Journal of Environmental Studies [JES] 2021. 24: 42- 52

Original Article

Fungus-Mediated synthesis of silver nanoparticles using Aspergillosis causing fungi

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doi:

ABSTRACT

Green synthesis of metal nanoparticles has gained great attention due to the need for safe, cost-effective and eco-friendly technologies for metal nanoparticles synthesis. Fungi have been represented as a novel field of study in nanotechnology. This study was focused on extra/intracellular synthesis of highly stable silver nanoparticles (SNPs) using aspergillosis causing fungi. Four strains of opportunistic fungi isolated from aspergillosis suspected patients at Assiut University Chest Department *A. flavus* KY609551, A. fumigatus MT994683, *A. niger* KY609552 and *A. terreus* MF852635 were kindly provided by Enas M. Amer, Faculty of Science of Assiut University tested for their ability to synthesize SNPs extra/intracellularly. Exposure of fungal cell filtrate to 1 mM of aqueous Ag^+ ions resulted in reduction of the metal ions and formation of silver nanoparticles. Visual observation showed the ability of the three different fungi namely *A. flavus* KY609551, *A. fumigatus* MT994683 and *A. terreus* MF852635 to produce SNPs. However, *A. niger* KY609552 could not gave SNPs at 1 mM of aqueous Ag^+ ions. The bioreduction of SNPs was monitored by ultraviolet-visible spectroscopy, and the SNPs obtained were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and X-ray diffraction. UV-visible spectra demonstrate a strong, quite narrow peak located between 435 and 450 nm was obtained in case of *A. flavus* KY609551 and *A. terreus* MF852635, respectively. Whereas a wide peak centering at 435 nm in case of *A. fumigatus* MT994683. To prepare the X-ray diffraction and FTIR for samples, a modified simple technique for extracting SNPs from aqueous solution using chloroform was used. It is the first time to be reported that used chloroform as an extract of SNPs from their aqueous solution, to facilitate FTIR and X-ray examination. X-ray diffraction confirmed the formation of metallic silver. FTIR spectroscopy analysis showed the presence of an amide group which appears to be responsible for biosynthesis and stability of nanoparticles for both *A. flavus* KY609551 and *A. terreus* MF852635.

KEYWORDS

Silver nanoparticles,

opportunistic fungi,

A. flavus,

A. fumigatus,

A. niger,

A. terreus.

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INTRODUCTION

Nanotechnology is concerned with the design and composition of nanoparticles in the nanometer range of 1-100 nanometers and controlling their size and shape (**Mansoori 2005**). The application of nanoscale materials and structures is an emerging area of nanotechnology.

Metal nanoparticles have been used in many scientific fields, in which silver nanoparticles of interest due to their surface resonance properties of the plasmon, which make it unique (**Stiufiuc *et al.* 2013**) and its antimicrobial effect (**Franci *et al.* 2015**). Silver nanoparticles are used in many fields such as chemical/biological sensors and biomedical

materials (**Evtugyn *et al*. 2014** & **Alon *et al*. 2014**), cosmetics (**Colvin *et al*. 1994**).

The manufacture of nanoparticles has several reported methods for the synthesis of SNPs using chemical, physical, photochemical and biological routes. Each method has advantages and disadvantages with common problems being, costs, stability, particle sizes and size distribution (**Aboul El-Nour *et al*. 2010**). Among the existing methods, biological methods are the most required method for producing nanoparticles as they are safe, low-cost technique and eco-friendly. Biological methods depend on the use of natural reducing agents such as polysaccharides, biological organism such as plants extract (**Shankar *et al.* 2003**, **Jha *et al*. 2009**, **Ahmad *et al.* 2011**, **Rao *et al.* 2014 & Elumalai *et al.* 2014**), fungi (**Mukherjee *et al.* 2001**, **Sastry *et al.* 2003**, **Bansal *et al.* 2005**, **Vigneshwaran *et al.* 2006**, **Ingle *et al.* 2009 & Verma *et al.* 2010**), yeast (**Kowshik *et al.* 2002, Jha *et al.* 2009, Arumugam and Berchmans 2011 & Kalathil *et al.* 2013**) and bacteria (**Saifuddin *et al.* 2009, Nanda and Saravanan 2009 & Sintubin *et al.* 2009**) to synthesize material either intra- or extracellularly. Thus, to be potentially utilized as eco-friendly nano factories (**Shankar *et al.* 2004, Mohanpuria *et al.* 2008**).

The fungal systems or myconanofactories have been exploited for the synthesis of metal nanoparticles. A large number of fungal strains are capable to synthesize SNPs extra/intracellularly, among which *Fusarium oxysporum* (**Mukherjee *et al.* 2001 & Ahmad *et al.* 2003**), *Neurospora crassa* (**Ingle *et al.* 2009**), *Aspergillus fumigatus* (**Bhainsa and D'Souza 2006**), *Aspergillus niger* (**Gade *et al.* 2008**), and *Aspergillus clavatus* (**Verma *et al.* 2010**) have been proven their ability to synthesize SNPs.

Fungi are much better compared to other microorganisms in many ways that they are easy to culture on a large scale by solid substrate fermentation, thus making a large amount of biomass available for processing; also, fungi can grow over the surface of inorganic substrate during culture. This leads to the metal being distributed in a more efficient way as a catalyst. Biosynthesis of nanoparticles of different elements is reported from both pathogenic and nonpathogenic fungi (**Vigneshwaran *et al.* 2007**). Therefore, the use of opportunistic fungi to produce nanoparticles because of having a high wall-binding capacity as well as intracellular mineral absorption capabilities, also produce large amount of enzymes per unit biomass.

The basis of the bio-synthesis process is the producing extra/intracellular biochemical compound that acts as a reducing agent and capping agent. The difference in the manufacturing mechanism between the extra/intracellular is related to the difference in biological factors used by the cell during the process. Where the fungal cell wall containing sugars play a role in the absorption and reduction of silver ions (**Meyer 2008**).

The extracellular secretions of reductive proteins are more and can be easily handled in downstream processing. And also, since the nanoparticles precipitated outside the cell is devoid of unnecessary cellular components, it can be directly used in various applications (**Narayanan and Sakthivel 2010**).

Therefore, it was of great significance to explore novel fungal strains for synthesizing SNPs based on the biodiversity specially aspergillosis causing fungi strains.

MATERIALS AND METHODS

I. Source of strains

The fungal Strains *A. flavus* KY609551, *A. fumigatus* MT994683, *A. niger* KY609552 and *A. terreus* MF852635 were kindly provided by Enas Amer from suspected patients at Assiut university hospital (**Enas *et al.* 2017**).

II. Biomass Preparation

II.1. Extra-cellular enzyme preparation

A. flavus KY609551, *A. fumigatus* MT994683, *A. niger* KY609552 and *A. terreus* MF852635 were grown in 250-ml Erlenmeyer flasks containing 100 ml potato dextrose incubated at 37°C. After 8 days, the mycelia mat was separated from media by filtration then extensively washed with sterile de-ionized water to remove any medium components from the biomass. Typically, 10 g of biomass (fresh weight) of each strain dependently was soaked in 100 ml de-ionized water and incubation at 37ºC on a rotary shaker (120 rpm) for 72 hrs. to obtain extra-enzymes under dark conditions. The cell filtrates were obtained by filtration using sterile Whatman filter paper no.1. The separated mycelia were re-used for detecting the intracellular products' activity as will be explained by (**Ahmad *et al.* 2003**).

II.2. Obtaining of the intracellular products using Bransonic® ultrasonic bath sonicator

The previously obtained mycelia of each strain were re-suspended (1 g/10 ml) in separate 250 ml sterile flasks and were held in Bransonic® ultrasonic bath sonicator at 35ºC. After 1 hr, the flasks were removed, and then filtered using the sterile Whatman filter paper no.1 to obtain the intracellular products from each fungal strain (**Aboul-Nasr *et al.* 2018**).

III. Biosynthesis of SNPs

For extra and intra-cellular biosynthesis of SNPs, 100 ml of cell filtrates derived from different fungal strains were mixed with 0.0169 g of AgNO3 (1 mM) and incubated at 37°C. A negative control (1 ml) contains a solution of 1 mM AgNO3 was exposed to the same experimental conditions. Also, one mL of each fungal filtrate was held as a positive control for comparison. All solutions were kept in dark to avoid any photochemical reactions during the experiment (**Ahmad *et al.* 2003 & Balaji *et al.* 2009**).

IV. Characterization of silver nanoparticles

Silver nanoparticles were characterized using visual-observation of change in color, UV–Vis spectrophotometer (JENWAY 7315 UV-Visible Spectrophotometer from 300-600nm), XDL 3000 powder X-ray diffractometer and Further characterization involved Fourier Transform Infrared Spectroscopy (FTIR) analysis of the dried powder of SNPs, by scanning it in the range 450–4000cm^{-1} at a resolution of 4 cm^{-1} (**Ahmad *et al.* 2003**).

RESULTS AND DISCUSSION

The color change of extra-cellular filtrate was observed visually from light yellow to reddish after two hours in case of *A. terreus* MF852635 and from four to five hours for *A. flavus* KY609551 and *A. fumigatus* MT994683, respectively (**Fig.1**). The color intensity of the cell filtrate with $AgNO_3$ was sustained even after 24 hrs. incubation, which indicated that the particles were well dispersed in the solution, and there was no obvious aggregation.

Whereas, when the intra-cellular filtrate was challenged with silver nitrate, a color change was observed after 4 hrs. in case of *A. terreus* MF852635, while intra-cellular filtrates of *A. flavus*

KY609551 and *A. fumigatus* MT994683 showed change in color after 6 hrs. However, there is no reduction of silver ions when the extra and intra-cellular filtrate was challenged with a concentration of (1mM) of silver nitrate in the case of *A. niger* KY609552. **Birla *et al.*** (**2013**) reported that the appearance of reddish color of fungal cell filtrate from light yellow due to excitation of surface plasmon after being challenged with silver ions resembles the formation of SNPs. Thus, color change of the solution clearly indicated the formation of SNPs. These results were consistent with the report of **Vigneshwaran *et al.*** (**2007**), **Jain *et al.*** (**2011**), **Li *et al.*** (**2012**) and **El Aziz *et al.*** (**2013**), which indicates ability of *A. terreus* and *A. flavus* in induced SNPs at concentration 1mM of $AgNO_3$. **Bhainsa** & **D'Souza** (**2006**) reported that SNPs can be formed extra/intracellularly by using *A. fumigatus*. **Sagar** & **Ashok** (**2012**) indicated the ability of *A. niger* to induce SNPs in a very high concentration of Ag^+, whereas **Gade *et al.*** (**2008**) reported that *A. niger* could produce SNPs at 1mM of silver ions concentration.

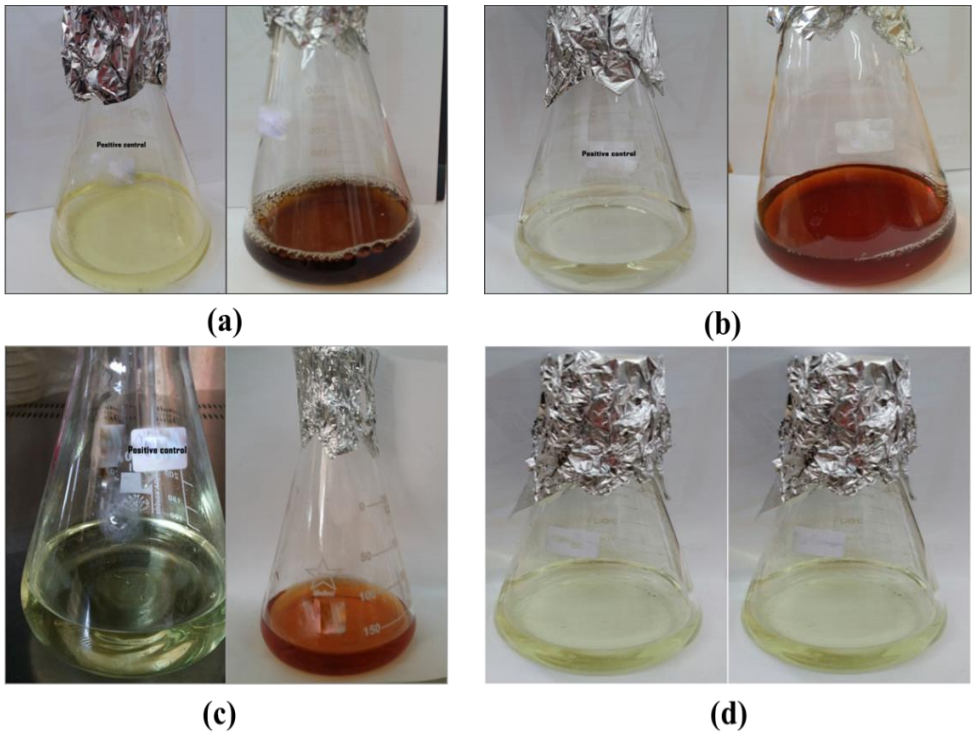

Figure 1: gradation in color four different fungal strains; a, b, c and d extra-enzyme filtrate in 24 hours after it was challenged with 1mM of silver ions from yellow to brown for *A.terreus* MF852635, *A. flavus* KY609551 , *A. fumigatus* MT994683 and *A. niger* KY609552, respectively

UV-Vis Spectrophotometers

Silver nanoparticle production was monitored by the change in color (**Fig. 1**) showed the uv–visible spectra of the silver nitrate solutions challenged with the four fungal strains. While no absorption band was observed in both controls (positive and negative). A characteristic surface Plasmon absorption band at 450 nm was observed in *A. terreus* MF852635. While in case of *A. flavus* KY609551 and *A. fumigatus* MT994683 intensity peak was at 440 nm and 435 nm, respectively in 24 hrs. which is characteristic surface plasmon resonance (SPR) peak of SNPs and hence confirmed their synthesis. After 72 hrs. of incubation, no change in intensity at 440 nm was observed indicating complete reduction of silver

ions (**Fig. 2**). The UV–visible spectra of the silver nitrate solutions challenged with the four fungal strains intra-cellular cell filtrate. *A. terreus* MF852635 the absorption spectra of nanoparticles were 445 nm after 24 hrs. and maximum absorption intensity over time was 450 nm after 48 hrs. while the intensity of absorption of silver nanoparticles synthesized by *A. flavus* KY609551, whereas the absorption of nanoparticles in case of *A. fumigatus* MT994683 was 440 nm and 445 nm, respectively after 24 hrs. (**Fig. 3**). Our results were matches with **Jain *et al.* (2011)** who reported that *A. flavus* NJP08 the UV-visible spectra of silver nitrate solutions when challenged with *A. flavus* NJP08, characteristic surface plasmon absorption band of SNPs was observed at 420 nm. **Bhainsa & D'Souza (2006)** reported that absorption maxima (λmax) of biosynthesized SNPs observed in the range of 420 nm in *Aspergillus fumigatus*.

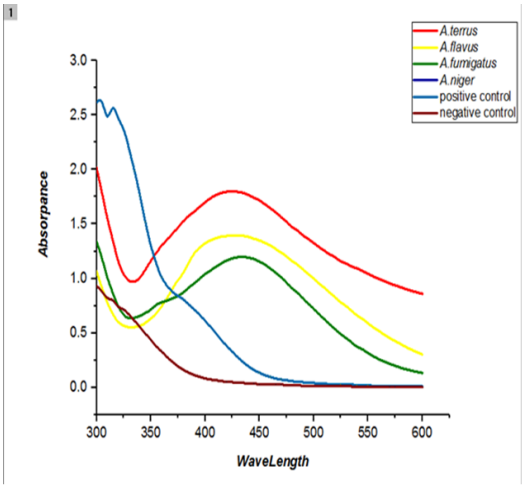

Figure 2 : UV-Vis. spectra of SNPs extract from extracellular enzyme of Aspergillus sp after 24h that *A.terreus* MF852635 SPR was (450 nm with OD=1.6) while *A.flavus* KY609551 UV-visible absorption spectra of biosynthesized SNPs was(440nm with OD=1.2) whereas *A.fumigatus* MT994683 was (435nm with OD=1.3).

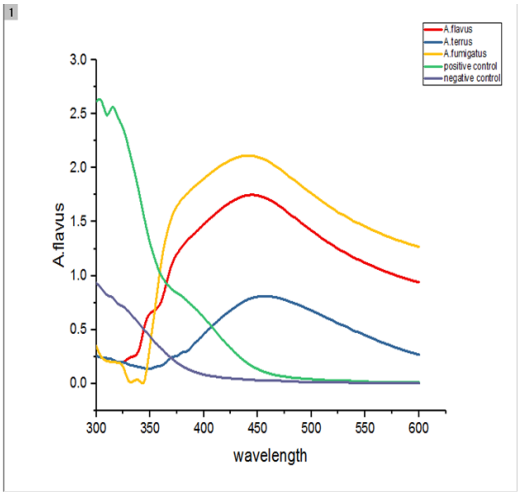

Figure 3: UV-Vis. spectra of SNPs extract from Intracellular enzyme of *Aspergillus* sp after 24h that *A.terreus* MF852635 SPR was (445 nm with OD=1) while *A.flavus* KY609551 UV-visible absorption spectra of biosynthesized SNPs was(440nm with OD=1.5) whereas *A.fumigatus* MT994683 was (445nm with OD=2) as OD values refer the concentration of silver nanoparticles, which gives a strong indication of the stability of these particles, as the more OD value greater than 1.5, the nanoparticles lose their stability.

Fourier transforms infrared spectroscopy (FTIR)

To identify biomolecules responsible for the reduction of the Ag^+ ions and stabilization (capping) bio-reduced silver nanoparticles synthesized by both fungal species at 37° C. Aqueous samples are commonly used to detect function groups that may exist and are responsible for nanoparticles synthesis. By using aqueous sample for FTIR analysis, it was found that (-OH) peak at 3306.90 cm^{-1} covered amines group (-NH) making it difficult to identify the characteristic protein groups responsible for biosynthesis nanoparticles, one peak at 1636.62 cm^{-1} refers to amide I (**Fig. 4**).

A simple modified method to overcome hiding an amides group in case of aqueous solution extract, a simple modified technique was used by extracting SNPs from the aqueous solution using chloroform. The chloroform extract was separated from the aqueous solution containing SNPs, collected by a separated funnel and let them to dry at room temperature **Aboul Nasr *et al.* (2018)**. FTIR spectrum revealed one peak at 1646 cm^{-1} that corresponds to the bending vibrations of the amide

I and amide II bands of the proteins, respectively; while their corresponding stretching vibrations were seen at 2918 cm^{-1}, respectively. While the peaks centered at 3351 and 3300 cm^{-1} are for N-H (amines group) stretching. It is well known that protein–nanoparticle interactions can occur either through free amine groups or cysteine residues in proteins and via the electrostatic attraction of negatively charged carboxylate groups in enzymes **Gole *et al.*** (**2001**). The peaks centered at 2960, 2871 cm^{-1} for (CH_3 mostly protein and lipid) and 2933 cm^{-1} are for (CH_2 mostly protein), while the peak in 1733.26 cm^{-1} is for C=O (carboxylic acid) stretching, typical of saccharides. The appearance of peak at 1376.40 and 1022.29 cm^{-1} can be assigned to the C–N stretching vibrations of the aromatic and aliphatic amines (Alkaline amine) (**Fig. 5**).

These observations indicated that the stability of silver nanoparticles is due to the presence and binding of proteins with silver nanoparticles. FTIR results revealed that the secondary structure of proteins has not been affected as a consequence of reaction with silver ions or binding with silver nanoparticles. It is important to understand, that it is not just the size and shape of proteins, but the conformation of protein molecules that plays an important role (**Jain *et al.* 2011**).

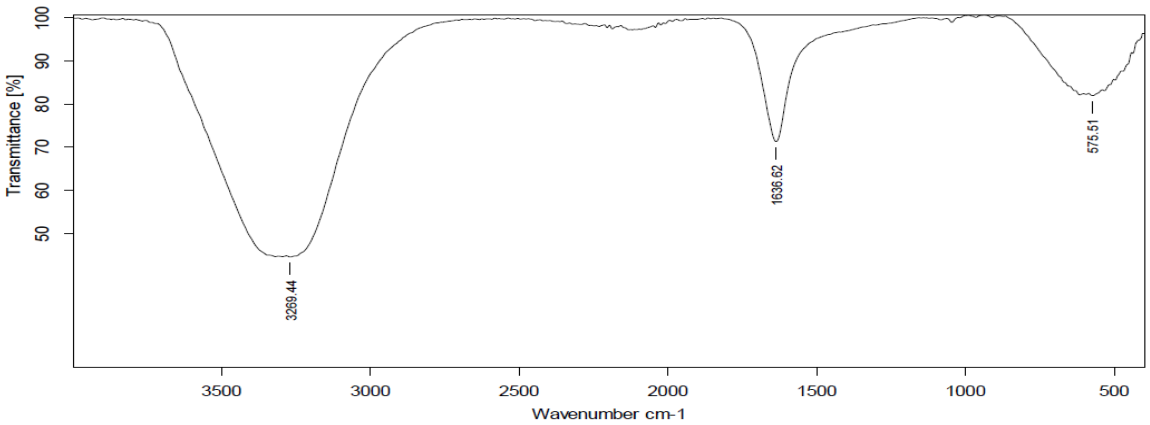

Figure (4) FTIR spectra of aqueous sample of silver nanoparticles at 37° C

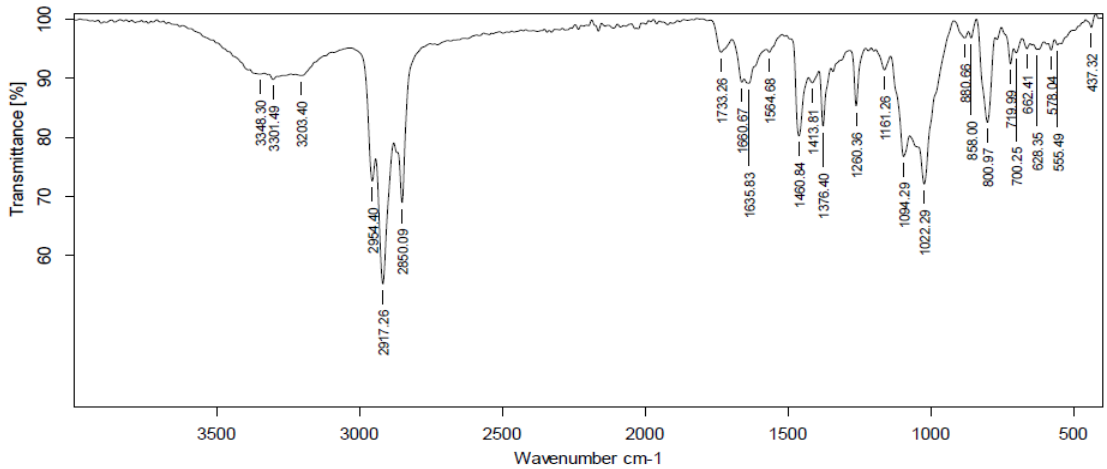

Figure (5) FTIR spectra of dried powder of SNPs extracted with chloroform at 37° C.

These results came in coincidence with **Fayaz *et al.*** (**2010**), **Jaidev** & **Narasimha** (**2010**) who observed that the stable and well dispersion of nanoparticles using FT-IR spectrum showed three distinct peaks, 3347.85, 1636.17 and 548.38 cm^{-1}. The peak at 3347.85 cm^{-1} indicated to the stretching vibrations of primary amines, while the peak at 1636.17 cm^{-1} indicated to carbonyl stretch vibrations in the amide linkage of proteins and 548.38 cm^{-1} is the fingerprint.

X-Ray diffraction

To prepare the sample in dried form, a simple modified technique was used by extracting SNPs from the aqueous solution using chloroform. The chloroform extract was separated from the aqueous solution containing SNPs collected by separated funnel and let them to dry by air gun **Aboul Nasr *et al.*** (**2018**).

The XRD pattern shows four intense peaks at 2Θ values (38.00, 44.28, 64.85, 77.44) corresponding to (111), (200), (220), (311), (222) planes of silver (**Fig. 6**). Thus, the XRD spectrum confirmed the crystalline structure of silver nanoparticles. These results correlated with **Prema** & **Rincy** (**2009**) and **Narasimha *et al.*** (**2011**).

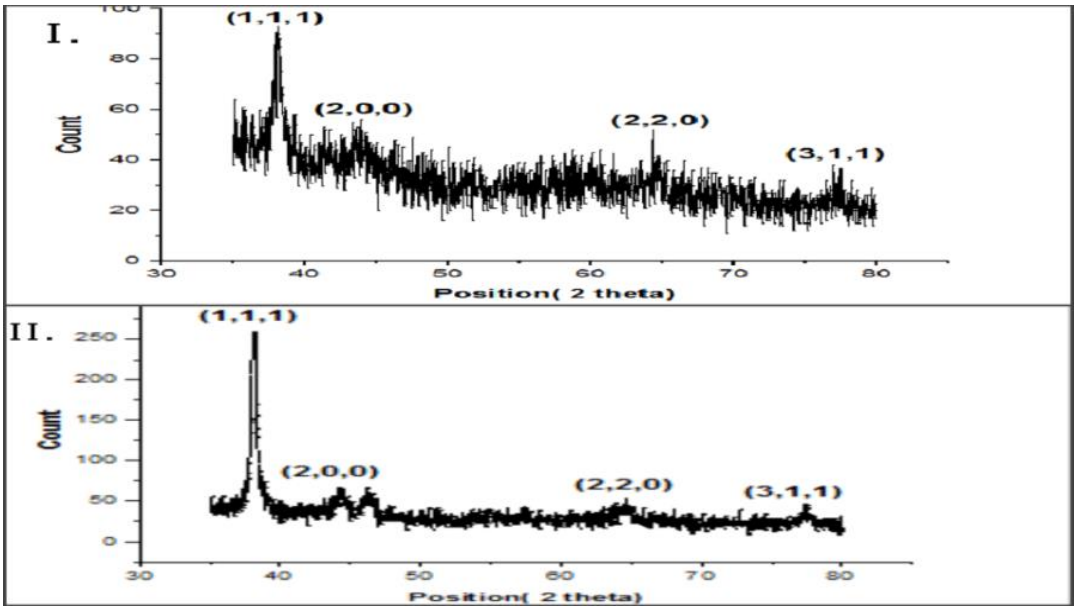

Figure (6). XRD pattern of silver nanoparticles synthesis by I.) *A. flavus* and II.) *A. terreus* at 37ºC. An intense peak corresponding to 2Θ = 38.00 indicates that the obtained SNPs are dominantly ruled by (111) planes.

CONCLUSION

In conclusion the enzymatic profile of opportunistic fungi makes them easily mediated SNPs induction extra/intracellularly in case of *A. flavus* KY609551, *A. fumigatus* MT994683 and *A. terreus* MF852635. Whereas *A. niger* KY609552 produced in high concentration of SNPs. Silver nanoparticles with higher stability was confirmed by using UV-visible spectra, which gives an indication of the constancy of the constituent particles, where SNPs induced by *A. flavus* KY609551 were more stable followed by *A. terreus* MF852635 and *A. fumigatus* MT994683. Extracting of the aqueous solution containing SNPs synthesized by *A. flavus* KY609551 and *A. terreus* MF852635 by simple methods modified with chloroform enhanced the results of FTIR by not hiding an amide group in case of using aqueous extract and gave better X-Ray diffraction identification of crystalline nature of the SNPs. The

chloroform extract was also the first to be used by the author to extract nanoparticles from its aqueous solution, which can be easily dried at room temperature and by an air gun, which gave a better result than the conventional methods.

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