Research Article

Green Extracellular Synthesis of the Silver Nanoparticles Using Thermophilic *Bacillus Sp*. AZ1 and its Antimicrobial Activity Against Several Human Pathogenetic Bacteria

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Received: September 20, 2015; **Revised:** December 16, 2015; **Accepted:** February 15, 2016

Background: Silver nanoparticles (AgNPs) are among the most effective antimicrobial agents that are used in the medicine and pharmaceutics. During the past decades, metal nanoparticles synthesis through application of the biological methods has increasingly been used, as the biologically synthesized particles are mostly non-toxic as well as effective.
Objectives: The main goal for undertaking the present investigation was to evaluate the extracellular synthesis of the AgNPs by a native thermophilic *Bacillus Sp*. AZ1 that was isolated from a hot spring in Ardebil province. Subsequently the antimicrobial potentials of the nanoparticle was evaluated against several human pathogenic organisms.
Materials and Methods: The biosynthesized AgNPs were confirmed visually by appearance of a dark brown color formation in the mixture as well as silver surface plasmon resonance band by using UV-Visible spectroscopy. The AgNPs were further characterized by SEM, EDX and TEM. The antimicrobial activity of the AgNPs was investigated using *Salmonella typhi*, *Escherichia coli*, *Staphylococcus epidermis*, and *Staphylococcus aureus*, by applying disk diffusion method.
Results: Identification of the strain AZ1 by the 16S rRNA sequence analysis showed 99% sequence homology between this strain and *B. licheniformis*. The obtained UV-Visible spectrum of the aqueous medium containing silver ion, showed a peak at 425 nm which indicates a correspondence to the plasmon absorbance of the silver nanoparticles. The biosynthesized AgNPs were found to be in the size range of ~7-31 nm with spherical the shape. Studies regarding the antibacterial effect of the particles showed the highest inhibitory effect against the two strains; *E. coli*, and *S. typhi*, respectively.
Conclusions: Our study presents a simple green synthesis process for the production of an extracellular nanoparticles which is environmental friendly. Biosynthesis of the AgNPs by a thermophilic bacillus from the hot spring (Qeynarjeh, Ardebil) in Iran with the highest similarity to *Bacillus licheniformis* is reported for the first time.

Keywords: Antimicrobial agents; Biosynthesis; Nanoparticles; 16S rRNA

1. Background

Resistance to human pathogens to antibiotics is a major challenge in different areas, such as biomedical and pharmaceutical researches. As examples in this regard, *Staphylococcus aureus* is resistant to the methicillin, *Salmonella typhi* to the ciprofloxacin. The problem extends and prevails for other pathogenic bacterial species as well (1-3). The new drug resistant pathogenic bacteria have led to many concerns about ineffectiveness of the antibiotics in addition to the emergence, or reemergence of the multidrug-resistant (MDR) pathogens (4). Several decades ago substances such as silver salts and elemental silver have been used in primary health and curative wound care (5, 6), however, with the appearance and development of new antibiotics, the interest to silver as an antimicrobial was drastically reduced (8, 7). The AgNPs can be used against a broad range of microbes (9). The AgNPs aims a broad range of targets in the micro-organisms. As well, it was found that, it is unlikely that microbes caould develop resistance against nanosilver as they need to develop a board range of mutations simultaneously to protect themselves (5). The AgNPs can be synthesized using different methods, such as chemical, physical, and biological. Synthesis of nanoparticles by chemical method is simple and economic since it requires short time for syn-

thesis. Also, synthesis by this method would result in the production of a large amount of the nanoparticles. But nowadays, development of the nature-friendly processes for the nanoparticles synthesis are essential and mainly considered. Thus, developing toxic chemical methods have not gained any point for the synthesis of the nanoparticles. Therefore, biological method of synthesis could be introduced as an alternative method for elimination of such problems (10-12). Until now, the AgNPs were synthesized by bacteria (13), plants (14), fungi (15), Algae (16), and yeasts (17). Inorganic nanoparticles can be generated using micro-organisms through either extracellular or intracellular activities (18). The extracellular biosynthesis of the nanoparticles is economic compared to the intracellular method, because of the simplicity of the production process. The intracellular biosynthesis method requires some additional steps such as application of suitable detergents or ultrasonic treatment for releasing of the synthesized nanoparticles (19, 20). In this study, we have used the culture supernatant containing silver nanoparticles of the thermophilic *Bacillus sp*. AZ1 isolated from hot spring of Qeynarjeh, Ardebil province in Iran. In the present article we report the antimicrobial activity of the AgNPs against a number of human pathogenic bacteria. The nanoparticles were quantified and characterized by UV-Vis spectroscopy, Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Analysis (EDX), and Transmission Electron Microscopy (TEM).

2. Objectives

The main goal of this project was to evaluate extracellular synthesis of the AgNPs by a native thermphilic *Bacillus Sp*. AZ1 which was isolated from a hot spring in Iran and its antimicrobial potentials against several human pathogenic organisms.

3. Materials and Methods

3.1. Isolation and Identification of the Microorganism

The Bacillus strain used in the present study was isolated from a hot spring in Qeynarjeh, Ardebil provice in the north-west Iran with a water temperature as high as 83ºC. Water samples were collected in a sterile flask and then incubated on nutrient agar plates at 37ºC for 48 h, subcultures were carried out to obtain single colonies. Identification of micro-organisms was performed according to the current physiological and biochemical assays and amplification of bacterial 16S rRNA genes was performed by applying the universal forward (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and reverse (5′-CTA CGG CTA CCT TGT TAC GA-3′) primers using an Eppendorf thermal cycler. Preparation of the Bacillus gnomic DNA was performed according to Cheng and Ning (2006) with minor modifications to the original protocol (21). The thermal profile was started with initial denaturation at 94ºC for 4 min followed by 30 cycles of 1min at 94ºC (denaturation), 1min at 57ºC (annealing), 1 min at 70ºC (extension), and 7 min at 70ºC (Final extension). Dendrogram of the phylogenetic tree was drawn using the Mega-6 software (www.megasoftware.net).

3.2. Biosynthesis and Characterization of the Silver Nanoparticles (*AgNPs*)

To obtain microbial biomass, small amount (one-loop) of the grown colonies were picked up and inoculated into each of Erlenmeyer flasks containing 100 mL of the liquid LB medium. The Erlenmeyer flasks were incubated in a rotary shaker incubator, with stirring speed of 150 rpm for 24 h at 40°C. At the end of each incubation period, the liquid medium was centrifuged at 10,000 ×*g* for 15 min for removing cell-debris. The cell-free supernatant obtained in the previous step was poured into five separate Erlenmeyer flasks equally; four flasks were supplemented with the silver nitrate salt up to 1 mM concentration (100 mL final volume). The 5th flask was stored as the negative control for the next step (The cell-free supernatant). One more flask (the 6th flask) was taken as positive control containing 100 mL of 1 mM of silver nitrate in the distilled water. The Erlenmeyer flasks containing the supernatant mixed with the 1 mM of silver nitrate, were incubated for 24 h at the same condition that was considered for the microbial biomass growth. To confirm the extracellular formation of the $AgNP_s$, bioreduction of the silver ions in the medium was monitored using changes in the color from yellow to dark brown. The absorption spectrum of the medium containing reduced silver ions was recorded by a UV-Visible spectrophotometer (Varian Carey 5000) from 300 nm to 800 nm at the regular time intervals. The supernatant that was exposed with the 1 mM of silver nitrate, was air dried and the remaining powder was scrapped out for the subsequent characterization of the silver nanoparticles. The morphological characterization and distribution of the nanoparticles was scanned using SEM (FESEM, Model: JSM-6160) and TEM (Philips model CM200) with an accelerating voltages of 20 and 200 KV, respectively. Also, the presence of elemental silvers were confirmed by, the air dried pow-

der was scanned with an EDX that was connected to a SEM (Philips model) instrument.

3.3. Antibacterial Activity

The bactericidal capacity of the AgNPs was examined according to the modified Kirby Bauer disk diffusion method (22). The antibacterial assay used against two groups of strains: *Staphylococcus aureus*, *Staphylococcus epidermidis* as Gram positive bacteria and *Escherichia coli*, and *Salmonella typhi* as Gram-negative bacteria. The suspension cultures of each tested strains were swabbed unevenly onto sterile Mueller-Hinton agar plates by sterile cotton swabs, using a micro pipette, 10 mm diameter wells were made on agar plate, 100 µL of the synthesized AgNP solutions (0.01 $mg.mL^{-1}$) were poured into each well and incubated at 37ºC. Twenty-four hours after inoculation, the diameter of inhibition zone around each well was recorded.

4. Results

4.1. Identification of Microorganism

The isolated micro-organism was identified following to the routine biochemical and morphological examinations. Results showed that the isolated strain resembles and relates to the Bacillus species. The isolated strain (AZ1) was a spore-forming, rod-shaped, optionally anaerobic, and Gram-positive bacterium. As well the bacterium was found to be Catalase and Oxidase positive. These tests were conducted according to Bergey's Manual of Systematic Bacteriology (23). PCR amplification showed a length of about 1500 bp of the product (data not shown). Furthermore, sequencing of the PCR amplified 16S rRNA was conducted. The sequence of the 16S rRNA gene was aligned against the other recorded sequences in the Gen Bank and it was found 99% homology to the sequence of the *B. licheniformis* (Figure 1). The phylogenetic tree was drawn by MEGA6 software according to the neighbor-joining method. Finally, the sequence of the isolate was submitted to the NCBI (http://www.ncbi.nlm.nih.gov) and saved under accession number of: KT281606.

4.2. Biosynthesis of the Silver Nanoparticles ($AgNP_s$*)*

The silver nanoparticle synthesis reaction was started when silver nitrate was added to supernatant and incubated at 37ºC. After 6h of incubation in the dark room conditions, first change in solution color was

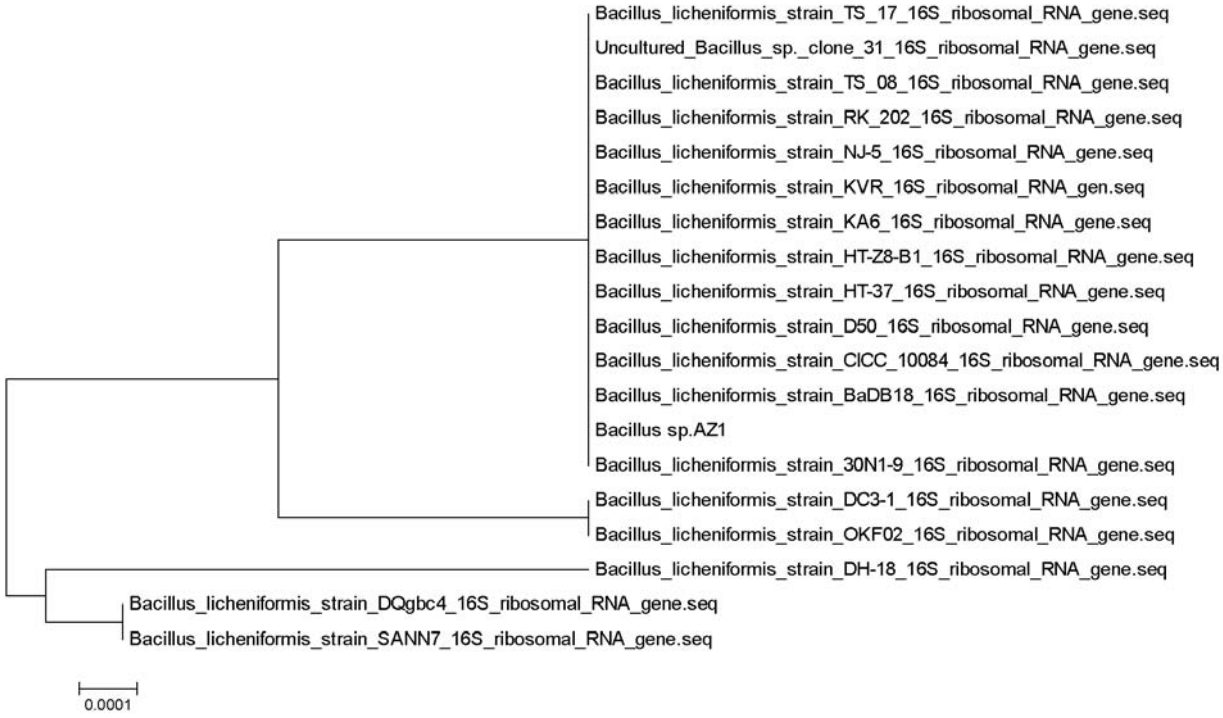

Figure 1. The dendrogram of phylogenetic tree. The dendrogram was drawn according to 16S rRNA genes of the *Bacillus* sp. AZ1 by Mega 6 software (www.megasoftware.net)

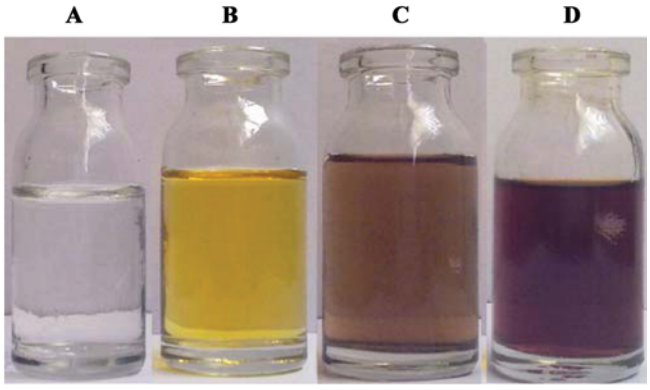

Figure 2. The differential change in the supernatant solution color formation following to the mixing with a solution of $AgNO_3$ (1×10^{-3} M): A: $AgNO_3$ solution (1 mM) only (as positive control), B: culture supernatant without $AgNO_3$ (as negative control), C: supernatant mixed with $AgNO_3$ 1 mM after 6 h, D: supernatant mixed with $AgNO_3$ 1 mM after 24 h

appeared and continued to develop up to 24 h. The color solutions changed from light yellow to dark brown (Figure 2). The UV-Visible spectrum of the aqueous medium containing silver ion showed a peak at 425 nm which showed correspondence to the Plasmon absorbance of silver nanoparticles (Figure 3A). Formation of the silver nanoparticles in range of ~7-31 nm was revealed by the SEM (Figure 3B). Much information about distribution and morphological features of AgNPs was obtained by the TEM analysis. TEM micrograph showed AgNPs particles with a wide range of shapes, although a large number of which were spherical in shape. Furthermore, the synthesized silver nanoparticles were found to be either aggregated or as a single granules at certain locations (Figure 3C). Also analysis by TEM has indicated a range of sizes for AgNPs of ~9-32 nm, which agrees well with the results obtained by SEM analysis. The presence of AgNPs was further confirmed by EXD analysis following to exposure to the four signals (the elements such as C, Ag, O, and Cl have shown the highest peaks), one of which was in the silver region (Figure 3D). The absorption of the metallic silver nanocrystallites generally shows an absorption peak approximately at 3 KeV (31).

4.3. Anti Microbial Activity

Antimicrobial activity of the biosynthesized AgNPs

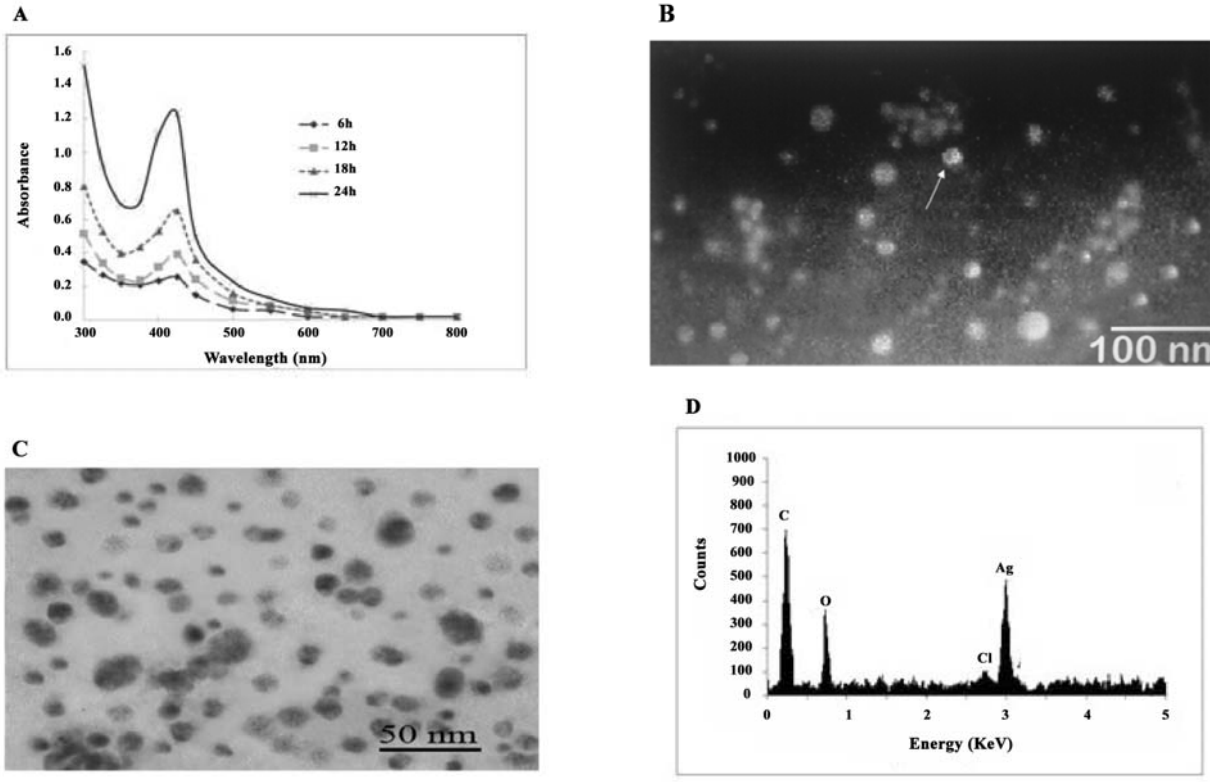

Figure 3. The UV-Visible spectra spectrum of the silver surface Plasmon resonance bands obtained at the different time intervals. B: SEM micrograph shows formation of AgNPs by culture supernatant mixed with the $AgNO_3$ (1×10^{-3} M) after 24 h. C: TEM micrograph obtained for the synthesized AgNPs by the bacterial culture supernatant supplied with the $AgNO_3$ (1×10^{-3} M) for a period of 24 h of the incubation. D: Energy dispersive X-ray spectrum of the AgNPs suspension revealing peaks of the elemental silver

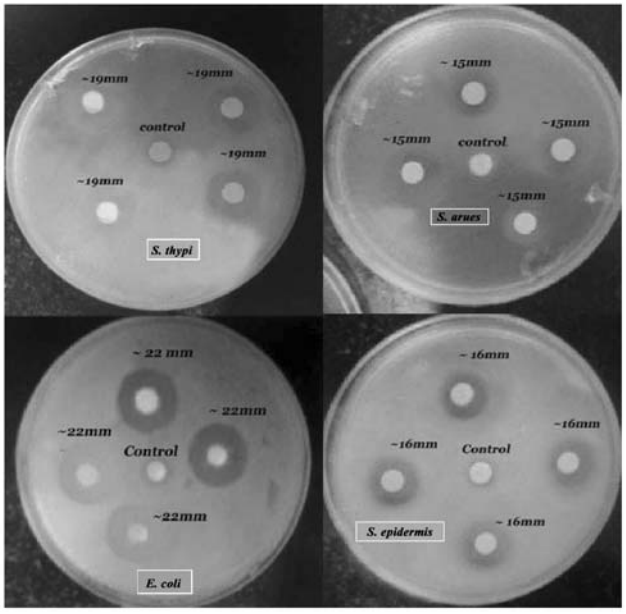

Figure 4. Anitbactiral activity of AgNPs against the four pathogenic bacteria strains by disk diffusion method

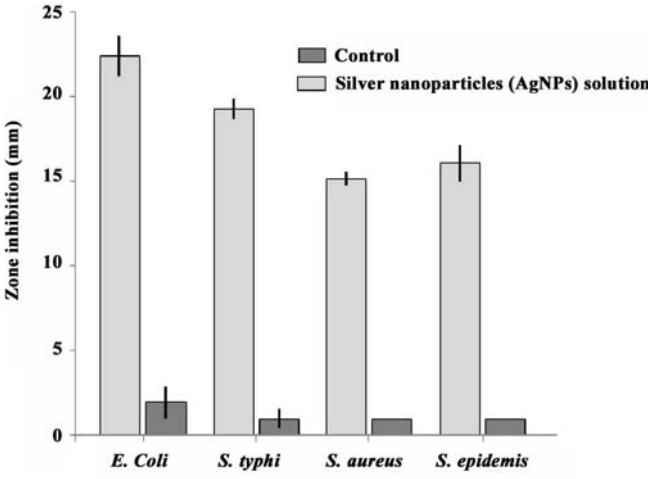

Figure 5. Comparison of the AgNPs growth inhibitory effect on four bacterial strains through measuring the of the zone of growth inhibition against the four pathogenic bacteria strains. Supernatant without $AgNP_s$ solution was used as the negative control

was studied against two groups of the Gram-negative (*E. coli* and *S. typhi*) and Gram-positive (*S. aureus* and *S. epidemis*) bacteria (Figure 4). The silver nanoparticles showed the highest activity against the *E. coli* with 22 mm of zone of growth inhibition. Other bacteria tested in this investigation were also affected by AgNPs, however with a lower range of inhibition. For example, the zone of growth inhibition for *S. typhi* was 19 mm, for *Staphylococcus epidermidis*, and *Staphylococcus aurues* were 16 and 15 mm, respectively (Figure 5).

5. Discussion

In recent years, much attention has been focused on microbes, in order to synthesis nano metal due to the fact that synthesis of the nano particles by microbes is more convenient than other approaches. Also different strains of the bacteria have been subject of studies for the synthesis of the nanparticles such as silver, iron, gold, etc. Moreover, bacteria could easily be geneticall-ly manipulated and easy to handle. Among all nanoparticles, the silver ions have always been shown to have an excellent antimicrobial properties. There are numerous reports showing that AgNPs have higher effective antibacterial activity against a number of pathogenic bacteria, fungi, and viruses at a very low concentration, and without any side effects on human body (4, 5, 7-9). Thus, such a history about capabilities have persuaded us with the idea to use thermophilic bacillus for biosynthesis of AgNPs in the present work.

Our study regarding the biosynthesis of silver nanoparticles have proven by ascending changes in the color intensity from yellow to dark brown following a direct correlation with the time of incubation. The change in the color intensity might be due to the excitation of the Surface Plasmon Resonance (SPR) of the silver nanoparticles or reduction of the silver nitrate (24, 25). Generally speaking, the AgNPs, due to the presence of the characteristic SPR band at 430 nm in UV-visible spectroscopy could be measured as an indicator for measurement the amount of AgNPs (26). As it could be seen in the (Figure 2), the appearance of the sharp peaks during color development period (i.e., 6-24 h) is due to the surface Plasmon, which is well documented for various metal nanoparticles with the particle sizes ranging from 2 to 100 nm (27). Furthermore, the optical characteristics, such as peak width and absorption are mainly depend on the other factors including: size, shape, medium, surface charge, the interaction between the particle composition, refractive index of the surrounding medium, particle stability, and surface-adsorbed species (28-30). There are numerous reports regarding extracellular synthesis of the AgNPs by Bacillus species generated with the different sizes (31, 32, 36, 37). Also, in 2008 two studies have been reported on the synthesis of AgNPs using *Bacillus licheniformis* by Kalishwaralal *et al*. (13, 19). They reported nanoparticles synthesized with an aver-

age particle size around 40 and 50 nm. The size of the nanoparticles obtained in the present study is much smaller than the nanoparticles synthesized using the same bacterial strain in the other reports. These results show that *Bacillus lichniformois* AZ1 could be used for the biosynthesis of the smaller AgNPs from silver nitrate. Although the mechanisms that are involved in the generation of the extracellular nanoparticles by microbes are not yet fully understood, but, many researchers believe that microbial synthesis of the metal nanoparticles might be through bioreduction of the metal ions by yet an unknown biochemical reaction. However, it was documented that nitrate reductase could be the main factor for oxidation of the silver ions nanoparticles by the *Bacillus licheniformis* (19, 20, 33). Studies on the antibacterial effects of the AgNPs have been subject of different reports. In this redard the effect of AgNPs on the growth of both Gram positive and Gram negative organisms were investigated. Chudasama *et al*. (34) and Ramgopal *et al*. 2011 (35) have reported that *S. aureus* (Gram positive) had a greater antibacterial activity compared to that of *E. coli* (Gram negative). Prakash *et al*. 2011 (36) have also reported a similar results for antibacterial effects of the AgNPs on Gram negative bacteria (*E. coli*) and Gram positive (*Streptococous pyogene*). Whereas, Dipak and Sankar *et al*. in 2014 (20) and Priyadarshini *et al*. in 2013 (37) have reported the highest and the lowest zone of inhibition formation against *E. coli* and *Staphylococcus* respectively. Our studies have shown a higher antibacterial activity against Gram negative organisms which might be due to the thick peptidoglycan layer in the bacterial cell wall, as the peptidoglycan layer of Gram positive bacteria are thicker than that of Gram negative bacteria. The peptidoglycan layers are consisted of linear polysaccharide chains crosslinked with short peptides and leading to the formation of a more rigid structure which provides a strong barrier against the penetration of the AgNPs (38). The detailed mechanism behind the antibacterial activity of AgNPs against other bacteria are not clearly established, but, it seems that the AgNPs penetration and the damage it impose to the bacteria might proceed according to the following steps: In the first step, the nanoparticles break through the permeable outer membrane, and in the second step, the AgNPs enter the internal plasma membrane of the bacteria and inactivate respiratory chain dehydrogenase. The end result of these steps are: leakage of the cell materials (in the first step), inhibition of the respiration and growth of the cell simultaneously, affects on phospholipids, denaturation of proteins and induction of the cell membrane collapse, and finally decomposition and cell death (in the second step according to the above scheme) (39). Various factors may influence the AgNPs toxicity such as: nanoparticle shape and size, surface chemistry, crystallinity, capping agents, as well as other factors such as ionic strength, pH, the presence of ligands, divalent cations, and macro molecules (as environmental factors). For example truncated triangular nanoparticles have stronger antibacterial activity than spherical and rod shaped AgNPs (40). Also, it has been found that there is a direct relationship between a decrease in the size of the nanoparticles and an increased effectiveness of the antibacterial properties. It could be due to the increased number of atoms which are exposed to the surface available for the redox reactions, biochemical, and photo chemical reactions together with the physicochemical interactions of the nanoparticls with bacterial cells (40). Our data show that silver nanoparticls can be produced by the *Bacillus licheniformis* AZ1 with the size range of ~ 7-31 nm. Thus, the silver nanoparticles presents a simple green synthesis process for production of extracellular nanoparticles which is environmentally acceptable.

Acknowledgements

Our thanks to the Department of Medical Biotechnology, Hamedan University of Medical Sciences, Iran, as well as Department of Biotechnology, Buali Sina University, Iran for providing us with the required facilities for undertaking this research. Authors also appreciate Ms. Ostad Ahmadi, the Biotechnology laboratory assistant to technician for his kind cooperation with us in doing the present research. The present study was financially supported by a research grant allocated by the Department of Biotechnology, Buali Sina University, Iran.

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