Comparative Evaluation of Chemical Reduction Methods by Ethanol and *Bacillus licheniformis* on The Physical Properties, Stability and Coating of Nanoparticles

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ABSTRACT

Background and Objectives: Size of silver nanoparticles synthesized by ethanol and *Bacillus lichenioformis*is 20 nm and 15 nm, respectively. Nanoparticles can be used in treatment of several diseases. Chemical and biological methods have been used to synthesize silver nanoparticles. The aim of this study was to compare the size, shape and coating of silver nanoparticles synthesized by the chemical and biological methods.

Methods: Ethanol was used in the chemical reduction method and *B. licheniformis* was used in the biological method. Physical evaluation (salt test), absorbance measurement at 450 nm and imaging by transmission electron microscopy were performed to compare nanoparticles in terms of size, shape and coating.

Results: Observed maroon color, maximum absorption at 400-450 nm range and electron microscopy images confirmed the presence of nanoparticles. The shape of nanoparticles synthesized by the two methods was spherical. However, biosynthesized nanoparticles were smaller and had protein coating.

Conclusion: Given the smaller size of biosynthesized nanoparticles and presence of coating confirmed by the electron microscopy images, biosynthesis is recommended because of enhanced nanoparticles properties and reduced toxicity.

Keywords: Nanoparticles, Coating, Toxicity.

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INTRODUCTION

Silver nitrate is often used as a precursor for a variety of silver nanoparticles. Silver nanoparticles have different shapes (spherical, rod and wire-like and triangular), coatings (citrate, polymer, peptide andcarbohydrate) and sizes (≥100 nm) (2).Silver nanoparticles are non-toxic, non-allergenic, highly stabile, hydrophilic, environmentally friendly and heat-resistant. Their antibacterial property has expanded their applications in areas such as the textile, paint, ceramics, pharmaceuticals, agricultural, animal husbandry and cosmetics industries (3, 4). Silver nanoparticles are produced by chemical and biological methods. The chemical methods consist of chemical reduction, photochemical method (radiation), electrochemical method, and pyrolysis or heating. Chemical reduction is the most commonly used chemical method (5). Biological methods (using bacteria, fungi and plants) have a huge potential for the production of metal nanoparticles such as titanium/nickel, titanate, zirconium, gold and silver. Synthesis of nanoparticles using microorganisms and plants is environmentally friendly, more cost-effective, highly stabile, non-toxic and heat-resistant while showing their size and protein coating. In most chemical methods, a chemical reducing agent (to reduce the metal ions) and a stabilizer (e.g. polyvinylpyrrolidone) are used to control growth of particles and prevent accumulation. In these methods of synthesis, the stability of the particles becomes controversial and large-scale production is difficult. Therefore, there is a demand for production of nanoparticles using alternative environmentally friendly methods such as the biological methods (8). Development of experimental biological processes for the synthesis of nanoparticles is evolving and has become an important branch of nanotechnology (9). Size, morphology and surface have great importance in determining the toxicity of nanoparticles. Biosynthesized silver nanoparticles are smaller than chemically synthesized silver nanoparticles (10).Therefore, comparative evaluation of size, shape and coating of the synthesized silver nanoparticles have been taken into account.

MATERIAL AND METHODS

Chemical synthesis of silver nanoparticles

For the chemical synthesis of silver nanoparticles, one gram of polyvinylpyrrolidone (PVP), 40 ml of ethanol and 0.5 g of silver nitrate were mixed until completely dissolved. The mixture was placed in a microwave at 80 watts for 4 minutes and then store for 24 hours. Production of silver nanoparticles was evaluated by examining the morphological characteristics (visual observation), salt test (in which a pinch of salt was added to the solution and lack of milky color indicates lack of silver ions' presence in the solution), reading absorbance at 350-550 nm (by a spectrophotometer) and imaging using a transmission electron microscope (TEM). A *Bacillus licheniformis*strain was obtained from the Iranian Research Institute of Plant Protection (IRIPP) (No. 1024-c) that was kept alive on surface of slanted trypticase soy agar (TSA) medium in screw-cap tubes at 2-5 °C. The bacterial strains were separately inoculated and streaked onto sterile TSA media in petri dishes. The media were incubated at 37 °C for 48 hours (instead of logarithmic phase, secondary metabolites produced in the liquid medium were considered that are used for reduction of silver ions into silver nanoparticles) and then evaluated in terms of growth and purity. Biomass of *B. licheniformis*strainswas prepared by separate inoculation of $1.5x10^8$CFU/ml of bacteria with 100 ml of sterile nutrient broth in 250 ml Erlenmeyer flasks, and incubation at 37 ° C for 48 hours in a shaking incubator at 200 rpm. After centrifugation at 3000 rpm for 20 minutes, the transparent supernatant was separated from sediment. The sediment was washed three times with distilled water. Then, the supernatant and cell mass of each strain were used separately to evaluate silver nanoparticles production under dark and light conditions. Next, 1.5×10^8CFU/ml of *B. licheniformis*strains that were capable of producing silver nanoparticles in closed systems were added per each ml of sterile alginate. The alginate containing *B.*

*licheniformis*droplets were added to 0.5% calcium chloride. After formation of alginate beads containing bacteria, the samples werewashed three times with distilled water and then used for biosynthesis of silver nanoparticles in the presence and absence of light. Five grams of the cell mass obtained in the previous stages were added to 5 ml of silver nitrate. The samples were separately placedunder dark and light conditions to evaluate the production of silver nanoparticles. In addition, 5 ml of the clear supernatant of strains were added to 5ml of silver nitrate (10^{-3}mM) to evaluate production of silver nanoparticles under light and dark conditions.

Investigating the presence of silver nanoparticles and their coatings using TEM:

Solutions of chemically or biologically synthesized nanoparticles were examined by TEM. Nanoparticles solution was subjected to ultrasonic waves with a frequency of 50 Hz for 20 minutes to create proper dispersion for nanoparticles and prevent their aggregation. Then, 15 ml of the solution was taken using pipette and tip-filtered samplers and poured on the electron microscopy grids. After drying, the grids were placed and examined in the electron microscope (Kafa Tehran Co.) with voltage of 100 kW.For evaluating the extracellular production of silver nanoparticles, 2ml of the supernatant from the previous stage were taken and the absorption was read in the 350-550 nm range. In order to evaluate the effects of culture medium's composition on silver nitrate, the control flasks without microbial inoculation were used under the same conditions and the absorption was measured in the 350-550 nm range. The antibiotic disks were soaked into the nano-solutions prepared by the chemical or biological methods. Bacterial strains (*Escherichia coli*) were cultured on Mueller Hinton agar medium, antibiotic disks were placed on the surface of the medium, and incubation was done for 48 hours at 37 ^{o}C.

RESULTS

Evaluation of silver nanoparticles production by salt test, observing the maroon color and absorption measurement at 400-450nm range indicated that the conversion of silver ions to silver nanoparticles was performed better in the presence of light (figure 1). TEM examination confirmed the production of silver nanoparticles as well as their coatings, spherical shape, singularity and size (figure 2).

Figure 1- Silver nanoparticles absorption by *B. licheniformis*(A) andPVP (B)

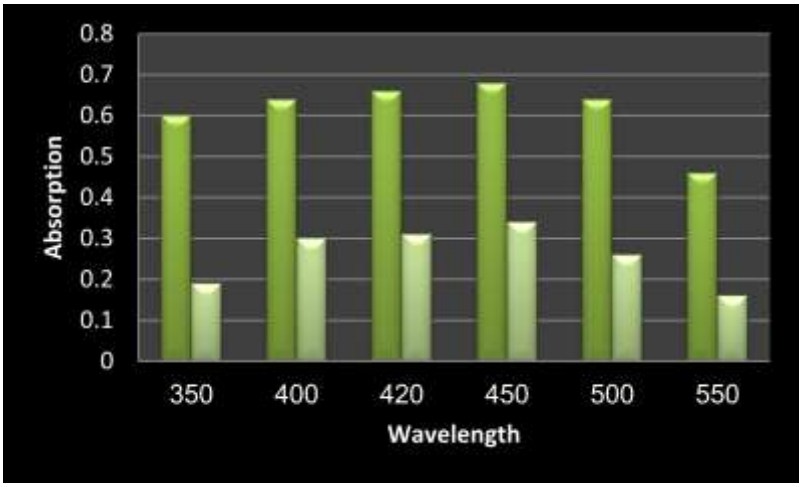
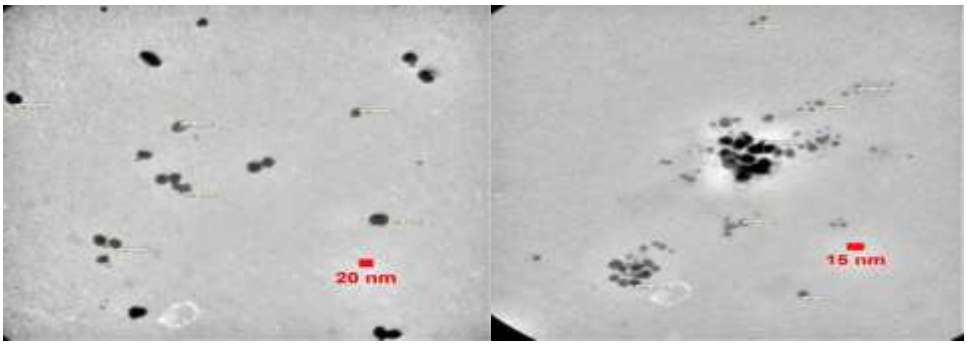


Figure 2- Electron microscopy shows the size, shape and coatings of silver nanoparticles



A: Nanoparticle containing *B. licheniformis*, B: Nanoparticles containing PVP
The diameter of growth inhibition zone of chemically and biologically synthesized silver nanoparticles was 2 mm and zero, respectively.

DISCUSSION

One of the most important requirements of nanotechnology is access to a safe method of producing various metal nanoparticles.As mentioned previously, various nanoparticles can be produced by the chemical and biological methods. The biological method is preferred because it is more energy-efficient and cost-effective (11).Different microorganisms including bacteria, fungi and viruses are capable of producing metal nanoparticles via reduction of metal ions in the environment. A variety of enzymes, reducing substances, extracellular polysaccharides and electron shuttles are effective in the reduction process. Intercellular synthesis occurs in the presence of reducing enzymes within the cell, while extracellular synthesis occurs when the enzymes are released outside the cell (11). In many cases, both sets of enzymes are involved in the process of metal ion reduction and inhibition of their toxic effects for the microorganisms. Among the microorganisms involved in the biosynthesis of nanoparticles, fungi usually have more reducing capability than bacteria (12, 13). The presence of silver nanoparticles due to the change in absorbance of the solution was detectable by a spectrophotometer. TEM was used to confirm the production of silver nanoparticles. Some studies investigated the effects of coating on nanoparticles. For example, Reidy et al. (2013) investigated the presence of coating and showed lower toxicity of coated silver nanoparticles. Accumulation is a factor that causes toxicity and coating has a major role in preventing the binding of silver nanoparticles to each other. Thus, presence of coating can reduce the toxicity of silver nanoparticles (14, 15). According to the results of the present study, silver nanoparticles synthesized by *B. licheniformis*have coating and are less toxic. Smaller nanoparticles have less surface area to volume ratio and improved antibacterial property (16, 17). Therefore, it is expected that According to previous studies, proteins are effective in stability of nanoparticles by preventing oxidation and aggregation. The nanoparticles synthesized in the present study had long-term stability (six months). The stability of chemically synthesized silver nanoparticles is due to PVP use, while the stability of bio-synthesized nanoparticles is due to their coating. The results of this study and other studies show that the diameter of growth inhibition zone of biosynthesized silver nanoparticles indicates their antibacterial property.Analysis of TEM images showed that chemically synthesized silver nanoparticles are uncoated, while biosynthesized silver nanoparticles are coated. Biosynthesis reduces aggregation of particles and cause less toxicity. In addition to the effects on the environment and humans, silver nanoparticles affect microorganism. Size and shape of these nanoparticles influence their toxicity and those smaller than 10nm can penetrate the cell wall. In this regard, studies on bacteria with bioluminescent properties showed that silver

nanoparticles could destroy the cell wall and affect bacterial DNA during replication or destroy the DNA. Increased use of nanoparticles in products has increased their release into the environment and beneficial soil microorganisms are among the species at risk. However, there is not enough information about the effects of silver nanoparticles on function of soil microorganisms. Nanosilver is the most common nanomaterial used in products that require to have bactericidal effects. The study of Njuguna et al. on the toxic effect of silver on beneficial soil bacteria (that play a major role in nitrogen fixation) reported that silver can inhibit the growth of these microorganism at a lower concentration compared to other heavy metals.

CONCLUSION

Implementation of new approaches with less cost and environmental pollution in the production of nanoparticles is of utmost importance. Thus, a great deal of attention has been given to the biosynthesis of nanoparticles in recent years.The antibacterial properties of surfaces coated with silver nanoparticles colloid synthesized by *B. licheniformis* are more favorable due to their smaller size compared to nanoparticles synthesized by the chemical method.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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