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BIOSYNTHESIS, OPTIMIZATION AND CHARACTERIZATION OF SILVER NANOPARTICLES BIOSYNTHESIZED BY *Bacillus subtilis* ssp *spizizenii* MT5 ISOLATED FROM HEAVY METALS POLLUTED SOIL

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ABSTRACT: Nanotechnology and nanoparticles (NPs) researches have attracted a lot of interest in recent decades, and there is growing attention to find more effective ways for their synthesis. The use of biological approach, (using various microorganisms), as bio-nanofactories provides a clean and promising alternative process for the fabrication of silver nanoparticles. This study confirmed the production of silver nanoparticles (AgNPs) by a cost effective, safe and environment-friendly technique using silver nitrate and supernatants of the bacterium *Bacillus subtilis* ssp *spizizenii* MT5 as a bio reducing agent. Supernatants of the tested microbe growing on nutrient broth (NB) were used for fabrication of AgNPs. Some parameters of optimization *i.e.,* incubation time, silver nitrate concentration, mixing ratio of culture supernatant and silver nitrate, media type, temperature degree and pH level were studied. The biosynthesis of AgNPs in the cell extract filtrate was confirmed and characterized by biophysical methods using the advanced available instruments. The determined conditions for the bioinspired synthesis of AgNPs revealed that incubation time was 40 h, silver nitrate concentration was 3mM, supernatant and silver nitrate ratio was 1:4, medium type was nutrient broth (NB), agitation speed was 160 rpm, temperature degree was 35°C and pH level was 7. Characterizations of the produced bio silver nanoparticles were done using the advanced available methods. The ultraviolet-visible spectrum showed an absorption peak at 420 nm. Transmission electron microscopy (TEM) showed that the mean diameter of the formed AgNPs was 38 to 49 nm. Powder X-ray diffraction (XRD) showed that the particles are crystalline in nature, with a face-centered spherical structure. Dynamic light scattering (DLS) and Zeta potential analysis showed that the average AgNPs size was 31.42 nm and the zeta potential was -20.8mV, Fourier Transform Infrared Spectroscopy analysis (FT - IR) confirmed the presence of elemental silver and the dual function of biomolecule responsible for the bio reduction and stabilization of AgNPs in the reaction mixtures. The scanning electron microscopy (SEM) micrograph indicated that produced AgNPs are spherical in shape. However, it also showed an indeterminate morphology. Energy-dispersive X-ray spectroscopy (EDX) exhibited strong signal in the silver region which confirms the formation of AgNPs.

Key words: Biosynthesis, silver nanoparticles, optimization, characterization, TEM, SEM, FT-IR.

INTRODUCTION

Bionanotechnology is considered a new branch of science creating a growing sense of excitement in life sciences, especially in the field of the modern medicine (**Xia *et al*., 2010**). Silver (S) metal is considered nontoxic, safe, inactive anti-bacterial agent used for several centuries. Application of nanoscale materials and structures usually ranges from 1-100 nm. (**Arora *et al*., 2008**). Silver nanoparticles (AgNPs) is defined as a silver mineral of very small size (10-100 nm). They attracted the attention of workers in different fields all over the world due to the unique chemical and physical properties since they are having a

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promising application in medicine, agriculture, environment remediation, food technology, water treatment..*etc*. **(Sastry *et al*., 2010; Singh *et al*., 2015)**. For example, in medicine, they have been intensively studied owing to their excellent antimicrobial activities against bacteria **(Petrus *et al*., 2011; Tripathi *et al*., 2017)** against fungi **(Alizadeh *et al*., 2014; Barzegar *et al*., 2018)** and as antiviral agents **(Galdiero *et al*., 2011; Narasimha *et al*., 2012)**. However, the properties of stability, sizing and dispersion of the formed AgNPs differed with the type of microbial species and strains used **(Sanghi and Verma 2009; Prakasham *et al*., 2012)**, therefore, the exploration the new bacterial strain showing the ability to fabricate AgNPs with certain characteristics is still at the forefront of studies in nanofields. In the last decades, several bacterial species such as *Staphylococcus aureus* **(Nanda and Saravanan, 2009),** *Ureibacillus thermosphaericus* **(Juibari *et al*., 2011)**, *Bacillus cereus* **(Sunkar and Nachiyar, 2012)**, *Bacillus thuringiensis* **(Pereira *et al*., 2015)** have been reported to produce AgNPs, and a great efforts have been made to optimize the growth conditions for their biosynthesis of AgNPs by the applied bacteria, *i.e.,* $AgNO_3$ concentration, pH, temperature, incubation time and others play important role in enhanced production of AgNPs. Synthesis of AgNPs requires silver salt (usually $AgNO_3$), reducing agents (NADH and $NADH^-$ dependent reductase enzymes) and a stabilizer (proteins and peptides) for controlling the synthesis of nanoparticles (NPs) and preventing them from aggregation **(Barua *et al*., 2013)**.

In the light of these information, the present study has been designed to use bacterial strain, namely *Bacillus subtilis* ssp *spizizenii* MT5 that was recovered from soil polluted with heavy metals for biosynthesis, optimization and characterization of AgNPs produced.

MATERIALS AND METHODS

Bacterial Isolation and Selection

Soil samples were gathered from industrialized area polluted with heavy metals at Abou- Hammad city (Wady El-Moulak village), Sharkia Governorate, Egypt. An obtained soil was transported to the laboratory in sterile polyethylene bags. Sterile saline solution (0.9% *W/V*) was used to dilute the soil samples and the bacterial isolates were recovered by spread plate technique on nutrient agar (NA) at 30˚C for 2 days. All isolated bacteria were individually grown in 250 ml conical flasks containing 50 ml sterile nutrient broth (NB) supplemented separately with either 0.1 mM $AgNO_3$ (as primary screening) or 1.0 mM $AgNO_3$ (as a secondary screening) for selecting their potential to produce AgNPs. The culture flasks were put for 48 hr., in a shaker incubator at 160 rpm, and 30ºC. Observation were recorded after 48 hr., and 24 hr., in the case of primary and secondary screening, respectively. The turbid appearance of the culture flasks ensured the growth and the intensity of developed color was considered for selecting the target microorganism. The progression of the bioreaction in the turbid flasks was monitored both by visual inspection as the color changed from yellowish to brown and by measuring the absorbance by UV-Vis spectrophotometer as recommended by **Elbeshehy *et al*. (2015)**

Preparation of Bacterial Cell Free Extract

For the biosynthesis of AgNPs, only one selected bacterial isolate was separately inoculated in 250 ml conical flasks containing 100 ml sterile nutrient broth (NB). The growth parameters were adjusted at pH 7 and incubation temperature 30ºC in shaker incubator at 160 rpm. Followed by incubation the enriched cultures were subjected to centrifugation at 10,000 rpm (Eppendorf) for 10 min. The supernatant material was separated out and utilized as crude source of NO_3^- reductase enzyme for the extracellular synthesis of AgNPs **(Shahverdi *et al.*, 2007)**.

Identification Bacterial Isolate

The selected isolate was identified due to its morphological, biochemical and physiological characteristics by the procedures outlined in Bergey's manual of systematic bacteriology **(Logan** and **De Vos, 2009)**, then it was subjected to identification process by Matrix-assisted laser desorption ionization time of flight (MALDI TOF) mass spectrometry **(Bille *et al*., 2012)**, as advanced and accurate tool to confirm the previously identification.

Biosynthesis of Silver Nanoparticles

For bio fabrication of AgNPs by the tested bacterium, 250ml conical flasks containing 20 ml of supernatant from bacterial culture were

separately mixed with 30 ml of 1mM aqueous solutions of filtered sterilized $AgNO_3$, were conducted (**Shahverdi *et al.*, 2007; Elbeshehy *et al.*, 2015)** with some modification. Then, the reaction mixture flasks were placed at 160 rpm in shaker incubator at 30°C up to 24 hr., and allowed for reduction process. Also, a set of flasks containing 20ml of NB and 30 ml of 1mM $AgNO_3$ solution were prepared to confirm that the biotransformation of Ag^+ ion to Ag^0 atoms was mediated only by the used bacterial cell free extract (**Elbeshehy *et al.*, 2015)**.

Optimization Factors Studied

To obtain maximum AgNPs production, different growth conditions and reactions for biotransformation of Ag^+ ion to atom Ag^0 by the tested microbe were optimized. These factors were incubation time (1, 8, 16, 24, 32 and 40 hr.), temperature degree (20, 25, 30, 35 and 40ºC), $AgNO_3$ concentrations (1, 2, 3, 4 and 5 mM), pH level (5.5, 6.0, 6.5, 7.0 and 7.5), mixing ratio of culture filtrate and silver nitrate solution (1:4, 2:3, 3:2, 2.5:2.5 and 4:1) and media type (nutrient broth and Luria-Bertani broth were used for producing the culture supernatants of the tested bacteria), and these experiments were performed according to **Safekordi *et al.* (2011) and Elbesheby *et al.* (2015)** with some modifications. In each experiment, the prepared conical flasks, 250 ml containing 50 ml of mixture reactions, were incubated in an orbital shaker at 160rpm, in light, taking into account the procedures of each parameter tested. At the end of the incubation period, the progression of the reaction was monitored both visually inspection by the change in color by naked eyes and by measuring the absorbance maximum at 420nm by UV. Visible absorption spectroscopy (Laxco™, Alpha-1502 Alpha Series Spectrophotometer, 200 - 1000 nm).

Characterization of Silver Nanoparticles

The particles of AgNPs (in reaction mixture, dried powder or annealed forms) were prepared due to the recommended procedures in each analysis and subjected to the following instruments: Ultraviolet–visible spectroscopy for UV-Vis spectroscopy (Laxco™ dual beam spectrophotometer (Laxco™, Alpha-1502 Alpha Series Spectrophotometer, 200-1000 nm) (**Forough and Farhadi, 2010)** for observing surface Plasmon resonance (SPR) absorbance peak of AgNPs, Fourier Transform-Infrared spectroscopy for FT-IR analysis (Bruker Tensor 37, Kaller Germany, due to **Aguillar *et al.* (2011)**, for detection the interaction between proteins present in the supernatants used and the AgNPs and to identify the potential biomolecules in the bacterial supernatants. PAN analytic X-Ray diffractometer for XRD analysis, due to **Kalabegishvili *et al.* (2012)**, to determine position, peak intensity and width of XRD. Scanning Electron microscopy for SEM analysis using SEM Quanta 250 FEG, FEI company, the Netherlands, due to **Pavani *et al.* (2013**) and Transmission Electron Microscopy for TEM images using TEM JEOL 1010, Japan, due to **Ganachari *et al.* (2012**), they were used for detection the size, distribution and morphology (crystal structure) the produced AgNPs. Zeta sizer analyzer (Nano Z2 Malven, Malvern Hills, UK, for Zeta potential analysis due to **Dash *et al.* (2014**) and Dynamic Light Scattering – DLS analysis due to **Chattopadhyay *et al.* (2013),** for Zeta potential and the average size of the formed AgNPs determination.

RESULTS AND DISCUSSION

Identification of Used Bacteria

Initial, only 4 bacterial isolates out of the total 29 isolates collected from the polluted soil samples were chosen, where 0.1mM $AgNO_3$ was used (first screening). On screening these isolates using 1mM $AgNO_3$ (second screening) only one bacterium out of 4 was selected for further studies. This bacterial isolate was coded MT5 and it was selected due to its ability and the intensity to change the color of reaction mixtures at the two tested concentrations (0.1 and 1.0 mM $AgNO_3$) and two tested incubation times (48 and 24 hr.). The pure isolate was Gram positive, motile, long rod and spore forming under light microscope and results showed that this bacterium resembles and relates to the *Bacillus* species. From the results of morphological, biochemicals and physiological testes carried out on the selected bacterium due to procedures outlined in Bergey's Manual (2009) **(Logan** and **De Vos, 2009)** it can be inferred that this bacterium is *Bacillus subtilis*. This bacterium was further subjected to rapid and accurate identification of bacteria and fungi by MALDI TOF Mass spectrometry. The result

showed its maximum identity of 98% to various *Bacillus* spp. mainly *Bacillus subtilis* ssp *spizizenii* DSM 15029T DSM **(Bille *et al*., 2012; Krasny *et al*., 2013)**. Thus, the local bacterial isolate, *Bacillus subtilis* MT5 is similar to *Bacillus subtilis* ssp *spizizenii* DSM 15029T DSM.

Biosynthesis of AgNPs

Experimental conditions had a strong effect on the properties, size, morphology and stability of the metal NPs. A major field of interest is how to create a biosynthesis method to control the morphology, properties, stability and size. The difference in color of reaction mix from shallow yellow to brown within a day of inoculation indicated the biosynthesis of AgNPs by bacterial cell extract from the tested microorganism. It is well known that excitation of surface plasmon trembling of metal NPs show yellowish brown color in water **(El-Shanshoury *et al*., 2011)**. An excitation of surface plasmon trembling which is distinguishing of AgNPs causing changing in the color **(Kalaiselva, 2013)**. An electromagnetic field in the observable variety is united to the collective oscillation of transmission electrons, the dipole oscillation arising the surface plasmon vibrations will happened **(Mubayi *et al*., 2012).** Control treatment displays no color change when incubated at the same condition, Fig. 1 shows the result. The decrease of Ag^+ ions thus the construction of AgNPs data is not yet clear, and the protein molecules and enzymes such as nitrate reductase are considered of a good adaptable agent in the reaction mixture of AgNPs **(Narayanan and Sakthivel, 2010)**.

Optimization of Growth Conditions

In these experiments, and in order to reach the best growth conditions for the highest yield of AgNPs by the tested microbe, primary temperature degree at 30ºC, incubation time at 24 hr., and agitation speed at 160 rpm were selected due to **Velmurugan *et al*. (2014)** for the biosynthesis of AgNPs as an appropriate value commonly employed in these experiments.

Incubation Time

The first factor considered for an optimization of the biosynthesis of silver nanoparticles was the incubation time. The absorption spectra of AgNPs formed in the reaction media at different durations every 8 hr., were studied. Fig. 2 shows the UV-Vis spectra of reduction of Ag to AgNPs using supernatant of cell culture at different reaction times. It was found that when the incubation time was less than 1hr., there was no formation of AgNPs, this might be because of the redox potential of the $AgNO_3$ is reduced. Moreover, by increasing the time of reaction, the absorption peak was increased, and more AgNPs were formed during the incubation time which was ranged from 1 to 40 hr. From the spectra, it was found that the optimum incubation time for the completion of reaction was 40 hr., were a maximum absorbance was recorded otherwise, the incubation time was over. In addition, it was observed that increasing the incubation time to more than 40 h. did not increase the absorption significantly, which indicates the stability of the AgNPs colloidal solution. In this respect, it can be notice that silver surface plasmon resonance (SPR) occurs at 420nm, and SPR with the function of time revealed at particle biotransformation by this strain is reaction time dependent **(Prakasham *et al*., 2012).** Recently, **Devi *et al*., (2017)** mentioned that the maximum AgNPs synthesis was achieved at an incubation time of after 24hr., using *Pseudomonas putida* strain LUA 15.1 followed by gradually decrease in activity up to 120 hr., whereas **Thamilselvi and Radha (2013)** mentioned that *Pseudomonas putida* NCIM 2650 has been found to produce maximum extracellular AgNPs synthesis after 28 hr., of incubation.

$AgNO_3$ Concentration

In this experiment, different concentrations of $AgNO_3$ solution (1, 2, 3, 4 and 5 mM) were utilized in order to maximize the yield of AgNPs, and the absorbance of the silver colloidal solution was monitored using the UV-Visible spectrophotometer. UV-Visible absorption spectra showed that by increasing the concentration of silver nitrate solution, the absorbance of AgNPs was amplified, and the maximum yield of AgNPs was obtained when the concentration of $AgNO_3$ solution was 3mM as seen in Fig. 3. In this connection, **Sarangadharan** and **Nallusamy (2015)** stated that the particle size of AgNPs range from 3 to 130 nm in supernatant with 1mM $AgNO_3$ and 45 -170 nm in supernatant with 3mM $AgNO_3$ when AgNPs were produced using *Bacillus licheniformis.*

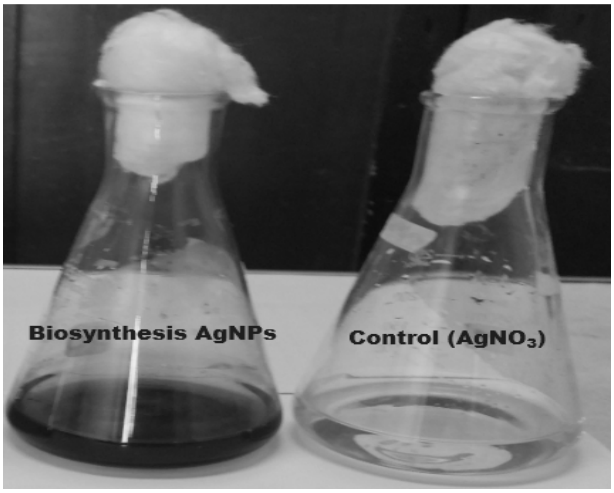

Fig. 1. Biosynthesis of AgNPs by *Bacillus subtilis* ssp *spizizenii* MT5

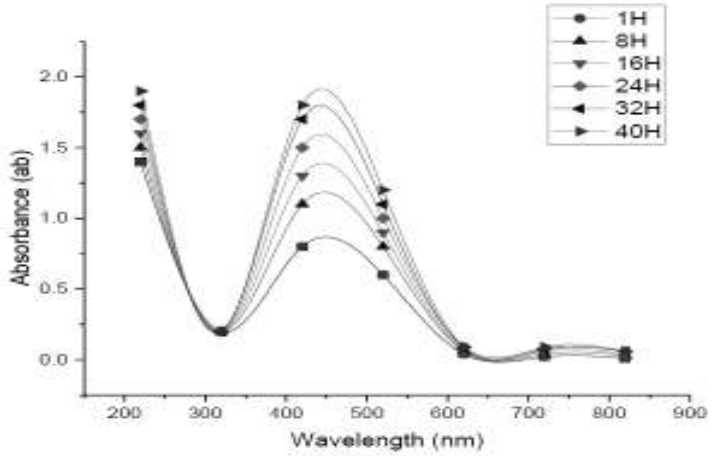

Fig. 2. UV-Vis spectra of incubation time of AgNPs biosynthesis by *Bacillus subtilis* ssp *spizizenii* MT5

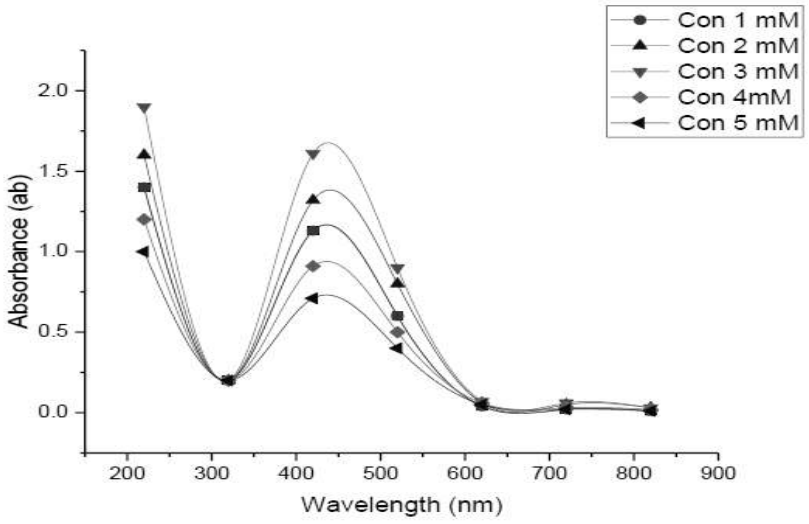

Fig. 3. UV-Vis spectra of $AgNO_3$ concentrations of AgNPs biosynthesis *by Bacillus subtilis* ssp *spizizenii* MT5

Mixing Ratio of Culture Supernatant and $AgNO_3$

After an incubation of different volumes of 1mM $AgNO_3$ for 24 hr., with 5 different volumes of supernatants of *Bacillus subtilis* ssp *spizizenii* MT5 (10:40, 20:30, 25:25, 30:20 and 40:10), UV- visible spectra of AgNPs were recorded. UV-visible spectra of AgNPs are shown in Fig. 4. UV readings showed that the absorbance of 0.53, 0.91, 1.11, 1.53 and 1.82 at 420 nm for the tested ratios, respectively. These results revealed that volume of supernatant and silver nitrate ratio 1:4 was considered to be the optimal ratio since it gave a maximum absorbance of 1.82 at 420nm. The increase in culture filtrate causes a decrease in absorbance. The reduction recommends reduction in NPs size. It seems that by increase of filtrate amounts of nanoparticles become smaller **(Safekordi *et al*., 2011)**.

Media type

Two different media were selected, nutrient broth and LB broth. UV readings were obtained after incubation for 24 hr., (Fig. 5). The absorbance of LB media was 0.71 whereas this absorbance was 0.91 for nutrient broth at 420 nm. Nutrient broth was found to be more favorable for the manufacturing of AgNPs by *Bacillus subtilis* ssp *spizizenii* MT5 in this experiment.

Temperature degree

Five different temperatures (20, 25, 30, 35, and 40^{o}C) were employed in this experiment. UV readings were recorded after incubation for 24 hr., (Fig. 6). UV spectra of AgNPs produced by *Bacillus subtilis* ssp *spizizenii* MT5 at the above-mentioned degrees showed absorbance of 0.52, 0.71, 0.91, 1.21 and 0.82 at 420 nm, respectively. The best temperature for the manufacture of AgNPs by the tested microbe was found to be at 35^{o}C since it gave an absorbance of 1.21 at 420 nm. Reaction rate increases causing consuming of reaction temperature increases most silver ions to be in the formation of nuclei and consequently the secondary reduction procedure on the surface of nuclei preformation has been stopped **(Safekordi *et al*., 2011).**

pH level

Five different pH levels (5.5, 6.0, 6.5, 7.0 and 7.5) were selected for optimization of biosynthesis of AgNPs in this experiment. UV-Vis readings were obtained after incubation at 30ºC for 24 hr., (Fig. 7). UV-Vis spectra of AgNPs produced by *Bacillus subtilis* ssp *spizizenii* MT5 at the pH levels tested showed absorbance of 0.64, 0.81, 1.21, 1.41 and 0.93 at 420 nm, respectively and pH 7 was found to be an optimum for the manufacturing of AgNPs by the tested microbe. In this respect, many studies have reported an increase in manufacturing of AgNPs at lower pH when a high absorbance was recorded at this pH. The reductase enzyme catalyzing the biosynthesis of AgNPs might be inactivated as a condition became more alkaline and this could be the reason why reduced synthesis and lower absorbance were noticed at higher pH values (**Koilparambil *et al*., 2016**).

Characterization of Silver Nanoparticles

Visual inspection

Initial, the greenish yellow color appearance in the reaction mixtures and mirror similar lighting on the Erlenmeyer flask walls obviously indicated the biofabrication of AgNPs in the interaction mix, Fig. 8. The change in color shows the creation of AgNPs, and the appearance of brown color was due to the excitation of surface plasmon vibrations, and the appearance of dark brown in color denotes that the good nanoparticles synthesis when compared to normal one.

UV-Vis spectra

In the UV-Visible absorption spectrum, a strong, broad peak, between 300 and 600 nm was recorded in this analysis. Surface plasmon band centered at nearly 420 nm which was shown by using the UV-Vis spectra of the AgNPs (Fig. 9), which is the distinguishing of AgNPs and visible pointed out the synthesis of nanoparticles in reaction mixture. The wavelength, at which the imaginary and real portions of the dielectric function of silver practically disappeared was ~320 nm. The plasmon bands are wide with an absorption tail in the longer wavelengths, which could be in practice because of the particle allocation size. Observation of this peak is due to surface plasmon which is well recorded for various metal NP ranging in size from 2 to 100 nm **(Sastry *et al.,* 1997; Sarangadharan and Nallusamy, 2015)**. This is consistent with previous reports showing that

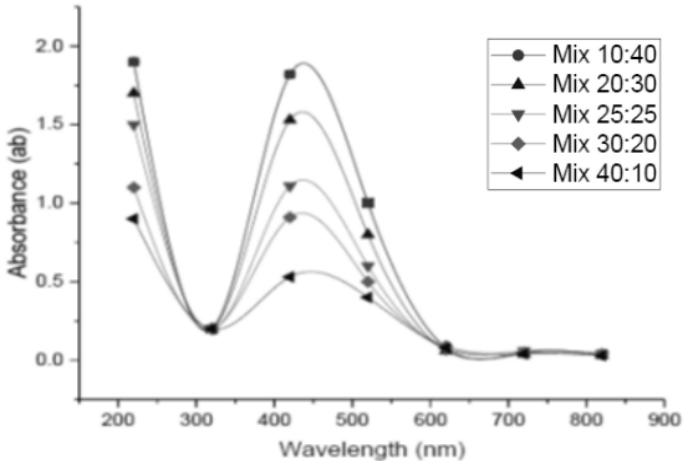

Fig. 4. UV-Vis spectra of mixing ratios of culture supernatant and silver nitrate of AgNPs biosynthesis by *Bacillus subtilis* ssp *spizizenii* MT5

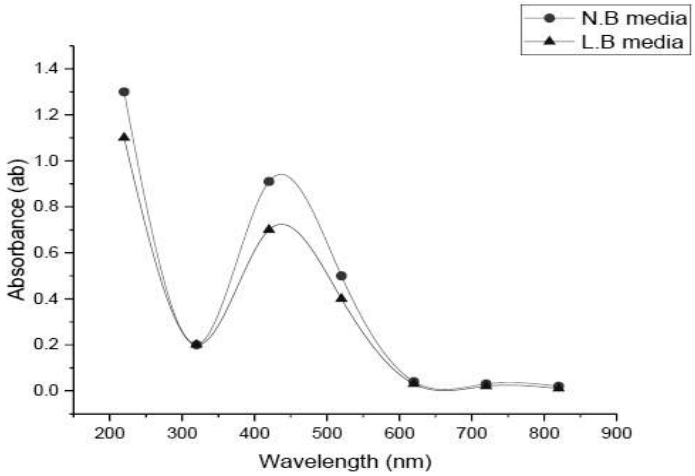

Fig. 5. UV-Vis spectra of different media of AgNPs biosynthesis *by Bacillus subtilis* ssp. *spizizenii* MT5 in Nutrient media broth and L.B media

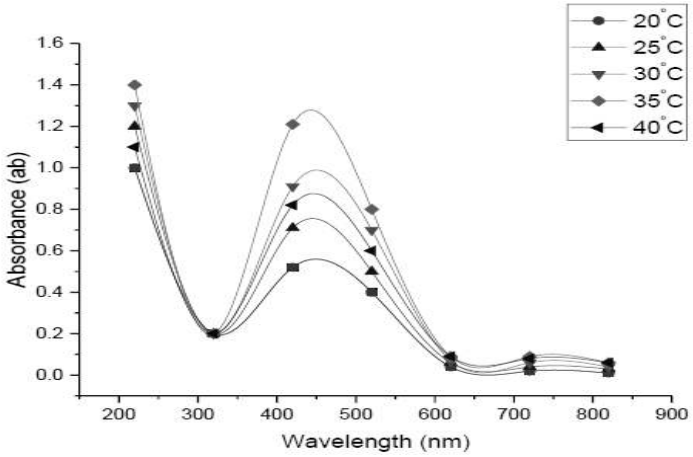

Fig. 6. UV-Vis spectra of different temperatures of AgNPs biosynthesis by *Bacillus subtilis* ssp *spizizenii* MT5

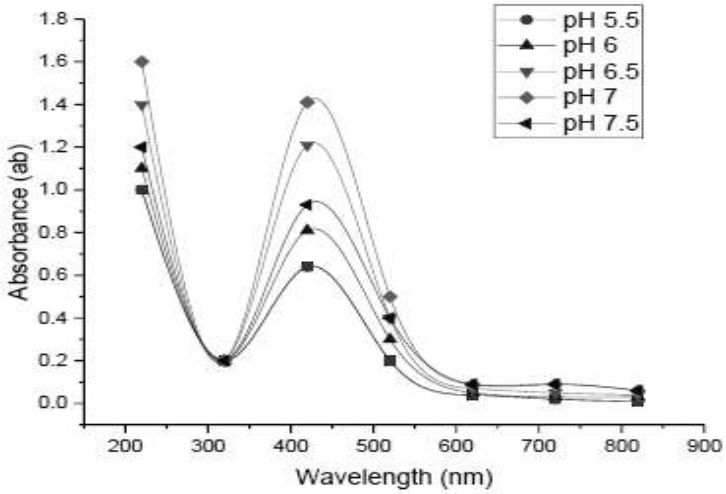

Fig. 7. UV-Vis spectra of different pH levels of AgNPs biosynthesis by *Bacillus subtilis* ssp *spizizenii* MT5

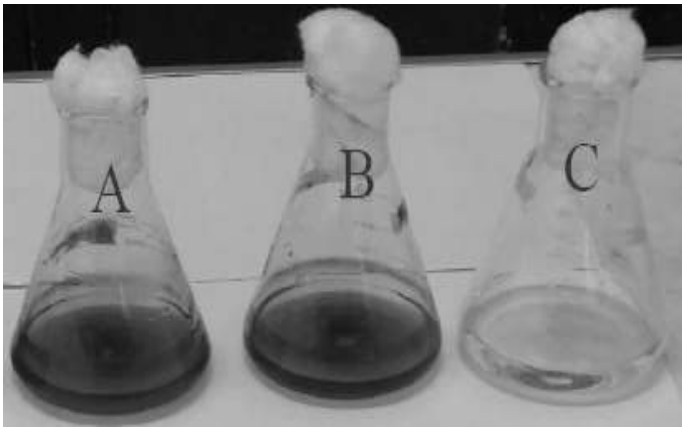

Fig. 8. Color change indicates the creation of AgNPs by the culture filtrate of the tested microbe (a) after 12 hr., (b) after 24 hr., (c) control flask ($AgNO_3$ solution)

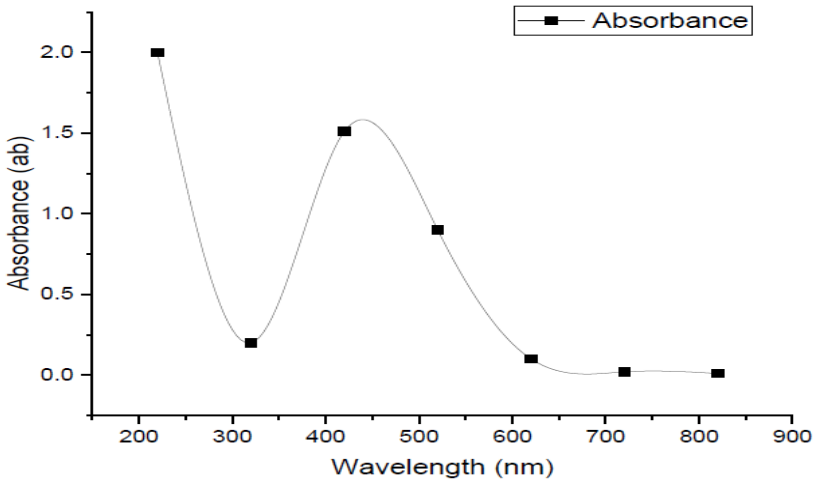

Fig. 9. UV-Vis Spectrum of AgNPs biofabrication by *Bacillus subtilis* ssp *spizizenii* MT5

the range of UV-Vis spectra of the Ag^+ colloid nearly from 200-700 nm. **Sileikaite *et al.* (2006)** reported that the absorption band in noticeable zone and plasmon peak at 445 nm is idealistic for AgNPs synthesis. The synthesis AgNPs was primarily characterized by UV spectrometer, and it is an important technique for the analysis of nanoparticles. Also, **Vithiya *et al.* (2014)** reported characterization of AgNPs synthesized by *Bacillus* spp. was observed in the UV visible spectra at 430 nm. As well, **Bhuvaneswari *et al.* (2016)** characterized AgNPs synthesized extracellularly by *Bacillus subtilis* using UV-visible spectroscopy and SEM. UV-visible absorption spectra detected a sharp absorption spectrum at 430 nm.

Dynamic Light Scattering (DLS) and Zeta Potential

From DLS data, it could be inferred that the average size of the synthesized AgNPs is 31.42 nm and 0.331 poly dispersity index (PDI) value. The single peak denoted that the synthesized AgNPs quality is good **(Mahl *et al*., 2011).** A single peak with the zeta potential of the nanoparticles value as (-20.8mV) showed that the repulsion between the synthesized nanoparticles is present. However, **Ahmad *et al.* (2003)** stated that the nanoparticles especially those produced by *B. licheniformis* were stable (Zeta potential ranged from 16.6 – 21.3 mV). A great positive or negative zeta potential of particles in suspension, this means that there will be no tendency of the particles to assemble together. But, low zeta potential values of particles this means that there will be no force to prevent the particles coming together Fig. 10. Recently, DLS analysis revealed that AgNPs synthesized by *Bacillus pumilus, B. persicus* and *B. licheniformis* were in the range size of 77 – 92nm, **Elbeshehy *et al*. (2015)**

Scanning Electrons Microscope (SEM)

The SEM micrograph of AgNPs synthesized by *Bacillus subtilis* ssp *spizizenii* MT5 is presented in Fig. 11. SEM presented more insight to seeking the size and morphology details of the AgNPs. The sample stubs with double-sided taps, covered with gold in a sputter cover were mounted with the freeze dried of AgNPs and SEM was used to examine at 20 kV with a tilt angle of 45°. Illustrative SEM micrograph of the *Bacillus subtilis* ssp *spizizenii* MT5 which synthesized silver nanoparticles were magnified by $5\text{-}10*10^3$ times. From the results, the size of the formed AgNPs due to SEM was ranged from 30 to 90 nm (Fig. 11). However, **Malarkodi *et al*. (2013)** pointed out most of the particles of AgNPs produced by *Bacillus* spp. are spherical shape and size ranges from 65 – 70 nm analyzed by SEM.

Transmission Electron Microscopy (TEM)

The TEM micrograph of AgNPs synthesized by *Bacillus subtilis* ssp *spizizenii* MT5 is presented in Fig. 12. It is well known that much information about morphological features and distribution patterns were obtained by TEM analysis. It was exposed that the particle is spherical and dispersed well without agglomeration. The particle size of AgNPs synthesized by the tested microorganism was ranged from 38 to 49 nm. **Das *et al.* (2014)** characterized silver nanoparticles synthesized extracellularly by *Bacillus* strain CS 11 using Transmission Electron Microscopy, and their results revealed that the AgNPs was spherical in shape and in the size ranged from 42 to 94 nm. However, **Kushwaha *et al.* (2015)** characterized AgNPs synthesized extracellularly by *Escherichia coli* using TEM micrograph and found the nanoparticles to be well-disperse with a size ranging from 20-50 nm. The worthwhile thing in nano fields, variation in nano shape and size of SNPs formed by biological system is commonly recorded due to growth and operation conditions as observed in these experiments.

EDX Spectroscopy Analysis

The graph obtained by the EDX analysis showed the attendance of the silver (Fig. 13). The EDX analysis assured that the existence of AgNPs in the bacterial supernatant, giving a characteristic peak at 3–4 keV in EDX image, which mentions the reduction of Ag+ to Ag^0 and Fig. 13 shows the EDX spectrum of the prepared AgNPs. Silver (Ag) signal comes from the AgNPs and the atomic percentage of silver was 79.96%. In this connection, the absorbance of the metallic AgNPs generally shows an absorption peak approximately at 3 KeV **(Deljou and Goudarzi, 2016)**. In addition to Ag, there were also some other peaks. The atomic

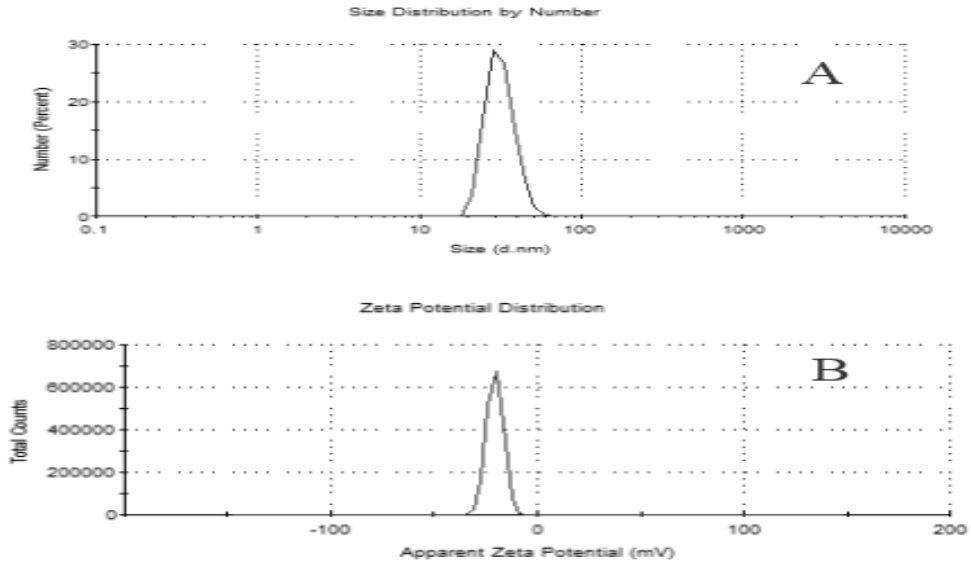

Fig. 10. a DLS, b zeta potential of AgNPs synthesis by *Bacillus subtilis* ssp *spizizenii* MT5

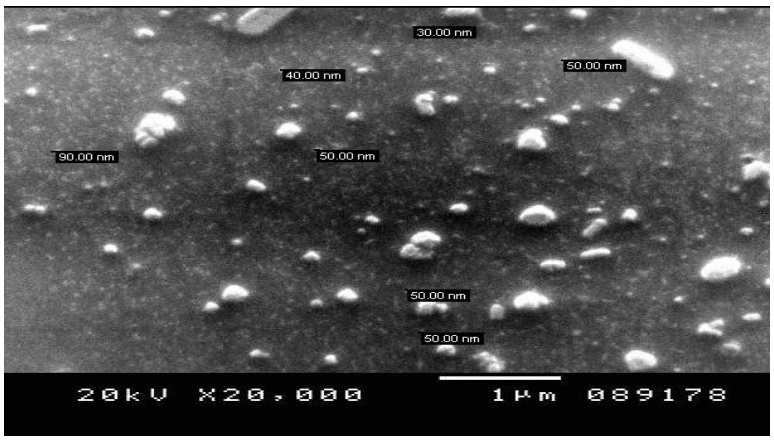

Fig. 11. SEM electron micrograph of AgNPs biosynthesized by *Bacillus subtilis* ssp. *spizizenii* MT5

Fig. 12. TEM electron micrograph of AgNPs produced by *Bacillus subtilis* ssp *spizizenii* MT5

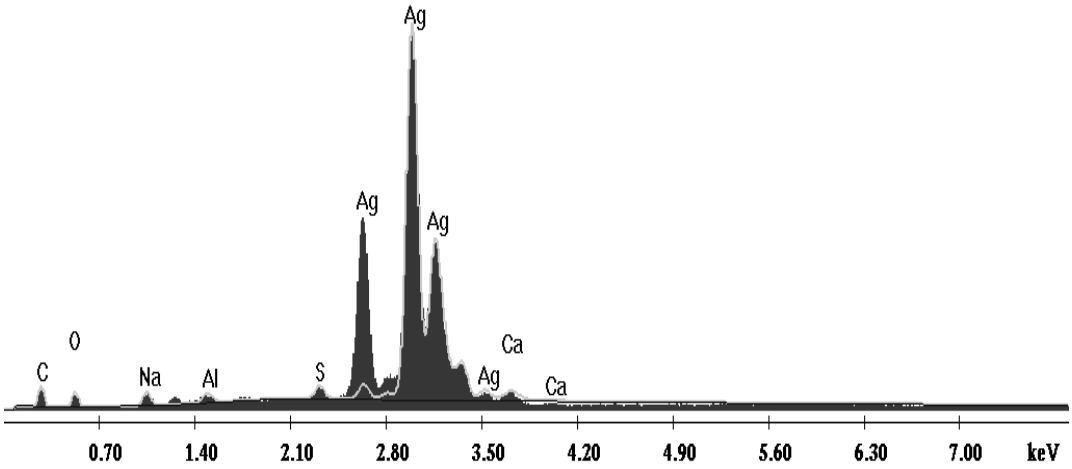

Fig. 13. EDX spectrum analysis of biosynthesized AgNPs by *Bacillus subtilis* ssp *spizizenii* MT5

percentages of Carbon (C) 5.45%, Oxygen (O) 7.84%, Sodium (Na) 3.22%, Aluminum (AL) 1.27%, Sulfur (S) 1.11% and Calcium (Ca) 1.16%. The carbon (C) signal comes from the adsorbed components of the microbe supernatant as well as coating material of the instrument. The signal of O may partly be from the atmosphere or -OH from the NaOH used for pH adjustment. Sodium (Na) signal may be produced from the NaOH which was used for pH adjustment during fabrication of AgNPs. Except (C), other elements have a very low atomic percentage compared to Ag, and suggest the fabrication of almost pure AgNPs.

Powder X-ray Diffraction (XRD)

XRD is commonly employed to explore the characteristic and structural details of the formed nanoparticles. Fig. 14 shows the XRD patterns of vacuum-dried AgNPs synthesized by *Bacillus subtilis* ssp *spizizenii* MT5. The XRD patterns indicate that the structure of AgNPs produced was spherical shape. In addition, all the AgNPs had a similar diffraction profile, and XRD peaks at 2θ of 33.13°, 45.11°, 56.09°, and 84.10° could be attributed to the 111, 200, 220, and 311 crystallographic planes of the spherical silver crystals, respectively, this indicates the biosynthesized AgNPs are well crystallized. The obtained results are in harmony with those of previous reports of characterization of AgNPs by XRD **(Ahmad *et al*., 2009; Litvin *et al*., 2012)**. The XRD pattern clearly indicated that the AgNPs formed in this study by the selected bacterium were crystalline in nature. The main crystalline phase was silver, and there were no obvious other phases as impurities were found in the XRD patterns (Fig.14). The XRD analysis shows that AgNPs produced are crystalline in their nature. These results support the reports of **Jeevan *et al*. (2012)** and **Manivasagan *et al*. (2013).** In general, from the results, all the samples contain four different sizes of AgNPs with size ranges from 30 to 70 nm. The overall results of XRD is correlated with the results of **Theivasanthi and Alagar (2012).**

Fourier Transform Infrared (FT-IR) analysis

The FT-IR reveal the presence of different functional groups like alkanes, amines and nitro compounds, the FTIR spectrum of biofabrication of AgNPs presented eight distinct peaks, measuring 3424.55, 2098.91, 1650.44, 1552.47, 1407.52, 1337.66, 1161,68 and 618.63 cm^{-1} as shown in the Fig. 15. The peaks at 3424.55 cm^{-1} denote NH stretch vibration of primary and secondary amides of protein. The peak at 2098.91cm^{-1} denote -C≡C- stretch vibration of alkynes. The peak at 1650.44 cm^{-1} mentions to N–H bend vibration of primary amines. The peak at 1552.47cm^{-1} refers to N–O asymmetric stretch vibration of nitro compounds. The peak at 1407.52 cm^{-1} indicates C–C stretch (in–ring) vibration of aromatics. The peak at 1337.66 mentions to C–N stretch vibration

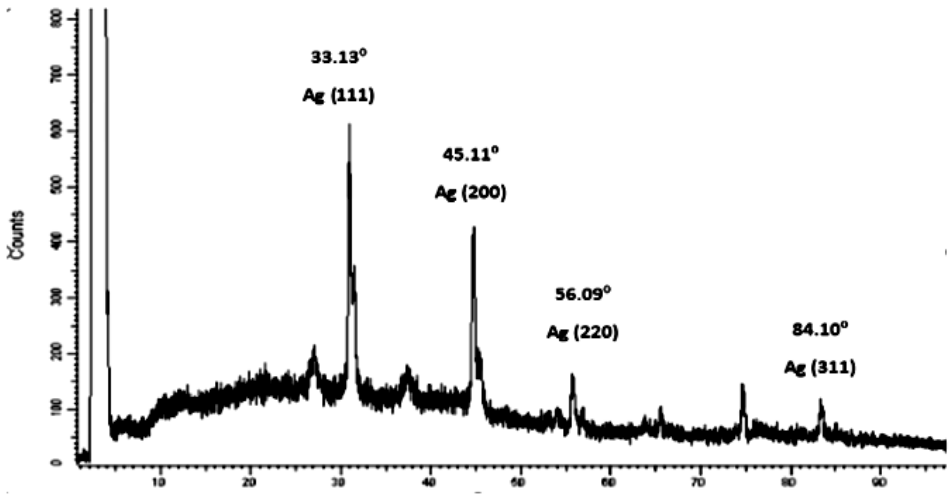

Fig. 14. XRD spectrum of biosynthesis AgNPs by *Bacillus subtills* spp *spizizenii* MT5

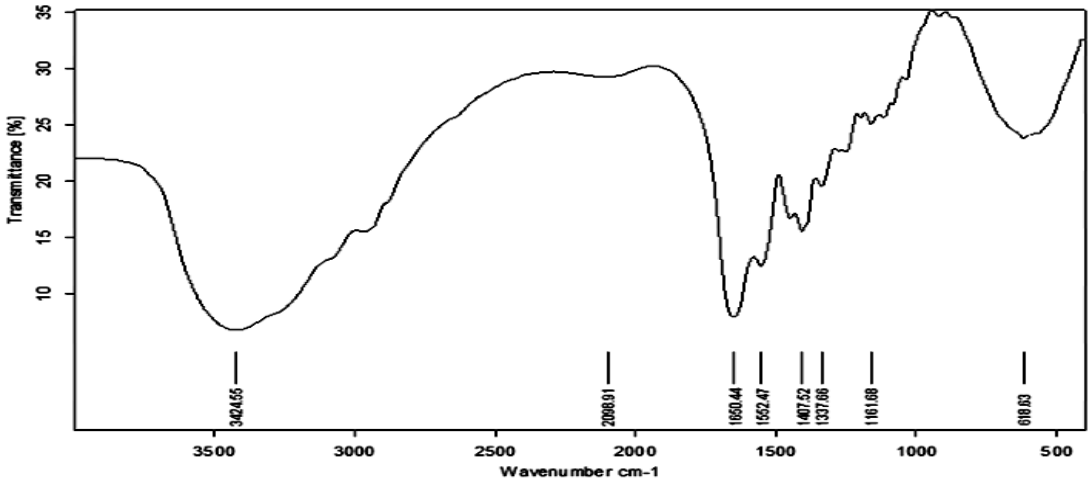

Fig. 15. FTIR analysis of biosynthesis AgNPs by *Bacillus subtills* spp *spizizenii* MT5

of aromatic amines. The peak at 1161.68 cm^{-1} refers to C–N stretch vibrations of aliphatic amines. The peak at 618.63 cm^{-1} refers to N–H wag vibrations of primary, secondary amines. The carbonyl groups of the amino acid residues and the peptides have strong facility to bind to the Ag **(Balaji *et al.*, 2009)**. The overall conclusion, hence confirmed the present of protein in the supernatant supporting the previous studies. Many researchers stated that the proteins can bind to NPs either through free amine or cysteine groups in proteins. These proteins which existing over the AgNPs surface might acts as capping agent for stabilization, **Mandal *et al.* (2005)**. Also, **Prakasham *et al.* (2012)** stated that FT -IR spectra of AgNPs showed N-H, C-H and C-N stretching vibrations denoting the presence of amino acid/peptide compounds in the surface of AgNPs produced by *Streptomyces albidoflavus*.

Conclusion

From the current study it can be inferred that the soil isolate of *Bacillus subtilis* ssp *spizizenii* MT5, has proved to be a good SNPs producers and their optimized growth conditions are incubation temperature (35°C), concentration of $AgNO_3$ (3mM), pH of the growth medium (7), incubation time (40hr.), agitation speed (160 rpm), the ratio of culture supernatant to $AgNO_3$ (10: 40) in the presence of nutrient broth. From

characterization process, the mean diameter of the formed SNPs was 38 to 49 nm and from dynamic light scattering (DLS) and Zeta potential analyses, the average SNPs size was 31.42 nm and the zeta potential -20.8 mV.

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التخليــق الحيــوى ومعظمــة وتـوصيف جـزيئــات الفضــة النانــو المتكــونـة بواســطة بكتـريا *Bacillus subtilis* ssp *spizizenii* MT5 المعزولة من تربة ملوثة بالمعادن الثقيلة

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إزداد فى الآونة الأخيرة إهتمام الباحثين بتقنية النانو والمواد النانونية وتركز الأهتمام بشكل واضح على إيجاد طرق أكثر فاعلية لتخليقها ويعد التخليق الحيوى الميكروبى من أفضل الطرق البديلة والواعدة لإنتاج جزيئات النانو وفى الدراسة الحالية تم إنتاج جزيئات الفضة النانو بواسطة بكتريا تم عزلها من تربة ملوثة بالعناصر الثقيلة وتعريفها بالطرق التقليدية وبتقنية حديثة وقد تم استخدام راشح خلايا بكتريا الـ *Bacillus subtilis* ssp *spizizenii* MT5 النامية على بيئة المرق المغذى لتخليق جزيئات الفضة النانو، وتمت دراسة بعض المعاملات لمعظمة عمليه التخليق الحيوى لجزيئات الفضة النانومثل: وقت التحضين، تركيز نترات الفضة، نسبة الخلط بين مستخلص الخلايا ونترات الفضة ونوع البيئة المغذية ودرجة الحرارة وقيم الأس الهيدروجينى، تم التأكد من عملية التخليق الحيوي لجسيمات الفضة النانو في خليط التفاعل باستخدام بعض الأجهزه المتطورة المتاحة، تبين من خلال الدراسة أن الظروف المثلى لعملية تخليق جزيئات الفضة النانو هى كالاتى: فترة التحضين لمخلوط التفاعل كانت ٤٠ ساعة وتركيز نترات الفضة المثالى ٣ ملى مول، نسبة الخلط المثالية بين نترات الفضة ومستخلص الخلايا ١ : ٤، ونوع البيئة المثالية بيئة المرق المغذى، ودرجة حرارة التحضين ٣٥ درجة مئوية وكان مستوى الأس الهيدروجيني هو ٧، تم عمل توصيفات لجسيمات الفضة النانو المنتجة باستخدام الطرق المتقدمة المتاحة حيث أظهرت نتائج التحليل الطيفى للاشعه فوق البنفسجية (UV–Vis spectrophotometer) أن أعلى قمة للأمتصاص كانت عند الطول الموجى ٤٢٠ نانو ميتر و أظهر أستخدام الميكرسكوب الإلكتروني النافذ (TEM) أن قطر جزيئات الفضة النانو المتكونة كان من ٣٨ إلى ٤٩ نانوميتر كما أظهرت حيود الأشعة السينية أن جزيئات الفضة النانو المتكونة ذات طبيعة متبللرة وأظهر تشتت الضوء الديناميكي (DLS) وتحليل زيتا أن متوسط حجم جزيئات الفضة النانو المتكونة كان ٣١,٤٢ نانومتر وأن شحنة جزيئات النانوالمتكونة كانت -٢٠,٨، كما تم قياس وجود البروتينات كعوامل مختزلة مسؤلة عن عملية التخليق الحيوى وعن ثبات جزيئات الفضة النانو وذلك بإستخام التحليل الطيفى للأشعه تحت الحمراء (FT–IR)، وأظهرت نتائج الفحص بالميكرسكوب الالكترونى الماسح (SEM) وجود جسيمات نانو فضة كروية متكونه ومتناثرة بصورة جيده، كما تم قياس العناصر المتكونة كما ونوعا وذلك باستخدام تحليل طاقة التشتت للاشعة السينية (EDX) وهذا يؤكد بصورة قوية على تكون جزيئات الفضة النانو.

المحكمـــون:

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