BENI-SUEF UNIVERSITY JOURNAL OF BASIC AND APPLIED SCIENCES 4 (2015) 225–231

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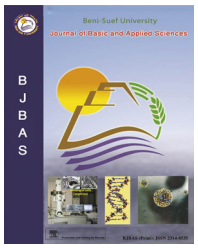

Full Length Article

Biosynthesis of size controlled silver nanoparticles by *Fusarium oxysporum*, their antibacterial and antitumor activities

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ARTICLE INFO

Article history:
Received 28 March 2015
Received in revised form 3 July 2015
Accepted 14 July 2015
Available online 5 September 2015

Keywords:
Biosynthesis
Controlled sized
Silver nanoparticles
Antibacterial
Antitumor activities

ABSTRACT

The biosynthesis method is thought to be clean, nontoxic and environmentally acceptable. Many microorganisms produce extracellular or intracellular metal nanoparticles with different efficiency, size and shape. The goal in this study is to control the size of silver nanoparticles. The preliminary screening of microorganisms, *Fusarium oxysporum* was selected to control size of silver nanoparticles. Parametric optimization showed smallest particle size when *F. oxysporum* treated with 10^{-2} M silver nitrate (metal ion concentration) at 50°C with 11 g wet biomass at pH 6 when fungal age 7 days when incubated for 72 h silver nanoparticles produced was characterized by TEM which revealed the formation of spherical, well-dispersed nanoparticles with size between 5 and 13 nm and FTIR gives the bands at 1619 and 1392.5 corresponding to the binding vibration of amide I and II bands of proteins, respectively. Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* showed maximum zone of inhibition of 2 mm and 1.6 mm, respectively, at 80 µL of silver nanoparticles. Cytotoxic activity was expressed as IC_{50} that is found to be 121.23 µg cm^{-3}.

1. Introduction

One of the most important aspects of nanotechnology is synthesis of nanoparticles (NPs) (one dimension less than 100 nm), which forms the core part of the nanomaterials. Nanoparticles possess more surface atoms than microparticles, which enhance their functional capabilities (Birla et al., 2013).

The nanoparticles of a wide range of materials can be prepared by a number of methods such as physical, chemical and biological. Generally, the physical methods have low yields and the chemical methods cause contamination due to precursor chemicals, use of toxic solvents and the generation of hazardous by-products (Wang et al., 2007).

Hence, there in a growing need to using environmentally friendly, safe, reliable and clean methods for the preparation

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http://dx.doi.org/10.1016/j.bjbas.2015.07.004

of nanoparticles (Zonooz and Salouti, 2011) that does not produce toxic wastes in their process synthesis protocol.

The metal microbe interactions have an important role in several biotechnological applications, including the fields of bioremediation, biomineralization, bioleaching and microbial corrosion (Bruins et al., 2000).

Unicellular and multicellular organisms are known to produce inorganic materials either intracellular or extracellular (Kumar et al., 2003; Peto et al., 2002; Sastry et al., 2004).

Examples: Bacteria for production of zinc sulfide (Labrenz et al., 2000), iron sulfide (Watson et al., 2000), silver (Klaus et al., 1999; Kowshik et al., 2003) nanoparticles and synthesis of nanoparticles of variable morphology using leaves of different plants, sprouts, roots (Shankar et al., 2003) and stems of live alfalfa plants (Gardea-Torresdey et al., 2003).

Three modes of AgNPs bioreduction were conducted namely: (i) bioreduction of silver ion by the tested fungi-secreted proteins in culture supernatant (CS), (ii) bioreduction of silver ion by adsorption of silver atoms on the mycelia pellet (MP), and (iii) bioreduction of silver ion from the mycelia pellet which was released into the silver nitrate solution (SN), respectively (Chan and Don, 2013).

The importance of bactericidal nanomaterial's study is because of the increase in new resistant strains of bacteria against most potent antibiotics. This has promoted research in the activity of silver ions and silver-based compounds, including silver nanoparticles (Singh et al., 2008). AgNPs have been proved to have great potential in anticancer activity because they are selectively involved in disruption of mitochondrial respiratory chain which leads to the production of reactive oxygen species (ROS) and interruption of adenosine triphosphate (ATP) synthesis, thereby causing nucleic acid damage. Excess free radicals generated in the body play a key role in many degenerative diseases of aging such as antitumor, antioxidant (Vasanth et al., 2014).

The purpose of this study was to screen a variety of bacteria and fungi for their ability to produce metallic nanoparticles. In addition, the potential to manipulate key parameters, which control growth and other cellular activities, to achieve controlled size of the nanoparticles was investigated.

2. Materials and methods

Fusarium oxysporum f. sp. *lycopersici* was obtained from Microbiological Resources Centre (Cairo, MIRCEN), Egypt. Potato Dextrose Agar medium (PDA) and Nutrient Agar medium (NA) were microbiological media for culturing fungus and bacteria.

2.1. *AgNPs biosynthesis by* F. oxysporum

F. oxysporum was grown up in Erlenmeyer flasks containing 100 ml PDA broth medium in incubator at 28 °C. After 5 days of incubation, the biomass was separated from the medium by filtration through Whatman filter paper no.1 and washed three times in sterile distilled water to remove any nutrient media that might interact with metal ions. Biomass was harvested and metal ions $AgNO_3$ was added to give an overall Ag-concentration of 1 mM (0.017/100 ml). The mixture was left for 5 days in incubator at 28 °C. The interactions were carried out in dark. The control was only metal ions without fungal biomass.

2.2. *Effect of parameters on controlling the size AgNPs*

To obtain the smallest size AgNps different concentrations of AgNO3 (10^{-1}, 10^{-2} and 10^{-3} M), temperatures 10, 25, 30, 35, 50, 60, biomass quantity, 3 g, 5 g, 7 g, 9 g, 11 g, 13 g, 15 g, 17 g and 19 g and 70°C, pH 5, 6, 7 and 8.

Particle sizing measurement of biosynthesized AgNPs was determined by Zeta Sizer nano-series.

2.3. *Characterization for silver nanoparticles*

Biosynthesized silver nanoparticles were characterized by Transmission Electron Microscopy (TEM) measurements. Particle sizing experiments were carried out by means of laser diffractometer using Zeta Sizer nano-series (Nano ZS). Measurements were taken in the range between 0.6:6000 nm. UV-Visible Spectroscopy at absorption range between 200-600 nm. The crystalline nature of AgNPs was confirmed by the analysis of XRD pattern. FT-IR spectra were recorded in the range 4000–500 cm^{-1}.

2.4. *Antibacterial assay*

The antibacterial activity of AgNPs synthesized by *F. oxysporum* was investigated against pathogenic bacteria *viz.*, the gram negative *Escherichia coli* and the gram positive bacteria *Staphylococcus aureus* by using agar well diffusion assay method. The test organisms were suspended in saline solution to give approximately OD 0.04 and OD 0.05 which were prepared on nutrient agar plates. Four agar wells were made on nutrient agar and each well was loaded with 20 µL, 40 µL, 60 µL and 80 µL, respectively, of AgNPs solution with AgNPs concentration 470.00 mg/L and incubated at 37 °C for 24 h. After incubation, the diameter of inhibition zone was measured using caliper.

2.5. *Antitumor activity*

MCF-7 Cell Culture: The human breast carcinoma cell line MCF-7 (MCF-7 is the acronym of Michigan Cancer Foundation -7, referring to the institute in Detroit where the cell line was established in 1973 by (Soule et al., 1973)) was cultured and used to evaluate the cytotoxic effect of the tested extracts at Nanotechnology & Advanced Materials Central Lab, Cairo, Egypt. Routine MCF-7 cell culture protocol was followed; in brief, cells were cultured in DMEM (Dulbecco's modified Eagle's medium, Lonza), which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/ml penicillin G sodium, 250 mg/ml amphotericin B and 100 units/ml streptomycin sulphate. The culture was maintained at 37 °C humidified with 5% CO_2 and for sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37 °C.

Water Soluble Tetrazolium salts (WST-1) assay: The cytotoxicological activity of various concentrations of the AgNPs under test (0, 20, 60, 100, 140, 180 and 220/μg cm^{-3}) were evaluated. The human breast carcinoma cell line (MCF-7). The selected doses were added to the cell monolayer in triplicate wells individual dose and its cytotoxicity was tested using a standard WST-1(4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate) assay as a fast and sensitive quantification of cell proliferation and viability (Ngamwongsatit et al., 2008).

The formazan dye produced by viable cells can be quantified by a multi well spectrophotometer (microplate reader) by measuring the absorbance of the dye solution at 450 nm. Data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50% of cell population IC_{50} (half maximal inhibitory concentration) was estimated exponentially. Cytotoxic activity was expressed as the mean IC_{50} of three independent experiments.

3. Results

Metal nanoparticles biosynthesis by *F. oxysporum*: The solution turned yellowish brown within a few hours after exposure to fungal biomass, an indication of extracellular nanoparticles synthesis. Control test without biomass addition stayed colorless, an indication that nanoparticle formation was mediated by the microbial culture.

In an attempt to achieve better size control, the effect of parameters such as metal concentrations, temperature, weight of fungus, pH and age of fungal biomass with different incubation time was studied by varying one parameter at a time, keeping the other experimental conditions the same.

3.1. *Effect of substrate concentration*

The smallest particle size was seen at a concentration of 10^{-2} M as shown in (Fig. 1A).

3.2. *Effect of incubation temperature*

As we increase the temperature, the particle size of AgNPs is decreased until it reaches the smallest size 30.24 nm at 50 °C (Fig. 1B).

3.3. *Effect of biomass wet weight of F. oxysporum*

The weight range of biomass from 3 g to 9 and from 13 to 19 leads to decrease in AgNps size. Eleven grams from biomass was found to be the optimal weight for smallest size (Fig. 1C).

3.4. *Effect of pH*

Figure 1D shows representative Zeta Sizer Nano histogram of NPs produced by *F. oxysporum* with smallest particle size at pH 6.

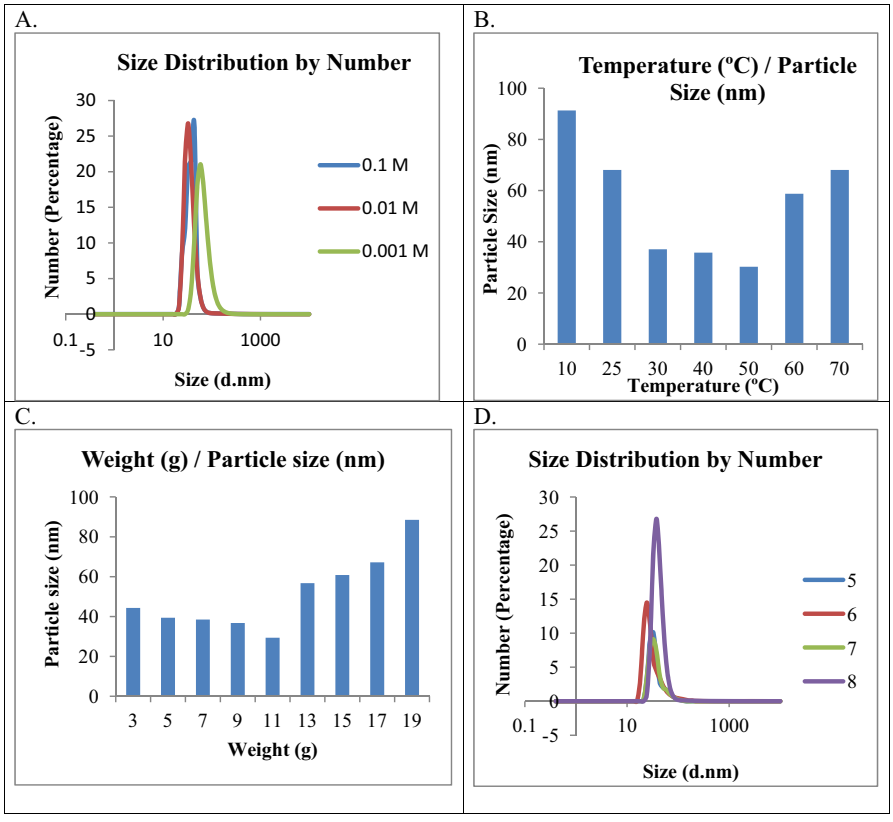

Fig. 1 – Parameters controlling size of biosynthesized AgNPs; metal ion concentrations (A), different temperatures (B), fungal biomass weight (C), effect of pH (D).

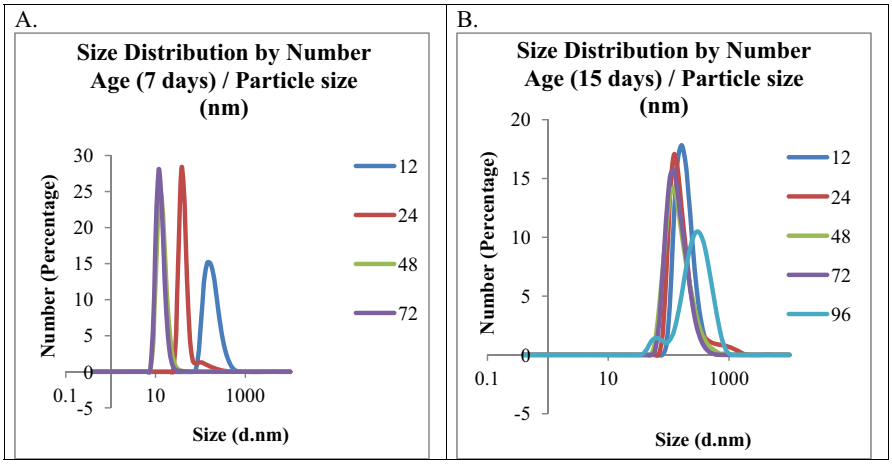

Fig. 2 – Effect of age of fungal biomass biosynthesis of AgNPs size, 7 days age (A), 15 days age (B).

3.5. *The effect of age of fungal biomass with different incubation time*

The result demonstrated that the particle size of AgNPs decreases as the fungal age in early stage with 7 days (Fig. 2A). But the old fungal biomass with 15 days (Fig. 2B) led to increase in AgNPs size. It is evident that maximum yield with smallest particle size was achieved at 72 h incubation time when the fungus age was 7 days.

3.6. *Characterization of silver nanoparticles*

3.6.1. *High resolution transmission electron microscopy (TEM)*

TEM measurements were used to determine the morphology and shape of nanoparticles. Fig. 3A showed that the difference in size and shape of AgNPs was biosynthesized before and after the optimization conditions. Revealed that the particles with *F. oxysporum* are spherical in shape and are uniformly distributed (mono dispersed) without significant agglomeration. The particle distribution before the optimization represented in the histogram shows that almost 70% of the particles are in the 15 to 40 nm range but after optimization it shows that almost 90% of the particles are in the 5 to 13 nm.

3.6.2. *Zeta sizer nano*

The analysis that was performed with Zeta sizer gives the particle size and size distribution.

3.6.3. *UV-visible spectroscopy*

The extracellular biosynthesis of AgNPs using *F. oxysporum* involves the bioreduction of silver ions in the filtrate. Reaction solution was given by periodic sampling of the reaction mixture at regular time intervals 12, 24, 48, 72 and 96 h by using UV-Visible spectroscopy. AgNPs synthesized shown maximum absorbance peak at 420 nm (Fig. 3B). The spectra clearly show the increase in intensity of silver solution with time, indicating the formation of increased number of AgNPs in the solution. According to this figure, there is no appreciable change in the UV-vis spectra of the reaction product after 72 h. An absorption band at ca. 270 nm is clearly visible and is attributed to aromatic amino acids of proteins.

3.6.4. *XRD spectrum*

The phase purity and composition of AgNPs are examined by XRD in Fig. 3C. The XRD spectrum showed four distinct diffraction peaks at 38.15°, 44.18°, 64.63° and 77.50° corresponding lattice plane value was indexed at (111), (200), (220) and (311) planes of face centered cubic (FCC) silver with a lattice parameter of a = 4.08 Å which were in good agreement with reference of FCC structure from joint committee of powder diffraction standard (JCPDS) Card No-087-0720.

3.6.5. *Fourier transform infrared spectroscopy (FTIR) analysis*

The nanoparticle samples were analyzed in FTIR to identify the possible biomolecules responsible for the reduction of the Ag^{+} ions to AgNPs by the cell filtrate. The FTIR spectrum is presented in (Fig. 3D). The bands of biosynthesized AgNPs from *F. oxysporum* were noticed at 1042.0, 1078.9, 1392.5, 1619.3, 2338.4, 2364.3, 2938.0, 3322.0 and 3654.0 cm^{-1} in the FTIR spectrum.

3.7. *Antibacterial assay*

Antibacterial assay of biosynthesized AgNPs was studied against pathogenic strains of *E. coli* and *S. aureus* using agar well diffusion method and zone of inhibition was shown in Fig. 4A and Table 1. The results showed that AgNPs were effective against both strains. In *E. coli*, the maximum zone of inhibition was 2 mm at 80 μL concentration. For *S. aureus*, showed 1.6 mm zone of inhibition at high AgNPs concentration 80 μL.

3.8. *Antitumor activity*

The WST-1 assay results revealed that fungal AgNPs synthesized by the present procedures have promising antitumor activity against human breast carcinoma cell line (MCF-7). The viability of the tested compounds in response to their concentration as illustrate in Fig. 4B. Cytotoxic activity was

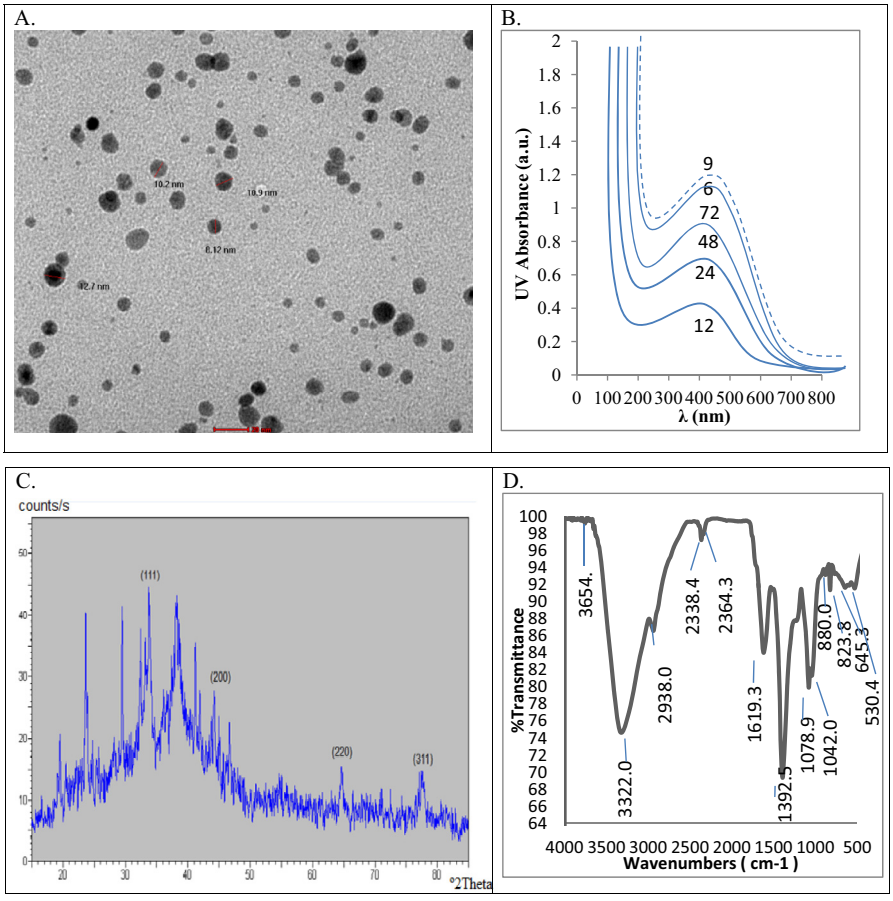

Fig. 3 – Characterizations of silver nanoparticles; TEM images (A), UV-Vis spectra at various times (12, 24, 48, 72 and 96 h (B), XRD patterns (C), FTIR spectrum (D).

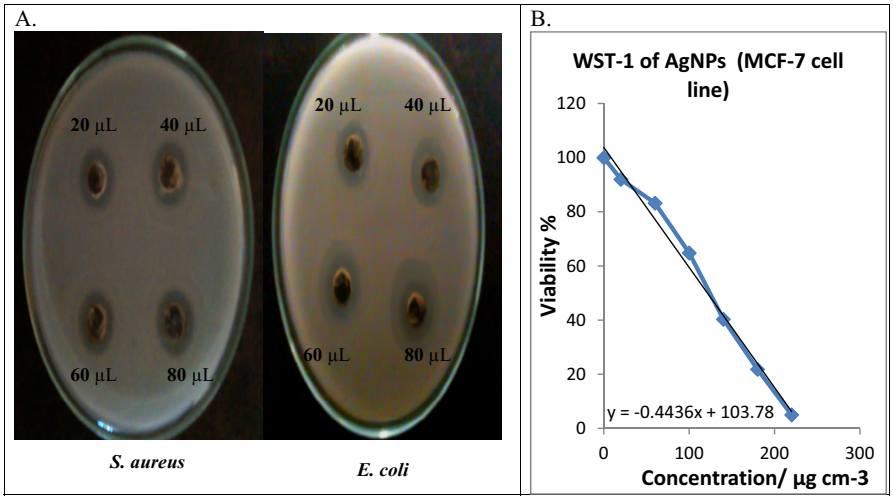

Fig. 4 – Application of AgNPs, Antibacterial activity of different concentrations of biosynthesized AgNPs (µL) against *E. coli* and *S. aureus* strains (A), viability chart of biosynthesized AgNPs against MCF-7 cell line (B).

expressed as the mean IC_{50} of three independent experiments; IC_{50} was found to be 121.23 µg cm^{-3}. The small values of IC_{50} for the fungal AgNPs prepared by the present method reveal higher and impressive efficiency as cytotoxic and anticancer drug.

4. Discussion

The present work aims to throw the light about the importance of microbes not only for NPs production but also indicates

Table 1 – Antibacterial activity in the term of mean diameter (mm) of AgNPs biosynthesized by *F. oxysporum* against *E. coli* and *S. aureus*.

Bacterial strains	Zone of inhibition (mm)			
	20 µL	40 µL	60 µL	80 µL
E. coli	1.6	1.8	1.9	2
S. aureus	1.4	1.5	1.5	1.6

the importance of them to control the size of NPs to be used in a wide variety of applications. In addition, apply these safety NPs as antimicrobial and antitumor.

Results recorded showed that at lower $AgNO_3$ concentrations, excess enzyme may have been available within the system, but higher production may not have occurred due to lack of substrate molecules. Alternatively, the presence of substrate in the medium may also serve to induce the release of enzyme from fungi, which may in turn reduce silver in the substrate to NPs. As more of the substrate molecules are supplied to the medium, the enzyme secretion by the fungi may proportionately increase until a threshold concentration which in this case was observed to be 0.01 M. Access addition of metal ions with concentration 10^{-1} M results in the presence of very large particles exhibiting irregularly shaped that most of the cell enzymes were consumed by reduction of particles, demonstrating the high capacity of the cells for silver reduction. This was in contrast with the results of many workers such as Prakash and Thiagarajan (2012) who reported 1.5 mM concentration for maximum AgNPs production.

At a temperature (50 °C), the majority of AgNPs were smaller. Further incubation at higher temperatures, the enzymes have denatured nature this lead to increase in particles size according to loss of enzyme activity. This result is close to result represented by Birla et al. (2013) which proved that 60 °C was the optimal temperature for maximum protein secretion.

By employing the variable weight of biomass, the effect of biomass amount on synthesis of small AgNPs was studied by Zeta-sizer analysis and provided evidence that increase in weight of biomass from 3 to 9 g increases the small particle size according to increasing the enzymes amount secreted by the biomass and with increasing the weight from 13 g to 19 g lead to increase in particle size. The amount of biomass plays a key role in synthesis or complete reduction of Ag^+ to Ag^0. The optimum weight for smallest particle size is 11 g.

The pH was found to be an important parameter affecting AgNPs synthesis in *F. oxysporum*. It was also proved that smallest size occurred at pH 6. At lower pH, protein structure gets affected and the protein becomes denatured and loses its activity so big size of NPs was observed (Banu and Rathod, 2011). The enzyme reductase catalyzing the synthesis is probably deactivated gradually as the conditions become alkaline, and this may be the reason for reduced synthesis and increase in size which is observed at higher pH values. A similar conclusion is reported on AgNPs production by *Penicillium fellutanum* (Kathiravan et al., 2014).

The results showed that the 7 days age of fungus as a young culture for 72 hours is better than 15 days of fungus age as the old culture for the same time of incubation.

Many reports provided evidence of synthesized AgNPs extracellularly by TEM images. Basavaraja et al. (2008) reported that the particles of AgNPs range from 8 nm to 60 nm with polydisperse and mostly spherical in shape using *F. semitectum* and Ahmad et al. (2003) also reported that the AgNPs in size range 5–50 nm, with spherical and triangular shape by *F. oxysporum*.

Our UV-Vis spectroscopy result showed that the strong surface plasmon resonance at ca. 413 nm that increases the intensity with time and reach the stabilization point after 48 h of reaction.

Many reports with agreement with our XRD result. Mahmoud et al. (2013) that good agreement with the unit cell of the face-centered cubic structure.

Basavaraja et al. (2008) showed agreement with FTIR spectrum using *F. semitectum* that the presence bands of amide I and amide II and arises due to carbonyl stretch and –N-H stretch vibrations in the amide linkage of the protein. This result showed that the protein molecules not only act as reducing agent but also act as stabilizing agent by binding the AgNPs through free amino groups or cysteine residues or through electrostatic attraction of negatively charged carboxylate groups in extracellular enzyme filtrate from fungal mycelia.

In summary, *F. oxysporum* has the ability to synthesize AgNPs with different size according to the factors affecting it. The smallest particle size was observed when the substrate concentration 10^{-2} M was incubated at 50 °C with biomass weight 11 g at pH 6 with young biomass culture with 7 days for 3 days. These nanoparticles can be used as antimicrobial agent against gram positive and gram negative bacteria. Small sized NPs showed more antibacterial activity than large size particles because small sized particles affect a large surface area of the bacteria (Vanaja and Annadurai, 2013). This was in agreement with the results of Das et al. (2013) and Singh et al. (2014) that proved gram positive bacteria are more susceptible to AgNPs than gram negative.

5. Conclusions

The extracellular synthesis of AgNPs of various morphologies and sizes in fungal culture *F. oxysporum* has been investigated. The size of the NPs was manipulated by controlling environmental and nutritional parameters. These AgNPs were proved to be powerful weapons as antibacterial and antitumor.

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