EXTRACELLULAR BIOSYNTHESIS, OPTIMIZATION, CHARACTERIZATION AND ANTIMICROBIAL POTENTIAL OF ESCHERICHIA COLI D8 SILVER NANOPARTICLES

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ABSTRACT

This study highlights the optimization of extracellular biosynthesis and antimicrobial efficiency of silver nanoparticles (AgNPs) using the crude metabolite of Escherichia coli D8 (MF06257) strain. The bacterial strain had been isolated from a sewage water stream located in Damietta City, Egypt. The optimum conditions for AgNPs production were at temperature 35°C, pH 7 and 1.5mM silver nitrate. The AgNPs biosynthesis was detected in culture filtrate within 1-2 minutes at room temperature (25±2°C) and sunlight. The characterization of AgNPs was studied by UV-Vis spectroscopy (maximum absorbance at 429 nm), X-ray diffraction (XRD) pattern (crystal planes were 110, 111, 200, 211, 220, and 311), transmission electron microscopy (TEM) (AgNPs were spherical in shape ranging from 6 to 17 nm), Fourier transform-infrared (FTIR) spectroscopy (the bands of symmetric and asymmetric amine were assigned at 3421.1 and 2962.13 cm⁻¹, the stretching vibration band of aromatic and aliphatic (C-N) exist at 1392.35 and 1122.37 cm⁻¹ bands), Zeta potential analyser (AgNPs had a negative charge value; -33.6 mV) and size distribution by volume (the presence of capping agent enveloping the AgNPs with a mean size of 136.0 nm). Nitrate reductase (NR) was assayed as an important partner in the optimized production (the rate of NR reached to 2.18 U/ml). The study demonstrated that AgNPs are potent inhibitors of Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Alternaria alternata, Fusarium oxysporum and Aspergillus flavus. The antimicrobial activity of AgNPs was studied by TEM. TEM micrographs showed an inhibition of S. aureus cell multiplication. In case of F. oxysporum, a reduction in the size of treated cells, formation of a mucilage matrix connecting the hyphal cells together, the appearance of a big vacuole, lipid droplets and a severe leakage of cytoplasmic contents were detected. AgNPs exhibited MIC values of 6.25μg/ml and 50 μg/ml against S. aureus and Candida albicans, respectively. In addition, AgNPs showed synergy effects by their combination with fluconazole that increased fold areas especially against A. niger, A. flavus and F. oxysporum.

Keywords: Escherichia coli, silver, nanoparticles, optimization, antimicrobial

INTRODUCTION

Among metallic nanoparticles, silver nanoparticles (AgNPs) have numerous applications in the field of nanobiotechnology due to their unique antimicrobial efficiency as growth inhibitors, killing agents or antibiotic carriers (Hamidi et al., 2019). AgNPs have widely attracted attention for the food, cosmetics and biomedical applications (Sondi and Salopek-Sondi, 2004). In the last few years, different chemical and physical methods had been included in AgNPs synthesis. These incorporated methods produced contaminated, toxic AgNPs in low yields. So, scientific researchers went to the biological synthesis of AgNPs using microorganisms (Wang et al., 2019). Through microbial biosynthesis, numerous scientists used bacterial strains in AgNPs biosynthesis due to their rapid growth rate and highly efficient enzymatic system (Galvez et al., 2019). The use of bacterial crude metabolites was embedded as reducing agents of silver ions into safe and ecofriendly AgNPs that called extracellular biosynthesis (De Souza et al., 2019). The extracellular production is more prioritized than the intracellular which requires extraction and purification of AgNPs from the microbial growth. In addition, the extracellular production was confirmed to include high amounts of proteins which acted as capping agents (Annamalai and Nallamuthu, 2016). One of the mechanistic aspects for AgNPs biosynthesis is the secreted enzymes by bacteria that act as reducing agents for silver ions (Quinteros et al., 2016). The shape and size of the biosynthesized nanoparticles (NPs) could be handled throughout controlling the production parameters such as concentration of metal ions, temperature, incubation period, pH and effect of solar irradiation (Sumitha et al., 2019). AgNPs have a strong bactericidal effect against a broad spectrum of bacteria such as Pseudomonas sp., Acinetobacter sp., Escherichia sp., Vibrio sp. and Salmonella sp. (Paul and Londhe, 2019). Furthermore, the biosynthesized AgNPs showed significance antifungal potential against Aspergillus flavus, A. nomius and A. parasiticus, Alternaria alternata, Fusarium sp., Candida tropicalis and C. albicans was reported (Bocate et al., 2019). This makes the AgNPs a potential candidate as a new generation of antifungal agents. The present work aimed to obtain a potent bioreductant bacterium possessing the ability to synthesize AgNPs extracellularly with efficient antimicrobial activity.

MATERIAL AND METHODS

Chemicals

Culture media was purchased from Oxoid Ltd., England. Silver nitrate was purchased from Panreac Quimica S.L.U, Barcelona, Spain. Fluconazole (Diflucan) was purchased from Pfizer Inc., New York, NY.

Microbial strains

The E. coli D8 strain was isolated from a sewage water stream located at Damietta City, Egypt). It was identified classically according to Bergey’s Manual of Systematic Bacteriology (Imhoff, 2005). The 16S rRNA gene sequence was also performed in order to confirm its identification and deposited in the database under accession number MF06257.

The bacterial and fungal strains used for the antimicrobial activity were kindly provided by the culture collection of Microbiology Laboratory, Faculty of Science, Damietta University, Egypt (Table 1).
dred µL of bacterial crude metabolites were
of the produced nitrite during 60
n of ZOI of each
. (2007)
amples were incubated at
- TEM Unit at
. (2019)
Clinical
thogenic bacteria and fungi. 30 μg
m tetroxide (2%, in the same buffer) for 90 minutes.
E. coli
h to
θ
has one unit of NR activity (U/ml).
NR activity was calculated pertain the amoun
et al
UK). A colloidal solution was used in this instrument by withdrawing 1 ml of
coated copper grid (Type G 200, 3.05 µm diameter, TAAP, USA) was prepared
Mansoura University, Egypt. The shape and size of the optimized AgNPs were
Characterization of biosynthesized silver nanoparticles
The X-ray diffraction (XRD) pattern of the AgNPs was recorded at 29 values
between 10° and 80° using a Cu X-ray tube at 40 kV and 30 mA with the X
The following characterizations of AgNPs were performed at TEM Unit at
Mansoura University, Egypt. The shape and size of the optimized AgNPs were
examined using TEM, a drop coating of nanocollodion solution into carbon-
coated copper grid (Type G 200, 3.05 µm diameter, TAAP, USA), and were grown on
\( \text{Mueller-Hinton broth (MHB)} \)
\( \text{RPMI broth medium} \)
respectively. Serial solutions of AgNPs, Penicillin G and Fluconazole (6.25-125 μg/ml in water) were tested. Mixtures were incubated at 37 °C and 35 °C for
\( \text{S. aureus ATCC25923 and C. albicans ATCC10231, respectively. After 48 hr, the growth turbidity was measured using a spectrophotometer against fungal species (Aspergillus} \)
\( \text{DOX} \)
\( \text{Penicillin G or Fluconazole) were used as antibacterial and antifungal positive control standard, respectively. Plates were incubated at 37°C or 28°C for bacteria and fungi, respectively. After incubation, zones of inhibition (ZOI) were measured in terms of millimetres after 24 hours and 7 days for bacteria and fungi, respectively.

### Minimal inhibitory concentration (MIC)
The MIC values for \( \text{S. aureus ATCC25923 and C. albicans ATCC10231} \) were measured using broth microdilution method according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) (Clinical Laboratory Standards, 2008; 2017). A 0.5 McFarland standard of \( \text{S. aureus ATCC25923} \) and \( \text{C. albicans ATCC10231} \) were grown on \( \text{Mueller-Hinton broth (MHB)} \)
\( \text{S. aureus ATCC25923} \) and
\( \text{C. albicans ATCC10231} \), respectively. After 48 hr, the growth turbidity was measured using a spectrophotometer against the growth control at 630 nm wavelength to determine n-values for each antimicrobial agent.

### Transmission Electron Microscopy (TEM) of nanosilver treated microorganisms
The exponential-phase cultures of \( \text{S. aureus ATCC25923 and F. oxysporum f. sp. lycopersici Fol4287} \) were subjected to silver nanocolloids (6.25, 50, 100 and 150 μg/ml and 50, 100 and 150 μg/ml, respectively) for 2 hours at 37°C and 30°C, respectively. Normal bacteria and fungi were included as controls. The cell cultures were centrifuged at 5000 rpm for 20 minutes, and then washed 3 times with distilled water. Fixative solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7) was added and left for 20 minutes at room temperature. The fixative was removed and then 0.1 M buffer was added for washing and post-fixed with osmium tetroxide (2%, in the same buffer) for 90 minutes. The fixed cells were dehydrated using graded series of ethanol. The dehydrated cells were embedded in Epon-Araldite (1:1) mixture for 1 hour that polymerized at 65°C for 24 hours. The cells were cut cross section using an ultra-microtome (50 µm), double-stained with uranyl acetate and lead citrate and exposed to observation on carbon-
coated copper grids (Type G 200, 3.05 µm diameter, TAAP, U.S.A.) using TEM (JEOL JEM-2100, Japan).

### Statistical analysis
The data were statistically analyzed using software system SPSS version 18. All values in the experiments were expressed as the mean ± standard deviation (SD) and were analyzed with one-way Analysis of Variance (ANOVA). The significant level was set at \( p \leq 0.05 \).

### RESULTS
Optimization of biosynthesized AgNPs
Escherichia coli D8 MF06257 biosynthesized AgNPs within 72 hours in dark conditions. The production of AgNPs was demonstrated by the peak at 429 nm in the UV-Vis spectra. Using 1% of bacterial supernatants and 1.5 mM
concentration greatly enabled AgNPs synthesis (Figure 1a). According to pH value, the brown colour appeared at pH (5-6) and its intensity was increased with the increase in pH value (Figure 1b). Stable and monodispersed AgNPs were synthesized at pH7. It was found that 35°C was the optimal temperature for AgNPs synthesis (Figure 1c). The brown colour appeared within 72 hours (Figure 1d) during incubation in dark conditions while biosynthesis occurred throughout a minute in case of the presence of solar irradiation (Figure 1e).

Characterization of the biosynthesized AgNPs

The XRD pattern for E. coli D8 (MF062579) crude metabolite and AgNPs were shown in Figure 2. It showed six characteristic peaks of AgNPs that appeared at 31.7°, 37.6°, 45.7°, 57°, 64.26°, and 77.4°, corresponding to respective crystal planes (110), (111), (200), (211), (220), and (311) (Galvez et al., 2019).

TEM images (Figure 3) show the spherical shaped and well-dispersed AgNPs. The particle size distribution analysis in the present study showed a mean size of 6-17 nm.
The Zeta potential study established the negative charge (-33.6) of the AgNPs (Figure 4a) and the size distribution by volume showed the presence of a capping agent surrounded AgNPs having mean size of 136.0-294.3 nm (Figure 4b).

Figure 5 illustrates the FTIR spectrum of *E. coli* D8 (MF062579) crude metabolite and AgNPs which confirmed the presence of proteins in the AgNPs biosynthesis. The stretch, primary and secondary amines vibrations bands were noted at 3421.1 cm\(^{-1}\) and 2962.13 cm\(^{-1}\), respectively. The stretching vibration of molecule appeared at 1658.48 cm\(^{-1}\) and 1596.77 cm\(^{-1}\) bands. The stretch C-N vibration of aromatic and aliphatic amines existed at 1392.35 cm\(^{-1}\) and 1122.37 cm\(^{-1}\) bands.

**Figure 3** Transmission electron micrograph of produced AgNPs (scale bar = 100 and 50 nm).

**Figure 4** (a) Zeta potential measurement analysis of AgNPs. (b) Size distribution by volume.

**Figure 5** FTIR spectrum of *E. coli* D8 MF062579 crude metabolite with and without AgNPs.

**Nitrate reductase activity**

The activity of NR was measured and calculated in the *E. coli* D8 (MF062579) crude metabolite, at the rate of 2.18 U/ml.
Antimicrobial Potential OF AgNPs

Agar well diffusion method

The AgNPs synthesized by E. coli D8 (MF062579) isolate crude metabolite were centrifuged and dried using freeze dryer (SIM international, USA, FFD-8T) and tested against different pathogenic bacterial and fungal strains. There are significant differences in antimicrobial effects between the samples with AgNPs, Penicillin G and AgNPs & Penicillin G treatment. Highly significant (P < 0.05) was observed between the microbial strains mainly S. aureus ATCC25923, E. coli ATCC25922, P. aeruginosa ATCC27853 (Table 2), A. alternata Fr. Keiseler, F. oxysporum f. sp. lycopersici Fol4287 and A. flavus Link ex Fries group (Table 3) and the diameter of inhibition zone.

Table 2: Antibacterial potential of AgNPs in comparison with benzylpenicillin (Penicillin G) as a standard drug in addition to the synergy action.

| Antibacterial agent | Concentration, μg/ml | Gram-positive bacteria | Zone of inhibition (mm, mean) * |
|---------------------|----------------------|------------------------|-------------------------------|
|                     |                      | Bacillus cereus ATCC6633 | Staphylococcus aureus ATCC25923 | Escherichia coli ATCC25922 | Pseudomonas aeruginosa ATCC27853 | Klebsiella pneumoniae ATCC33495 |
| AgNPs               | 50                   | 12 ± 0.03**            | 20 ± 0.14**                   | 18 ± 0.14**                 | 17 ± 0.03**                  | 14 ± 0.14**                |
|                     | 100                  | 15 ± 0.03**            | 24 ± 0.06**                   | 21 ± 0.14**                 | 20 ± 0.06**                  | 18 ± 0.14**                |
|                     | 150                  | 19 ± 0.06**            | 27 ± 0.14**                   | 24 ± 0.14**                 | 24 ± 0.06**                  | 22 ± 0.14**                |
| Penicillin G        | 50                   | 12 ± 0.03**            | 10 ± 0.03**                   | 36 ± 0.06**                 | 11 ± 0.06**                  | -ve                        |
|                     | 100                  | 14 ± 0.03**            | 12 ± 0.03**                   | 38 ± 0.03**                 | 14 ± 0.03**                  | -ve                        |
|                     | 150                  | 16 ± 0.03**            | 15 ± 0.14**                   | 40 ± 0.03**                 | 21 ± 0.03**                  | -ve                        |
| AgNPs & Penicillin G| 50                   | 13 ± 0.03**            | 18 ± 0.03**                   | 19 ± 0.06**                 | 18 ± 0.03**                  | 13 ± 0**                   |
|                     | 100                  | 15 ± 0.03**            | 21 ± 0.03**                   | 23 ± 0.03**                 | 20 ± 0.03**                  | 18 ± 0**                   |
|                     | 150                  | 17 ± 0.03**            | 25 ± 0.14**                   | 27 ± 0.03**                 | 25 ± 0.03**                  | 21 ± 0**                   |

*Mean surface area of the inhibition zone was calculated for each from the mean diameter ± SD.

Table 3: Antifungal potential of AgNPs in comparison with Fluconazole as a standard drug in addition to the synergy action.

| Antifungal agent | Concentration, μg/mL | Aspergillus niger van Tiegh | A. flavus Link ex Fries group | A. fumigatus Fresenius | Alternaria alternata Fr. Keiseler | Fusarium oxysporum f. sp. lycopersici Fol4287 | Candida albicans ATCC10231 |
|-----------------|----------------------|-----------------------------|--------------------------------|------------------------|---------------------------------|-----------------------------------------------|-------------------------------|
| AgNPs           | 50                   | 44 ± 0.06                   | 31 ± 0.03                      | 35 ± 0                  | 22 ± 0.14                       | 11 ± 0.06                                  | 11 ± 0.03                     |
|                 | 100                  | 47 ± 0.03                   | 35 ± 0.06                      | 37 ± 0.03               | 25 ± 0.03                       | 15 ± 0.03                                  | 13 ± 0                        |
|                 | 150                  | 52 ± 0.14                   | 39 ± 0.06                      | 40 ± 0.03               | 27 ± 0.06                       | 17 ± 0.03                                  | 15 ± 0.03                     |
| Fluconazole     | 50                   | 11 ± 0.06                   | 12 ± 0.03                      | 13 ± 0.03               | 15 ± 0.03                       | 10 ± 0.03                                  | 11 ± 0                        |
|                 | 100                  | 13 ± 0                      | 14 ± 0.03                      | 15 ± 0.03               | 19 ± 0.03                       | 12 ± 0.03                                  | 13 ± 0                        |
|                 | 150                  | 18 ± 0.14                   | 17 ± 0.03                      | 19 ± 0.03               | 22 ± 0.06                       | 15 ± 0.06                                  | 16 ± 0.14                     |
| AgNPs & Fluconazole | 50 | 49 ± 0.06 | 38 ± 0.06 | 27 ± 0.03 | 19 ± 0.03 | 16 ± 0.03 | 10 ± 0.03 |
| & Fluconazole   | 100                  | 53 ± 0.03                   | 41 ± 0.04                      | 33 ± 0.03               | 22 ± 0.03                       | 18 ± 0.06                                  | 12 ± 0                        |
|                 | 150                  | 55 ± 0.06                   | 44 ± 0.03                      | 36 ± 0.03               | 24 ± 0.06                       | 22 ± 0.06                                  | 15 ± 0                        |

*Mean surface area of the inhibition zone was calculated for each from the mean diameter ± SD.

The less inhibition effects of Fluconazole (15, 17 and 18 mm) were against the pathogenic fungi F. oxysporum f. sp. lycopersici Fol4287, A. flavus Link ex Fries group and A. niger van Tiegh, respectively. However, the biosynthesized AgNPs revealed significant synergistic effects when compared with Fluconazole in addition to its antifungal activities showing higher fold areas (Table 4).

Table 4: Synergistic effect of AgNPs and Fluconazole.

| Fungi                        | Zone of inhibition (mm, mean) * | Increase in fold area |
|------------------------------|---------------------------------|-----------------------|
| Aspergillus niger van Tiegh  | 13                              | 53                    | 15.62                 |
| A. flavus Link ex Fries group| 14                              | 41                    | 7.58                  |
| Fusarium oxysporum f. sp. lycopersici Fol4287 | 12 | 18 | 1.25 |

*Mean surface area of the inhibition zone was calculated for each from the mean diameter.

Figure 6 (a) Effect of AgNPs and Fluconazole on the growth of C. albicans ATCC10231. (b) Effect of AgNPs and Penicillin G against S. aureus ATCC25923.
TEM of nanosilver treated microorganisms

Antimicrobial activities of AgNPs against *S. aureus* ATCC25923 and *F. oxysporum* f. sp. *lycopersici* Fol4287 were easily demonstrated by TEM analysis as shown in Figures 7 and 8. TEM micrographs showed the morphological changes of the treated *S. aureus* ATCC25923 cells and inhibition of cell multiplication. The treated *F. oxysporum* f. sp. *lycopersici* Fol4287. TEM micrographs showed many changes, including reduced size of treated cells, the formation of a mucilage matrix connecting the hyphal cells together, the appearance of big vacuole and lipid droplets with severe leakage of cytoplasmic contents.

![Figure 7](image1.png)  ![Figure 8](image2.png)

**Figure 7** The bactericidal effect of AgNPs on the ultrastructure of *S. aureus* ATCC25923. (a) is a negative control (without nanosilver). Note normal cell division (arrow) and DNA replication. (b), (c), (d) and (e) are treated samples, there is no cell division observed at 150 μg/mL of AgNPs. The amount of DNA appeared to be less than those of untreated ones (arrow).

**Figure 8** The antifungal activity of AgNPs on the ultrastructure of *F. oxysporum* f. sp. *lycopersici* Fol4287. (a) is a negative control (without nanosilver). Note normal cell wall (W), plasma membrane (PM), Vacuole (V) and compact cytoplasm (Cy). (b), (c) and (d) are treated samples, note the formation of a mucilage substance (M) connecting the hyphae together. Note also big vacuole (V) and lipid droplets (L).
DISCUSSION

Bacteria are considered as an excellent source for the extracellular biosynthesis of nanomaterials. There is a bigwig whack to discover novel bacterial strains having motivated biological potential (Galvez et al. 2018). The crude metabolite of E. coli D8 MF06257 was used as a reducing agent, solvent toxicity and capping agent in the NPs extracellular biosynthesis. This type of synthesis is safe, renewable, simple, eco-friendly and cost-effective (Saiﬁuddin et al., 2009). This biosynthesis was performed within 1-2 minutes at room temperature and sunlight and light was found to be the best source of energy for the reduction of metallic plasmon vibrations in the AgNPs (Balasouha et al., 2006). The reduction of silver ions may be resulting from the NADH dependent enzymes activity present in the crude metabolite and/or some redox agents such as sulfur-containing proteins (Krishnaraj et al., 2012). The present study further reported the ability of E. coli D8 crude metabolite to reduce AgNPs and AuNPs and its brown color due to the excitation of surface plasmon vibrations in the AgNPs (Balasouha et al., 2006). The reduction of AgNPs by the crude metabolite of E. coli D8 showed three water soluble quinones; menaquinone, demethylmenaquinone and ubiquinone. The Specrophotometer analysis of the plasma absorption of the produced AgNPs showed maximum peak at 429 nm indicating to the good dispersion of AgNPs in sunlight after 1 minutes indicating NR is not the only factor in the silver ion reduction. Duan et al. (2016) suggested quinones act as an electron shuttle compound in the presence of sunlight and reduced silver ions into AgNPs. Sharma et al. (2012) believed that the crude metabolite of E. coli contains three water soluble quinones; menaquinone, demethylmenaquinone and ubiquinone.

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