION

The use of fungi in the synthesis of nanoparticle is a relatively recent addition to the list of microorganisms. The shift from bacteria to fungi as a means of developing natural "nanofactories" has the added advantage that downstream processing and handling of the biomass would be much simpler. The use of eukaryotes is potentially exciting since they secrete large amount of secondary metabolites. Therefore, the present study has reported the biological process for the synthesis of silver nanoparticles extracellularly using *Aspergillus deflectus and* intracellularly using *Penicillium pinophilum.* Parametric optimization study showed maximum absorbance at 2mM of $AgNO_3$ and pH 9.0. Further characterization was made by UV-Visible absorption spectroscopy which shows maximum absorption at 419 and 420 nm. High Resolution Transmission Electron Microscope (HRTEM) revealed the formation of spherical nanoparticles with and the particle size ranges from 6.57 to 23.7 nm.

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