Microorganism-mediated green synthesis of silver nanoparticles using *Aspergillus niger* and *Bacillus megaterium*

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Research in the field of nanotechnologies, the development of reliable and environmentally friendly processes for the synthesis of metallic nanoparticles constitutes an important element in this field. In this study, biosynthesis of silver nanoparticles using *Bacillus megaterium* and *Aspergillus niger* is reported. The results show that the characteristic UV-vis absorbance peak of AgNPs synthesized using *Aspergillus niger* was observed in the 433 - 448 nm range and around 475 nm with *Bacillus megaterium*. Most of the particles were spherical in shape and within a size range of 1 to 10.5 nm using *Aspergillus niger* and 3 to 15 nm with *Bacillus megaterium*. The FTIR analysis of AgNPs showed five absorbance bands at 3446, 1645, 1373, 1080, and 790 cm^{-1}. These AgNPs have potential antibacterial activity against *Escherichia coli*. The results of our study could help to improve the silver nanoparticle synthesis since our method makes them small, stable, and with a high antibacterial efficiency.

(Received November 14, 2021; Accepted March 18, 2022)

Keywords: Nanotechnology, Silver nanoparticles, Nanomaterials, Green synthesis, Bacillus megaterium, Aspergillus niger

1. Introduction

In the recent years, the applications of silver nanoparticles (AgNPs) have been of great interest in many scientific fields including biotechnology, biomedicine, ecology, agriculture, veterinary medicine, food, or cosmetics. This is due to their high chemical and thermal stability, their ecofriendly nature and their superior antibacterial and antifungal properties [1]. Many physical, chemical and biological methods are used to synthesize AgNPs. The chemical and physical methods are expensive, require large amounts of energy and generate toxic by-products, so that it has a lasting effect on the ecosystem. In contrast, the synthesis of AgNPs by biological methods is simple, fast, cost-effective, non-toxic and environmentally friendly [2].

Many studies have shown that biomaterials such as bacteria, fungi, and plants can be used to synthesize AgNPs [3]. By changing the AgNPs synthesis parameters like microorganism type, growth stage of microbial cells, growth medium, synthesis conditions, pH, substrate concentrations, source compound of target NPs, temperature, reaction time, and the addition of nontarget ions, we might be possible to obtain sufficient control of particle size and monodispersity [4]. In addition, biological methods have several other advantages, such as low toxicity, low cost, biocompatibility, high stability, good dispersion, and non-agglomeration [5].

An important consideration in microbial nanosynthesis is the selection of an appropriate microbial strain that can synthesize the targeted NPs with a specific particle shape and size range. The majority of the reported bacterial synthesized AgNPs are spherical, but a few others are triangular, hexagonal, cuboid, bullet-shaped, rhombic, rectangular, or in nanoplate form [6]. Fungi are also interesting agents for biogenic synthesis of silver nanoparticles since they can be produced while controlling their size and morphology [4, 7]. Fungi have advantages over other microorganisms, in that they produce large quantities of proteins and enzymes, some of which can be used for fast and sustainable synthesis of nanoparticles [4, 8].

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https://doi.org/10.15251/DJNB.2022.171.359

The strong antimicrobial activity is a major reason for the increased development of products that contain AgNPs. In particular, the antibacterial activity of AgNPs has been studied more because it inhibits the growth of microorganisms more effectively than other NPs [9, 10] and they are non-toxic to the human body at low concentrations [11]. AgNPs kill a wide range of Gram positive, Gram negative and antibiotic resistant bacteria and enhance the effectiveness of several antibiotics [10]. The antimicrobial activity of AgNPs can also affect fungal species, such as *Aspergillus* sp., *Candida* sp., *Saccharomyces* sp., [10] or *Penicillium* sp. [12]. Recently, it has been suggested that AgNPs bind with external membrane of lipid enveloped virus to prevent the infection. Although their interaction with viruses is still a largely unexplored field [13].

The main challenge in nanomaterials synthesis is the control of their physical properties, in order to obtain the desired particle size, morphology, chemical composition, crystal structure, and ability for monodispersity in solution. Many studies have shown that, the smaller the particle size, the larger the numbers of atoms on the surface, and, therefore, the electrons capable of exerting their antibacterial activity on pathogens. This is why synthesizing AgNPs with a small size is key to obtain better performance in many areas, especially in antibacterial activity. In this study, microbial production of AgNPs was investigated. Using the bacterial strain *Bacillus megaterium* and fungi strain *Aspergillus niger* as a reducing agent, this research work focuses on the production of AgNPs and their characterization using UV-vis spectrometer, FTIR, SEM, TEM, XRD analysis, and study their inhibitory action against *Escherichia coli*. Our goal in this study is to synthesize stable silver nanoparticles with a small size and a potent antibacterial effect.

2. Materials and methods

2.1. Materials

Silver nitrate ($AgNO_3$) was obtained from Sigma Aldrich (China). Luria-Bertani broth, LB Agar, Yeast Malt Broth (YM Broth) used for bacterial growth study were the products of HiMedia, India. Cultures of *Bacillus megaterium*, *Aspergillus niger* and *Escherichia coli* were obtained from Department of Microbiology, Vietnam National University, Hanoi. All other reagents and solvents used were purchased from Merck (Mumbai, India) and were of analytical grade with maximum purity.

2.2. Production of Biomass and Biosynthesis of AgNPs

The bacterial strain *Bacillus megaterium* was cultured in Luria-Bertani broth and the fungi strain *Aspergillus niger* was cultured in Yeast Malt Broth medium to produce the biomass for biosynthesis. The cultures were incubated at 35 °C for 72 h on a rotary shaker at 200 rpm. The culture supernatant was obtained following centrifugation at 11.000 rpm for 10 min. The supernatant was subsequently used for AgNPs biosynthesis.

$AgNO_3$ (0.2 mM or 0.4 mM of final concentration) was added to the 100 ml of collected cell-free supernatant and was kept for 72 h in a shaker at 150 rpm in the dark, at 60 °C for *Bacillus megaterium* and 70 °C for *Aspergillus niger*. The bioreduction of the silver ions in the solution was monitored by sampling the aqueous solution (2 ml) and measuring the UV-vis absorption spectrum of the solution.

2.3. Characterization of AgNPs

UV-vis spectrophotometry - Synthesized AgNPs were monitored by acquiring the UV-vis spectra of the reaction medium at wavelengths ranging from 300 to 800 nm using UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan).

SEM - The morphology and size of the synthesized AgNPs was studied by scanning electron microscopy (SEM) using ZEISS Sigma scanning electron microscope with 10 kV accelerating voltage.

TEM - The size and morphology of the AgNPs were determined by using TEM model JEOL electron microscope JEM-100 CX. The AgNPs were put on a carbon-coated TEM grids and allowed to dry, the extra solution was removed using a blotting paper. The AgNP size was calculated from the TEM images by measuring the diameter of approximately 50 nanoparticles.

XRD analysis - The formation of silver nanoparticles was checked by X-ray diffraction (XRD) using an X-ray (Bruker D2 diffractometer equipped with a Cu Kα radiation source). The full widths at half maximum (FWHM) values of X-ray diffractions were used to calculate particle size using the Debye-Scherrer formula [14].

FTIR spectroscopy - FTIR was used to recognize the conceivable biomolecules charge of the reduced Ag ions and capping of the bio-reduced AgNPs produced by the microbial extract. In order to detect the functional groups and their possible partnership in the biogenic of AgNPs, the freeze-dried produced nanoparticles was detected using FTIR spectroscopy (IRAffinity-1S Shimadzu, Japan).

2.4. Determination of Antimicrobial Activity

The AgNPs synthesized from *Bacillus megaterium* and *Aspergillus niger* were tested for antimicrobial activity by well-diffusion method against pathogenic microorganism *Escherichia coli*. The pure cultures of organisms were subcultured on LB agar at 35 °C. Wells of 6 mm diameter were made on agar media plates using gel puncture. 20 µl of the sample of AgNPs synthesized from *Bacillus megaterium* and from *Aspergillus niger* were loaded into the well using a micropipette. After a 24 h incubation at 35 °C, the different levels of zone of inhibition were by measuring the diameter of the zone of inhibition around each disc to the nearest mm.

2.5. Statistical analysis

Testing the significance of antimicrobial activity of silver nanoparticles was carried out by standard analysis of variance (ANOVA) (GraphPad Software 5.0, San Diego CA). The determinations were done in triplicate and the mean values ± SD were presented.

3. Results and discussion

3.1. Synthesis of AgNPs

In this study, we show for the first time the role of *Bacillus megaterium* and *Aspergillus niger* in the synthesis of AgNPs. The formation of AgNPs by their culture supernatants was described by the color change of the reaction mixture. The color change of the reaction mixture is the result of the excitation of surface plasmon resonance oscillations of AgNPs present in the reaction mixture [15,16] and has been observed by many researchers before [17]. We show that the cell filtrate of the tested bacterial and fungal strains was incubated with silver ions and all the selected organisms synthesized AgNPs successfully. Addition of $AgNO_3$ (0.2 mM and 0.4 mM) into the cell-free culture, in the dark, resulted in a color change from yellow to dark brown, indicating AgNPs synthesis due to the reduction of $AgNO_3$ (Fig. 1). Control (without $AgNO_3$) showed no color formation in the culture when incubated under the same conditions (data not shown). The intensity of the color of the solution increased further during incubation.

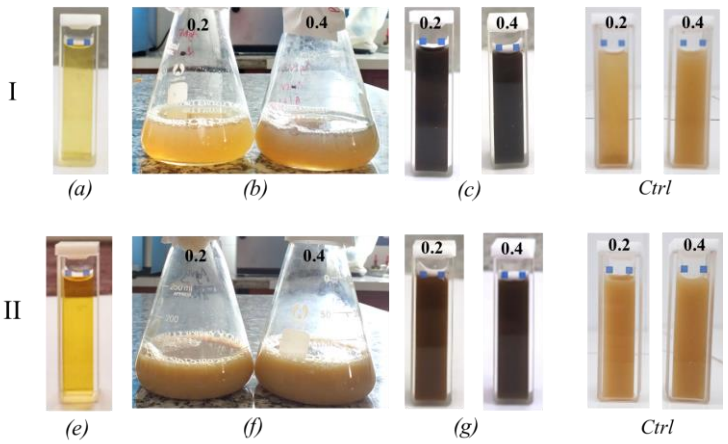

Fig. 1. Erlenmeyer flask containing cell-free filtrate of Aspergillus niger (I) and Bacillus megaterium (II) without (a) (e) and with (b) (f) silver nitrate (c) (g) with silver nitrate after 72 h of reaction. Ctrl: without silver nitrate after 72 h of reaction.

3.2. Characterization of AgNPs

The confirmation of stability and formation of AgNPs in the reaction flask was observed by UV-vis at wavelengths ranging from 300 to 800 nm (Fig. 2). Our results show that the *Bacillus megaterium* cell filtrate treated with $AgNO_3$ had the plasmon resonance peak noted around 475 nm (Fig. 2a). Our results are similar to the findings of Alsamhary [18], who reported an absorption peak at about 400 - 470 nm when studying *Bacillus subtilis*. It was indicated that the peak at 470 nm is due to the excitement of longitudinal plasmon vibrations. However, in the UV-vis spectrum of *Aspergillus niger* cell filtrate treated with $AgNO_3$, a broad peak was observed between 433 - 448 nm, the widening of the peak indicating that the particles were polydispersed (Fig. 2b). AgNPs are known to exhibit a UV-vis absorption maximum in the range of 400 - 500 nm because of surface plasmon resonance [19]. The peak intensity increasing along the exposure time under the experimental conditions in Fig. 2 indicates the increased synthesis of AgNPs in the reaction mixture and thus that of the amount of AgNPs [20, 21]. After 72 h of incubation, no further increase in intensity was recorded, indicating complete reduction of precursor silver ions (data not shown). Furthermore, the absorption peak of the produced AgNPs is not changing after 72 h, indicating their high stability. A change in the peak position of the samples could lead to an undesirable change in the characteristics of the synthesized nanoparticles [22]. The stable position of the absorbance peak indicates that new particles do not aggregate. The control solution (without $AgNO_3$) shows no evidence of absorption in the range of 300 to 800 nm.

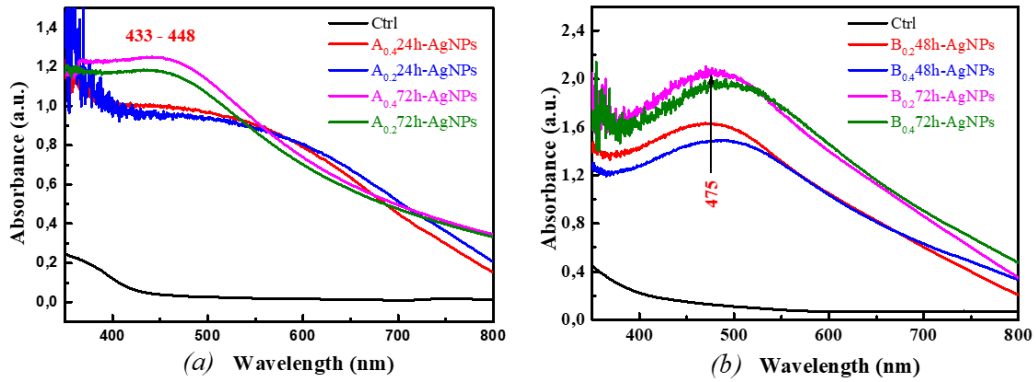

Fig. 2. UV-vis spectrum of AgNPs produced from cell-free culture of Aspergillus niger (a), Bacillus megaterium (b). $A_{0.2}$ or $A_{0.4}$: addition of $AgNO_3$ into 100 ml cell-free culture from Aspergillus niger to 0.2 mM or 0.4 mM final concentration; $B_{0.2}$ or $B_{0.4}$: addition of $AgNO_3$ into 100 ml cell-free culture from Bacillus megaterium to 0.2 mM or 0.4 mM final concentration.

The FTIR analysis of A-AgNPs revealed five prominent bands at 3446, 1645, 1373, 1080, and 790 cm^{-1}. B-AgNPs had FTIR bands at 3446, 2127, 1645, 1373, and 790 cm^{-1} (Fig. 3). These data provide evidence of specific functional groups constituting organic molecules, which are responsible for the reduction of silver ions to AgNPs as well as their stability [23, 24]. The absorbance peaks at 3446 cm^{-1} can be assigned to N–H stretching vibrations present in the amide linkages of primary and secondary amines of proteins [25] and O-H stretching vibrations of phenols/alcohols [26]. The peaks at 2127 cm^{-1} correspond to C=O stretching vibrations of carboxylic acids [26]. The band at 1645 cm^{-1} may indicate –C=O carbonyl groups and –C=C– stretching [27]. The peak 1373 cm^{-1} shows the presence of nitrocompounds from proteins or enzymes [28] and that at 1080 cm^{-1} can be assigned to –C–N– stretching vibrations [15]. The peak at 790 cm^{-1} can be assigned to the C–C stretch vibration of alkyl halides. This shows that proteins have a beneficial effect on AgNPs stabilization. By binding to AgNPs through free amino acids or

cysteine groups, and by encapsulating them, proteins help to stabilize biosynthesized AgNPs by creating a protective layer around them that prevents agglomeration [29, 30].

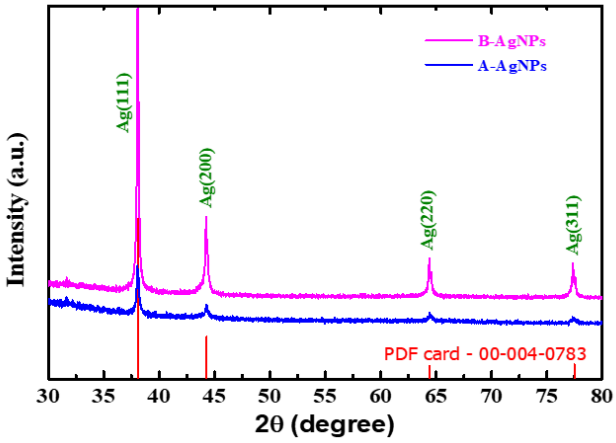

Fig. 3. XRD spectrum of synthesized AgNPs from cell-free culture of Bacillus megaterium (violet), and Aspergillus niger (blue) after 72 h.

The phase structure and purity of the AgNPs were examined by Power X-ray diffraction studies. The powder XRD pattern of the obtained AgNPs is shown in (Fig. 4). The recorded XRD spectrum of AgNPs confirmed their crystalline nature by 2θ values of 38.11°, 44.27°, 64.42° and 77.47° that can be indexed to the (111), (200), (220) and (311) Bragg's reflections of cubic structure of silver (PDF card-00-004-0783), respectively (Fig. 4). The formation of nanoparticles was indicated by the broadening of Bragg's peaks. The average size of AgNPs was calculated using Debye-Scherrer's equation [14]; $D = k\lambda/\beta\cos\theta$; where D is the crystallite size, k the Scherrer coefficient, λ the wavelength of X-rays, β the full width half maxima (FWHM), θ Bragg's angle (half of 2θ). The average crystalline size of the synthesized AgNPs was approximately 9.77 nm from *Bacillus megaterium* and approximately 8.37 nm from *Aspergillus niger*.

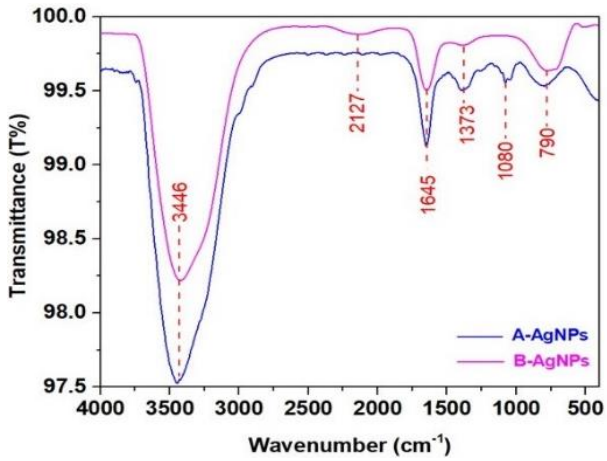

Fig. 4. FTIR spectral analysis of AgNPs synthesized using Bacillus megaterium (violet), and Aspergillus niger (blue) after 72 h.

3.3. SEM and TEM Analysis

SEM and TEM analysis gave us information about structural morphology, size, and shape of the synthesized AgNPs (Figs. 5 and 6). AgNPs were found to be of spherical or roughly spherical forms in most of the cases. The structural morphology, size, and shape of AgNPs found

in SEM measurement may be due to the combination of smaller particles and aggregates forming clusters (Fig. 5). The diameter of these nanoparticles lied between 3 to 15 nm from *Bacillus megaterium* and between 1 to 10.5 from *Aspergillus niger* (Fig. 6). Similar results were obtained in other studies that AgNPs synthesized from *Bacillus subtilis* or *Bacillus cereus* are spherical and in the size range of 2 to 20 nm [18, 31] Peiris et al. also reported in 2018 that AgNPs synthesized by *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, *E. coli* ATCC 25922 and *A. baumannii* are less than 20 nm [31] or in the size range of 4 - 24 nm [6, 27]. However, the study of Saravanan et al. reported that the size of AgNPs synthesized from *Bacillus megaterium* (NCIM 2326) was in the range of 80 - 98.56 nm [9], a much larger size than that of AgNPs synthesized from *Bacillus megaterium* using our method. Other studies on AgNPs synthesis from other bacteria strains showed the fabrication of NPs larger than 50 nm [32-34] or > 100 nm, [35, 36] and > 80 nm when obtained from fungi [16, 37, 38] Many studies reported that the temperature, pH, or $AgNO_3$ concentration in medium can affect nanoparticle size and stability. In a couple of studies, the rise in temperature was found to lead to a nanoparticles size increase, [29, 37] but in other it was found to lead to a size decrease [39, 40]. These contradictory results indicate that the effect of temperature on the size and stability of the nanoparticles synthesized varies according to the microorganism's species used. Adjustment of the synthesis pH can also help to control certain characteristics of the nanoparticles because the conformation of nitrate reductase enzymes could be altered depending on the concentration of protons in the reaction medium, leading to alteration of the morphology and size of the nanoparticles [41]. In some cases, a low $AgNO_3$ concentration resulted in a smaller nanoparticle size and an improved dispersion, [42, 43] depending on the species used. In general, it has been found that reducing the size of AgNPs enhances their stability and biocompatibility [44].

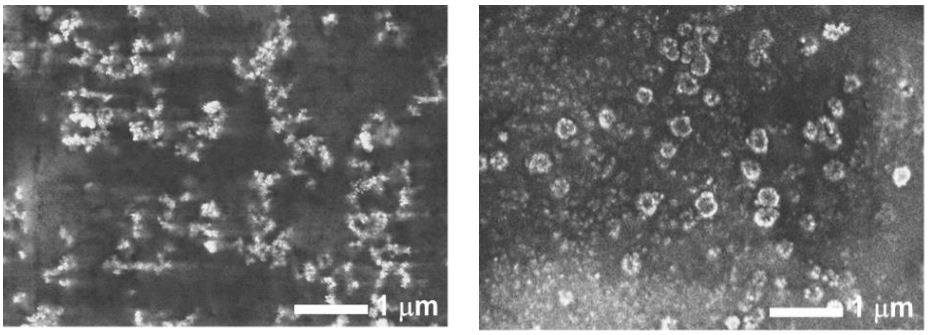

Fig. 5. SEM image of AgNPs produced from cell-free culture of Aspergillus niger and Bacillus megaterium after 72 h.

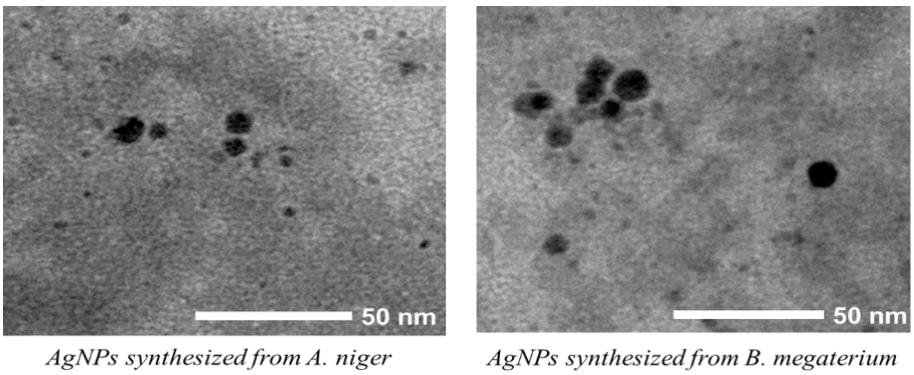

Fig. 6. TEM image of AgNPs produced from cell-free culture of Aspergillus niger and Bacillus megaterium after 72 h.

3.4. Antimicrobial efficacy

The antibacterial property itself of the AgNPs having already been established by numerous studies, that of the synthesized AgNPs was evaluated using disk diffusion method against selected pathogens. Zones of inhibition were observed for AgNPs synthesized by *Aspergillus niger* (A-AgNPs) and *Bacillus megaterium* (B-AgNPs). The visible clear zone produced by AgNPs against *Escherichia coli* pathogens is showed in Fig. 7. Data shows that the antibacterial efficacy of AgNPs tested against *Escherichia coli* after 24 h, 48 h, 72 h of reaction is not different. This mean shows that the antibacterial efficacy against *Escherichia coli* remained stable after 72 h, which corresponds to our expectation to find a method that produce a small size and stable AgNPs with long-term antibacterial activity.

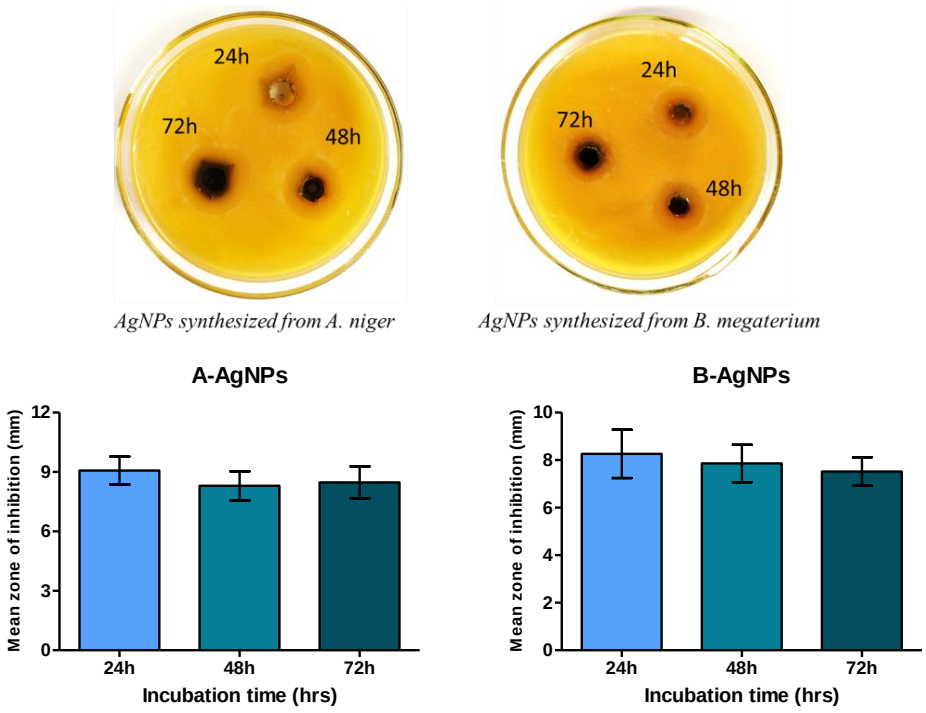

Fig. 7. Antibacterial activity assay against Escherichia coli by the disk diffusion method. A-AgNPs (synthesized from cell-free culture of Aspergillus niger), B-AgNPs (synthesized from cell-free culture of Bacillus megaterium).

Silver has been used for many years for its antibacterial properties in the medical field [3]. Recently, nanosilver has been used as an antimicrobial agent. However, the exact mechanism by which silver ions and AgNPs exert their antibacterial effects remains to be determined. Many studies have shown that the bactericidal effect of silver nanoparticles is size dependent [43-46]. The size dependence of the bactericidal potential of nanoparticles was investigated by Panacek et al. (2006) [47]. Smaller AgNPs with a large surface area available for interaction will have stronger antimicrobial effects than larger AgNPs [48]. Silver nanoparticles disrupt cellular function by attaching to the surface of cell membranes [45]. Panacek et al. (2006), reported that nanoparticles with the size of 25 nm had the highest antibacterial activity [47]. AgNPs not only interact with membrane surfaces, but can also penetrate inside bacteria [48] and can prevent cell division and DNA replication, ultimately leading to cell death [45].

4. Conclusion

We have found that the use of *Aspergillus niger* and *Bacillus megaterium* can be used to synthesize AgNPs with a size of 1 - 15 nm and a spherical shape. This was confirmed by a

plasmon resonance peak at 475 nm with *Bacillus megaterium* and a 433 - 448 nm range with *Aspergillus niger*, and by SEM, TEM and XRD analysis. These AgNPs were successfully tested for removing *Escherichia coli* pathogens. This simple selection method is ecofriendly in that it does not produce toxic chemicals during the biosynthesis process. Compared to other methods, it is also a cheaper and faster way of producing AgNPs, but further studies are required regarding the antimicrobial activity of AgNPs used for clinical applications.

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