Acta Scientific Pharmaceutical Sciences

Volume 1 Issue 4 October 2017

Research Article

Instantaneous Biosynthesis of Extracellular Silver Nanoparticles Using *Emericella quadrilineata* and its Antibacterial Efficiency

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Received: June 19, 2017; **Published:** September 29, 2017

Abstract

Microbiological methods are considered good alternative to both chemical and physical procedures for synthesis of silver nanoparticles (AgNPs). In this work, extracellular AgNPs has been biosynthesized by a safe and fast way using cell-free filtrate (CFF) from the fungus *Emericella quadrilineata*. The process was completed instantly after incubating CFF with 1 mM silver nitrate ($AgNO_3$) at 100°C and pH 10. The absorption spectrum of the synthesized NPs showed single-band absorption with peak maximum at 425 nm corresponding to the plasmon resonance absorption of AgNPs.

Biosynthesis conditions those activate nitrate reductase (NR) enzyme caused parallel enhancement of NPs formation. Fourier transform infrared (FTIR) spectral analysis indicated that certain proteins in the CFF are responsible for synthesis and/or stabilization of the biosynthesized AgNPs. The micrographs obtained from the high-resolution transmission electron microscope (HRTEM) showed the production of monodispersed NPs. They are of spherical shape with average size of 6 nm in the range of 2 - 26 nm. These NPs revealed reasonable antibacterial activity. When added to the antibiotic ciprofloxacin its antimicrobial activity was enhanced suggesting that they can be used as adjuvant for the treatment of bacterial infections.

Keywords: Instantaneous biosynthesis; Emericella; Silver nanoparticles; Antibacterial efficiency

Introduction

Nanotechnology has emerged as a rapidly growing field with various applications in science and technology. Since the early 1990s, the number of publications on NPs has grown exponentially with no sign of slowing down. Metallic NPs are being viewed as the future material and represent the starting point for nanostructured materials and devices [1]. Traditionally, NPs can be produced through physical and chemical protocols [2-4] although their low efficiency with regard to environmental and economic parameters. The development of biologically inspired experimental process for synthesis of NPs is evolving into an important branch of nanotechnology [5]. Biological methods for NPs synthesis have been avoided drawbacks of the chemical and physical methods and suggested as possible eco-friendly alternatives to them. However, biosynthesis of NPs is generally more time consuming. It requires several hours [6-8] or days [9-11] to form NPs. Some fungi were recorded in the last years to have good ability of synthesizing the extracellular AgNPs using their CFFs including species of *Aspergillus* [8,9], *Fusarium* [12-14], *Penicillium* [15,16] and *Trichoderma* [17].

Biosynthesis of AgNPs attracted attention of some investigators due to their diverse properties and uses. AgNPs have many applications in various areas including pharmaceutical and other biomedical applications [18,19]. Resistance in human pathogens is a big challenge in the biomedicine. Improvement of the potentialities of the antimicrobial agents is the main interest of some researchers who shifted their research works toward NPs in general and AgNPs in particular to solve the problem of bacterial multidrug resistance [20]. Because of their change in physiochemical properties, AgNPs have emerged as antimicrobial agents owing to their high surface-area-to-volume ratio and the unique chemical and physical properties [21]. Some researchers studied the antimicrobial efficiency of AgNPs synthesized by fungi and reported the possibility of utilizing them in fighting both bacterial and fungal pathogens causing human diseases [8,14,22].

The previous review reveals that there have been tremendous developments in the field of microorganism-produced AgNPs over the last decade. However, much work is needed to improve the biosynthesis efficiency especially shortening the period required to complete the process and the present work is a trial in this respect.

Materials and Methods

Chemicals and Glass Wares

All chemical used were of analytical grade. The reagent solutions were made with deionized water. $AgNO_3$ was purchased from Sigma. The glass wares were washed with aqua regia (3:1 HCl-HNO_3) and then thoroughly rinsed with deionized water to remove any metal contaminant.

Organism and Cultivation Conditions

The fungus *Emericella quadrilineata* (Thom and Raper) Benjamin AUMC 8636 is one of our lab collections. It was isolated in our lab from soil samples collected from the western province of Saudi Arabia [23] and identified by Assiut University Mycological Centre (AUMC) where it deposited with its accession number. This fungus was selected in a preliminary study to access potentiality of the available fungi on the biosynthesis of AgNPs. It was routinely grown on Czapek's- agar medium at 30°C and sub-cultured whenever required. The fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml MYPG medium having the following composition (g/100 ml): malt extract, 0.3; yeast extract, 0.3; peptone, 0.5 and glucose 1.0 [12]. The flasks were sterilized, left to cool, the fermentation medium was initially adjusted to pH 9 and inoculated with one ml of spore suspension (10^7 conidia) obtained from 7-day-old culture. The cultures were incubated on rotary shaker adjusted to 150 rpm at 30°C for 96h.

The fungal pellets were separated from the metabolic solution by centrifugation at 3000 rpm for 10 min and washed thrice with sterilized deionized water to get rid of the remaining biological molecules. Ten grams of the wet biomass was brought in contact with 100 ml of sterilized deionized water at 30°C, agitated at a velocity of 150 rpm for 72h and the mixture of biomass and CFF was separated as above.

Extracellular Biosynthesis of AgNPs

A triplicate set of 250 ml Erlenmeyer flasks each containing 90 ml of the CFF and 10 ml of 10 mM $AgNO_3$ in deionized water was added and mixed well so the final concentration of $AgNO_3$ would be 1mM. A positive (CFF) and negative (1 mM $AgNO_3$) controls were also checked for comparison. All sets were kept under agitation at 150 rpm in the dark. Change in the solutions toward the brown color was taken as preliminary sign of AgNPs formation but their formation was confirmed spectrophotometrically.

Optimization of the Reaction Conditions

Optimization of parameters affecting the biosynthetic process was achieved by the traditional one-variable-at-a-time strategy involving changing one independent variable. To study the effect of reaction temperature on formation of the AgNPs, 9 ml of CFF in test tubes were put separately in water bathes with different temperatures ranging from 30 to 100°C. To each tube one ml of 10 mM $AgNO_3$ in deionized water was added to obtain a final concentration of 1 mM, pH was adjusted to 9 in all cases and incubation completed as above. In the same way, different tubes containing the above reaction mixture but incubated at 100°C were used for studying the effect of pH value in the range of 6-12. In the last step, tubes containing the CFF and different concentrations of $AgNO_3$ were adjusted at pH 10 and incubated at 100°C.

Characterization of the Synthesized NPs

UV-Visible Spectroscopy Analysis

The bioreduction of Ag^+ in the $AgNO_3$ solution incubated with CFF was monitored by measuring the UV-Vis spectrum of the reaction medium. Absorption measurements were carried out at wavelengths from 300 to 800 nm using a double beam spectrophotometer (Metash UV-Vis, model UV-8500) at a resolution of 1 nm. UV-Vis analysis of several weeks old samples was also carried out to check stability of the biosynthesized AgNPs.

For the additional characterization tests, the produced AgNPs were separated out by centrifugation at 17000 rpm for 15 min. The settled AgNPs were washed three times with deionized water. When required the precipitated AgNPs either air-dried or re-dispersed in deionized water by ultrasonication (Chem Tec Ultrasonic Processor UP-500, SN: UH005-0076) to get rid of any uncoordinated biological molecules.

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR is a powerful tool for identifying types of chemical bonds in a molecule by producing an IR spectrum that is like a molecular fingerprint[1]. The AgNPs as well as the bio transformed products present in the CFF were separated by centrifugation and dried. FTIR spectra of the dried samples were recorded using FTIR Nicolet IS10 Spectrometer. All measurements were carried out in the range of 500-4000 cm^{-1} at a resolution of 4 cm^{-1}.

High Resolution-Transmission Electron Microscopy (HR-EM)

The morphology and size of AgNPs were performed in nanotechnology and advanced material central lab (NAMCL), Agriculture Research Center, Cairo. For this purpose, an aliquot of an aqueous suspension of AgNPs was transferred onto a carbon coated copper grid. Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. The grid was then scanned using a Tecnai G20 (Fei, Netherland) HR-TEM operated at a voltage of 200 kV.

Zeta Potential Measurement

Zeta potential of AgNPs was evaluated using a Malvern Zetasizer Nanoseries Nano ZS (Malvern Instruments Ltd, Malvern, UK). An aqueous suspension of AgNPs was filtered through a 0.22 µm syringe driven filter unit before measurement. Data obtained were analyzed using Zetasizer software.

Nitrate Reductase (EC 1.6.6.4) Assay

The bioassay technique was that adopted by Harley [24] depending upon potentiality of nitrate reductase (NR) in converting nitrate to nitrite. The enzyme activity was calculated based on the increase in nitrite over 60 min for the amount of sample 10 ml. One unit of enzyme activity as tentatively defined as the amount of enzyme that activates formation of one µmol nitrite/h/ml.

Effect of Temperature and pH on Enzyme Activity

Optimum temperature of the enzyme activity was determined by incubating the assay at various temperatures ranging from 30 to 100°C and adjusting the pH to 9. The optimum pH of the enzyme activity was investigated by taking measurements in different pH buffers (0.2 M) to cover the pH range of 6 - 12. These buffer solutions were phosphate (pH 6 - 8), Tris-HCl (pH 9 - 10) and glycine-NaOH (pH 11 - 12) at the optimum temperatures obtained in the previous step.

Antibacterial Assay

The AgNPs suspended in deionized water were examined for its antibacterial activity by a standardized single disk method [25]. Three gram-positive bacterial strains i.e. *Bacillus subtilis* MTCC 736, *Micrococcus luteus* MTCC 2522, *Staphylococcus aureus* MTCC 737 and the gram-negative *Escherichia coli* MTCC 1687 were used for this assay that was performed in triplicates. Petri plates are used with 20 ml of melted nutrient agar medium (5 - 6 mm in depth). The bacterial strains were grown on nutrient broth medium for 24h prior to the experiment. One ml of the bacterial suspension containing 106 cells was mixed well with the medium by gently swirling the plates on the table top. Sterile filter paper discs approximately 5 mm in diameter were impregnated with solutions of AgNPs and the antibiotic ciprofloxacin as positive control, dried, placed on the surface of the solidified seeded medium and gently pressed down to ensure contact. The plates were incubated at 37°C for 24h and diameters of the inhibition zones were measured and expressed as the mean values.

Results and Discussion

Biosynthesis of AgNPs

AgNPs are well known to exhibit a brown color in aqueous solutions [26] due to excitation of surface plasmon resonance (SPR). The aqueous $AgNO_3$ when exposed to the CFF from the fungus *E. quadrilineata* was reduced and the color of the mixture was changed from yellowish to brownish after1:45h at 30°C of shaking at 150 rpm in the dark (Figure 1).

Figure 1: Color change of CFF on addition of 1 mM $AgNO_3$. (No change in color observed in negative (a) and positive (b) controls).

The change of color is a preliminary indication for the formation of AgNPs. UV-Vis absorption spectroscopy is an important tool to confirm the formation of metal NPs [27]. Monitoring the resulting color by UV-Vis spectroscopy showed a SPR absorption band with a maximum of 425 nm suggesting the formation of spherical NPs in the reaction mixture [28,29].

Optimization of AgNPs biosynthesis

Increasing the yield and rapidity of formation are important requirements for efficient production of the metal NPs. The length of time required for the formation of NPs is the main drawback of the biological procedures. Optimization of the conditions affecting this process was explored in the hope of shortening the time of AgNPs biosynthesis using CFF from *E. quadrilineata* as well as increasing their yield. Two group of factors were investigated, the first concerned with the reaction conditions (reaction temperature, pH and $AgNO_3$ concentration) and the other with production of CFF (age of the biomass, incubation temperature, pH and velocity of shaking).

Effect of Reaction Conditions

Reaction temperature

The results (Figure 2) reveal that increasing the reaction temperature was accompanied by a decrease in the time required for the biosynthesis until reached few seconds at 100oC realizing the shortest time for

any studied fungal solution. This can be accounted to the high activation energy and speed reduction activity of reducing agents present in the CFF at 100oC as reported earlier [30]. Since 100oC showed the best results, further studies were all carried out at this temperature.

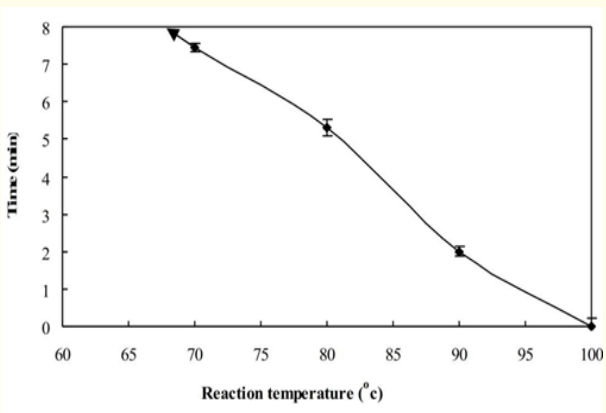

Figure 2: Effect of Reaction Temperature on the Time Taken by CFF to Biosynthesize Agnps.

pH value

Biosynthesis of AgNPs using CFF from *E. quadrilineata* was greatly affected by change of pH value of the reaction mixture. The results (Figure 3) demonstrate that AgNPs were efficiently synthesized in the alkaline solutions with pH values 9-12 especially pH 10 where the highest yield was obtained. At pH values 6 and 7 there was no change in color hence no production of NPs was observed. At pH 8 slight coloration with feeble spectrum was recorded. The activating effect of the alkaline pH values on the biosynthesis of AgNPs was previously recorded [31] using lactic acid bacteria. They attributed this finding to the increase in competitions between protons and metal ions for negatively charged binding sites. Moreover, the alkaline conditions were reported to increase the ability of the enzymes responsible for the synthesis of metal NPs [32].

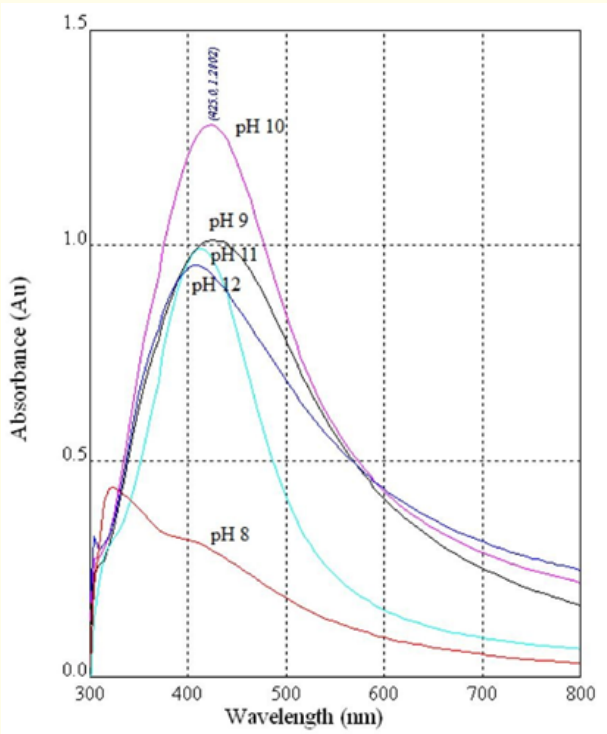

Figure 3: UV-Vis absorption spectra of AgNPs synthesized at different pH values.

Concentrations of $AgNO_3$

Concentration of $AgNO_3$ in the reaction mixture plays an important role in the bioreduction of Ag+ to Ag0. Figure 4 depicts the absorption spectra of AgNPs biosynthesized in presence of different concentrations of $AgNO_3$. The yield of AgNPs was increased with increasing salt concentration up to 1 mM where the maximal yield was achieved. An intense peak centered at 425 nm was observed in presence of 1 mM $AgNO_3$. Higher and lower concentrations changed dramatically this ideal picture. In presence of 0.1 and 0.5 mM a blue shift of 20 nm was detected indicating less stability of AgNPs. On the other hand, the presence of 5 mM com-

pletely prevented the bio reduction process. A precipitate (Inset to Figure 4) was clearly observed in presence of this high concentration suggesting precipitation of certain important proteins. These proteins may include NR enzyme and other proteins acting as reducing and/or capping agents.

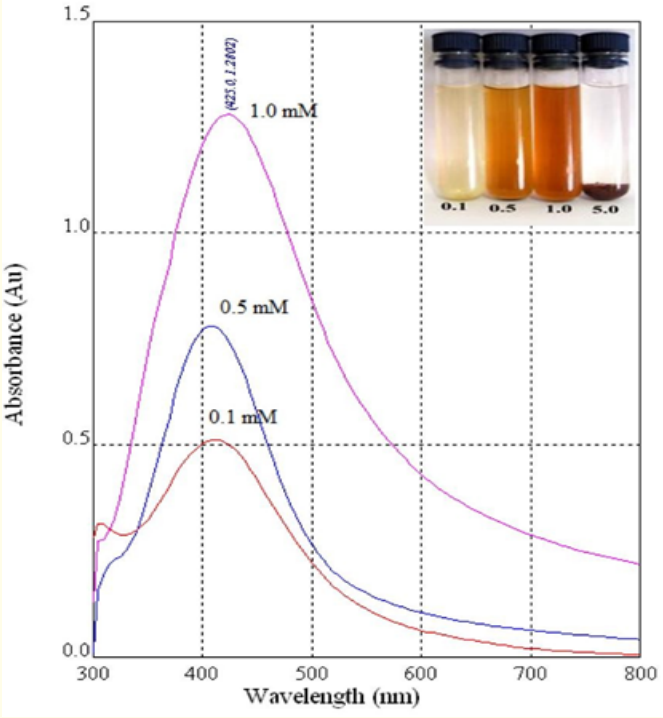

Figure 4: UV-Vis absorption spectra of AgNPs produced in presence of different concentrations of $AgNO_3$. Inset shows color change of CFF on addition of different concentrations of $AgNO_3$

Activity of NR

Activity of the enzyme NR was detected in the CFF with a maximal activity at 90oC (Figure 5a) and pH 9 (Figure 5b). Since these conditions are very near those required for the highest biosynthesis of AgNPs, it can be expected that the enzyme may have a role in this process.

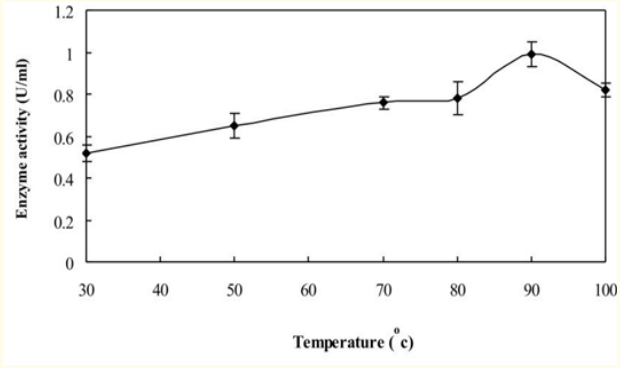

Figure 5a: Activity of NR from E. quadrilineata CFF at different temperatures.

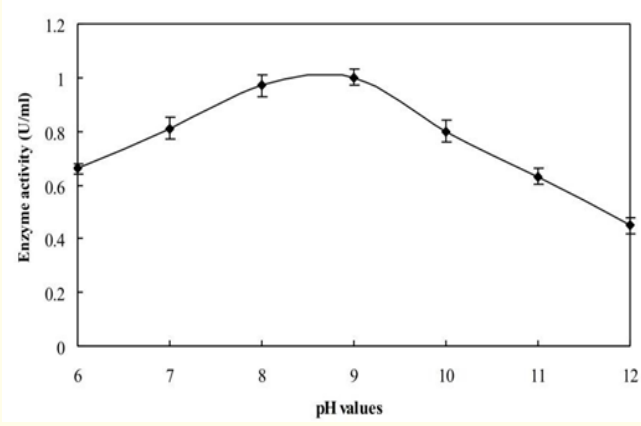

Figure 5b: Activity of NR at different pH values.

Effect of culture conditions

Effect of age of the biomass, incubation temperature, pH values, velocity of shaking on formation of CFF and in turn on the biosynthesis of AgNPs as well as on NR activity was then studied and the results are collectively presented in Figure 6. The results obtained in this work showed that the CFF formed by the fungal pellets under the studied conditions produced a variable effect on the yield of the biosynthesized AgNPs. The highest yield was obtained using the CFF secreted after 72h of shaking using 150 rpm at pH 9 and 30°C.

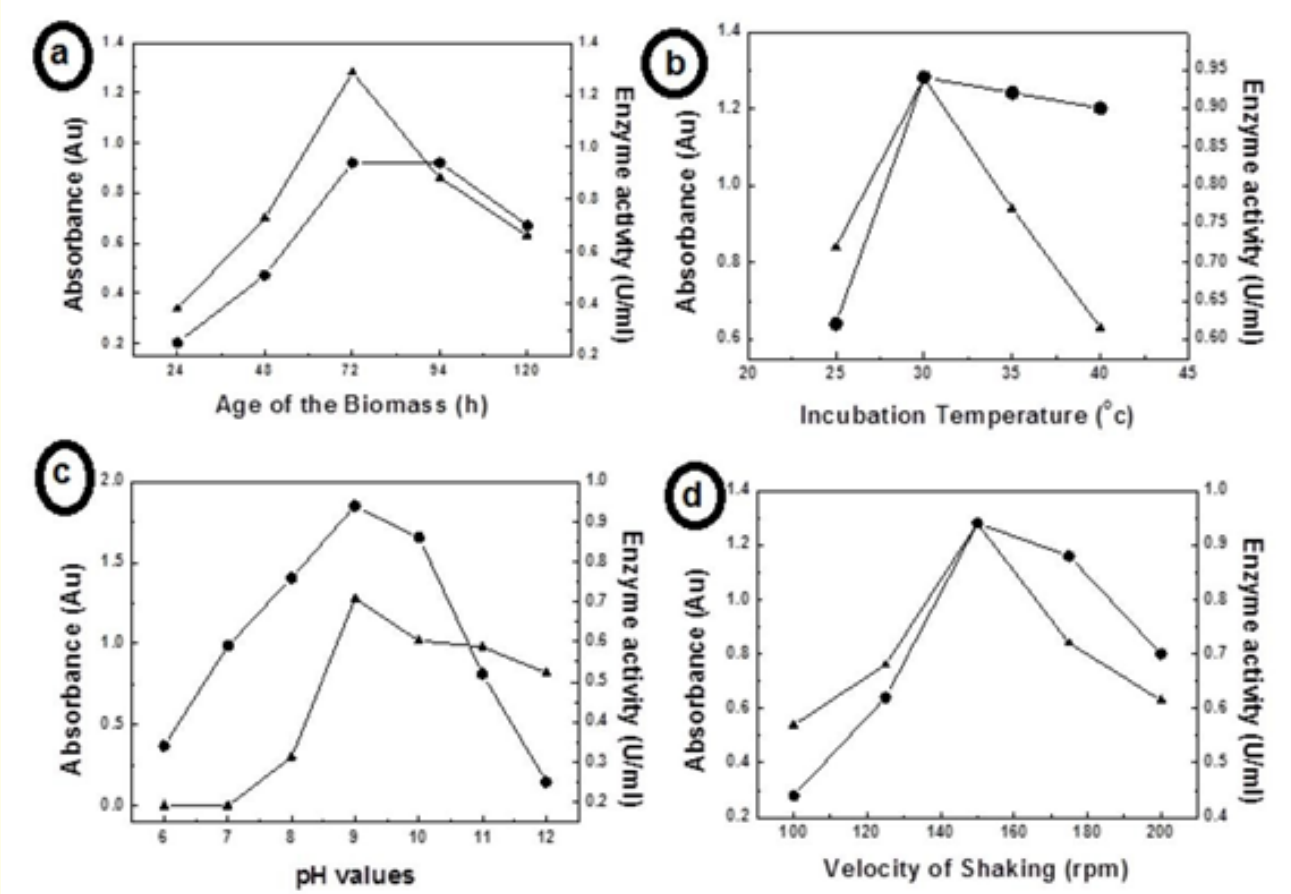

Figure 6: Biosynthesis of AgNPs (AU, —▲—) and NR activity (U/ml, —●—) using CFF from E. quadriliuneata as influenced by age of the biomass (a) incubation temperature (b) pH values (c) and velocity of shaking (d)

The fluctuation effect of the CFF mostly was a result of the change of its composition under various conditions. Under the best favorable conditions for the biosynthesis of AgNPs, activity of NR was at its maximum confirming its expected role. These findings accord with those reported previously [33] on the production of certain concentrations of enzymes and proteins that are actively involved in accumulation and reduction of the metal ions. Moreover, other workers [34] pointed out to the importance of optimizing NR activity to bring about enhanced NPs synthesis by *Bacillus licheniformis*.

FTIR Spectrum of CFF

In addition to the enzyme NR, certain biomolecules were suggested to play a role in the biosynthesis process. The FTIR spectra were recorded to identify potential biomolecules that contributed to the reduction of the silver ions and/or to the capping of the bio reduced AgNPs [35]. FTIR spectrum of CFF from *E. quadrilineata* (Figure 7) manifests the presence of many functional groups that may play a role in both reduction and /or capping of the AgNPs.

The band appearing in the region of 3000 - 3600 with a peak at 3442.41 cm^{-1} assigned to the starching of N-H of amide and amino groups in the protein [32]. The broadening in this band is the summation of association intermolecular hydrogen bonds arising from -NH2 and -OH groups in protein molecules which becomes much broader after the reaction with silver ions. The band observed at 2919.71 cm^{-1} is assigned to stretching of aliphatic C-H in the protein [32]. The FTIR showed the presence of two bands at 1646.43 and 1553.88 cm^{-1} correspond to the amide I and amide II those arise due to carbonyl stretch and N-H stretch vibrations in the amide linkages of the proteins, respectively [36]. Existence of the peak at 1405.03 cm-1 relates to symmetric stretching vibrations of -COO groups of amino acid residues with free carboxylate group in the protein [37]. The peaks at 1084.39 and 1021.10 cm^{-1} showed aliphatic amines with C-N vibration [7] and C-O single bond [38], respectively. The band at 706.28 cm^{-1} can be assigned to aromatic C-H bending. In addition, two new bending peaks were observed at 1384.35 and 800.33 cm^{-1} after formation of the Ag-

NPs. The amide and carboxylate bands in the spectrum of the CFF were shifted to lower wavenumbers after formation of AgNPs, due to coordination of these function groups to silver atoms [39]. These evidences suggest that the proteins in the CFF could perform the function for the formation and/or stabilization of the AgNPs.

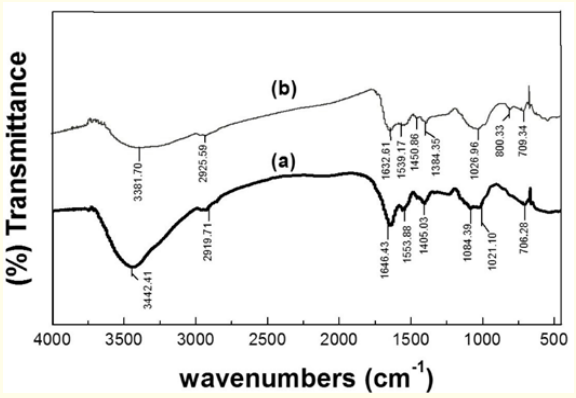

Figure 7: FTIR spectra of CFF from E. quadrilineata before (a) and after (b) interaction with $AgNO_3$

Characterization of the Biosynthesized AgNPs

Transmission electron microscopy provided insight into the morphology (Figure 8) and size (Figure 9) details of the biosynthesized AgNPs. The average size of AgNPs obtained from TEM analysis was about 6 nm on average and in the range of 2 - 26 nm. They are spherical, monodipersed and uniformly distributed.

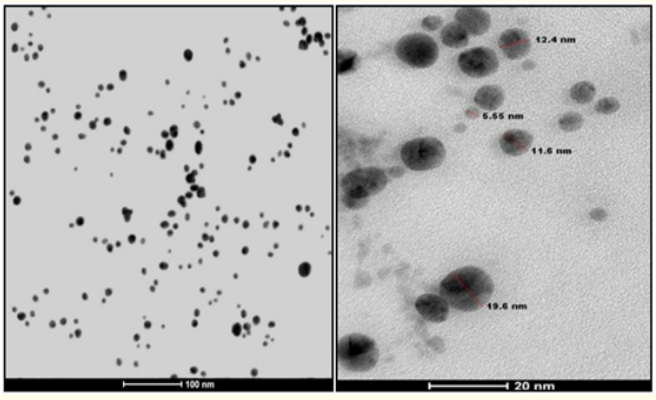

Figure 8: TEM micrographs showing spherical uniformly distributed NPs. Left: low magnification image (Scale bar 100 nm); Right: high magnification image (Scale bar 20 nm).

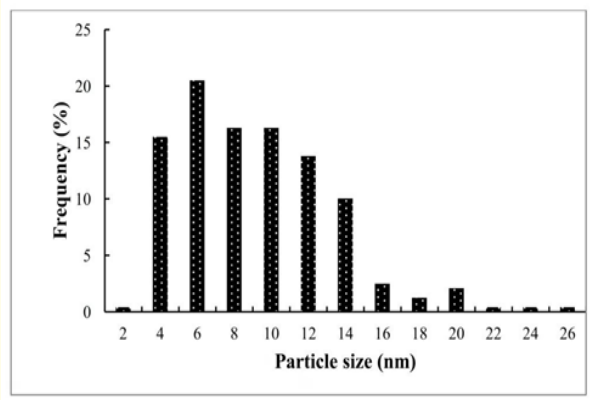

Figure 9: Particle size distributions of AgNPs from TEM analysis.

The particles are not aggregated after three months of periodical analysis showing good stability of these NPs. This might be confirmation to presence of the capping agents in the CFF in accord with previous explanation [40] Moreover, zeta potential of the biosynthesized AgNPs was measured and found to be -13.6 mV (Figure 10) in comparison with-13.7 mV for AgNPs from *A. niger* [41] and -21.7 mV from *Cunninghamella phaeospora* [42]. The zeta potential is a key indicator of the stability of colloidal dispersions. Its negative value indicates electrostatic repulsion [43] among the particles and thereby increasing the stability of the formulation.

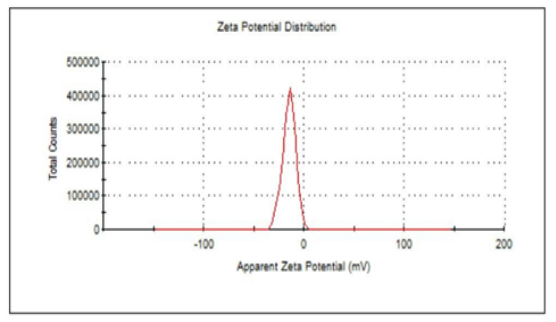

Figure 10: Zeta potential measurements of the biosynthesized AgNPs using CFF from E. quadrilineata

Antibacterial Efficiency

The progress in nanotechnology applications led to introduction of AgNPs into the medical field. Previous results [44] showed a very low antibacterial efficacy of AgNPs but on contrary, others

[45] indicated the very high efficacy of AgNPs. The antibacterial activity of the AgNPs biosynthesized in this work (Figure 11) was near to that of the antibiotic ciprofloxacin. The results demonstrate a fluctuation effect of both NPs and the antibiotic ciprofloxacin. This fluctuation is due to the variation in cell wall composition of the test organisms [1].

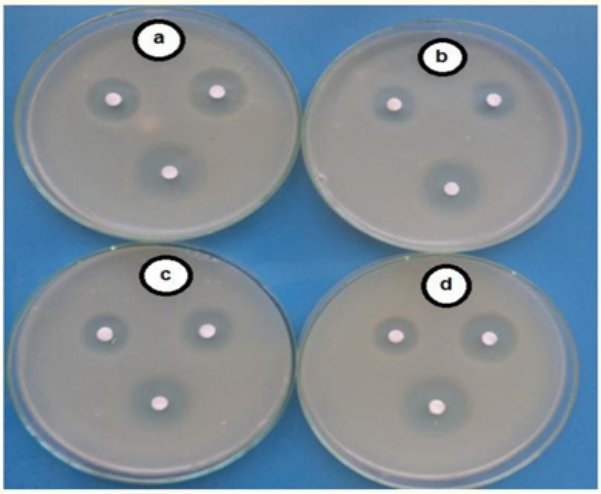

Figure 11: Antibacterial activity of AgNPs biosynthesized using CFF from E. quadrilineata against B. subtilius (a), E. coli (b), M. luteus (c) and S. aureus (d). In each image, NPs (upper left), ciprofloxacin (upper right) and NPs +ciprofloxacin (lower)

Although the mechanism of AgNPs against bacteria is still unclear, certain investigators suggested some explanations including attachment of the AgNPs to the surface of cell membrane leading to disruption of its power function such as permeability and respiration [46] and interaction with the nitrogenous bases in the DNA causing inhibition of the cell division [47]. When the AgNPs were combined with the ciprofloxacin (1:1), an enhancement of the antibacterial activity of both NPs and antibiotic was recorded especially against the human pathogen *Staphylococcus aureus*. This finding accord with the earlier report on the possibility of utilizing a combination of antibiotics and AgNPs therapeutically for the treatment of bacterial infections [48]. As the mode of action of the ciprofloxacin is known to be the inhibition of the A subunit of DNA gyrase which is essential in replication of the bacterial DNA thereby inhibiting cell division [49], the main role of the AgNPs in this combination possibly be the interference with the functions of the cell membrane.

Conclusion

A safe and fast biosynthetic approach for the formation of extracellular silver nanoparticles has been investigated in this work using cell-free filtrate from the fungus *Emericella quadrilineata* that not recorded in this regard before. The procedure is affected by the prevailing reaction and culture conditions. Role of the enzyme nitrate reductase and other proteins in the bio reduction process was suggested. The biosynthesized silver nanoparticles are characterized with their monodispersed, stability and reasonable antibacterial activity.

Conflict of Interest

The authors declare that they have no competing interests.

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