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Optimization of Silver Nanoparticles Production by *E. coli* Bacterium (DH5α) and the Study of Reaction Kinetics

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In this research, silver nanoparticles formation by *E. coli* bacterium (DH5α) is investigated. Effect of silver nitrate concentration, mixing ratio of filtrate of bacterium culture to silver nitrate, temperature and pH on size and production efficiency was studied. In order to identify and analyze nanoparticles, UV-VIS spectroscopy, atomic absorption spectroscopy, transmission electron microscopy and dynamic light scattering were used. It was found that initial concentration of silver nitrate play a key role in formation of nanoparticles. In addition, it was found that in lower temperatures, the size of silver nanoparticles is smaller and their distribution is narrower. Increase of pH causes increase in the size of nanoparticles and their accumulation. Further, the kinetics of Ag nanoparticles formation was studied. It was found that in similar physical conditions, the reaction of naoparticles formation and the reduction of silver ions are faster in the less mixing ratio of filtrate

Key Words: Biosynthesis, Silver nanoparticles, Optimum, Kinetics of reaction, *E. coli*.

INTRODUCTION

Nanotechnology plays an important role in many key technologies of the new millennium[1]. The application of nano-scale and nano-structure materials within range of 1 to 100 nanometers is an emerging area of nanoscience and nanotechnology. Nanomaterials may provide solutions to technological and environmental challenges in the areas of solar energy conversion, catalysis, medicine and water treatment[2].

The noble metals especially gold and silver due to their innumerable applications in different branches such as catalysis, photonics, photography and more importantly in the field of medicine as antimicrobial factors have drawn much attentions to themselves[3,4]. In addition, colloidal silver is of particular interest because of distinctive properties, such as good conductivity, chemical stability, catalytic and antibacterial activity[5].

Silver nanoparticles have many important applications which include, spectrally selective coating for solar energy absorption and intercalation material for electrical batteries, as optical receptors, polarizing filters, catalysts in chemical reaction, biolabelling and as antimicrobial agents[6].

For production of nanoparticles, one needs to know physical and chemical principles of nano-scale materials and also know-how to commercialize them. Broadly speaking, there are two approaches to nanoparticle production *i.e.*, top-down and bottom-up. The former makes a material decrease its size from large to nanoscale, whereas the latter produces nanomaterials by starting from the atomic level[7].

Generally, metal nanoparticles can be prepared and stabilized by chemical, physical and biological methods. The chemical approach, such as chemical reduction, electrochemical techniques, photochemical reduction[2,8] and pyrolysis[9] and physical methods, such as arc-discharge and physical vapour condensation (pvc)[10] is used. Living organisms have huge potential for the production of nanoparticles/nanodevices of wide applications. However, the elucidation of exact mechanism of nanoparticles production using living organisms needs much more experimentations[11].

Studies show that the size, morphology, stability and properties (chemical and physical) of the metal nanoparticles are strongly influenced by the experimental conditions, the kinetics of interaction of metal ions with reducing agents and adsorption processes of stabilizing agent with metal nanoparticles. Therefore, the design of a synthesis method in which the size, morphology, stability and properties are under control is a major field of interest[2,12].

As mentioned, living organisms like bacteria, fungi and plants *etc*. have huge capability in synthesis of metal nanoparticles. Recently it has been noticed that micro-organisms are usable as a bio-factory for synthesis of metal nanoparticles such as cadmium sulfide, silver and gold[6].

Use of micro-organisms in nanoparticles synthesis is considered as an exciting method and eco-friendly[13,14]. On the other side, researchers prefer biological synthesis, because distribution control of particles obtained from this method is better than other methods[15]. In addition, this method involves no environment toxicity which is usually accompanied with other chemical methods[2,16].

The first synthesis of silver nanoparticles by bacterium has been reported in 2000. Joerger and his coworkers[17] used *P. stutzeri* bacterium (AG259) for silver nanoparticles synthesis with a size smaller than 200 nm. Bacteria were grown on Lennox L (LB) agar substrate, containing 50 mM $AgNO_3$, at 30 ºC for 48 h in the dark. In another study, silver nanocrystals biosynthesis was performed using the bacterium *Bacillus licheniformis*. Aqueous silver ions were reduced to silver nanoparticles by adding the biomass of *B. licheniformis*. This was indicated by the change in colour from whitish-yellow to brown. The probable mechanism for the formation of silver nanoparticles includes the enzyme nitrate reductase[18].

Silver nanoparticles were biosynthesized by *Bacillus licheniformis* supernatant through extracellular method by means of silver nitrate solution. Silver nanoparticles size was found 40 to 50 nm and the required time for reaction completion was 24 h[19]. In 2009, it was investigated different visible-light irradiation's effect on the formation of silver nanoparticles from silver nitrate using the culture supernatant of *Klebsiella pneumonia*. Also, the study experimentally investigated the liquid mixing process effect on silver nanoparticle synthesis by visible light irradiation. That study successfully synthesized uniformly dispersed silver nanoparticles with a uniform size and shape in the range of 1-6 nm with an average size of 3 nm[20]. Another report focused on the synthesis of metallic bio-nanoparticles of silver using a reduction of aqueous Ag^+ ion with the culture supernatants of *Staphylococcus aureus*. The observation indicated that the reduction of the Ag^+ ions took place extracellularly. Also, the reaction between this supernatant and Ag^+ ions were carried out in bright conditions for 5 min[21].

It seems that many works have been done with regard to production of silver nanoparticles using various bacteria, but optimal conditions for silver nanoparticles formation leading to production in large scale have not yet been provided. Undoubtedly, one of the most important factors in production of every material including nanoparticles is the time of its formation (production). For this reason the bacterium *E. coli* was used. *E. coli* bacterium is a member of Enterobacteria family and in environmental conditions has a fast growth. In addition, within a few minutes it leads to Ag^+ ions reduction and eventually to formation of nanoparticles. For this reason, this bacterium from a list of bacteria (microorganisms) was selected for optimization. By means of this bacterium, optimal conditions for production and formation of silver nanoparticles were studies. By achieving the best temperature, pH, mixing ratio of bacterium filtrate to silver nitrate, the kinetics of reaction as well as silver nitrate concentration, one can create desirable conditions for optimal production of nanoparticles.

EXPERIMENTAL

Effect of mixing ratio on the formation of silver nanoparticles: First, culture medium of Muller-Hinton-Broth was prepared according to the conditions provided from the factory. *E. coli* bacterium was cultivated for a period of 1 day in Muller-Hinton-Broth's medium at temperature of 37 ºC. After 1 day bacterium suspension was prepared and was made ready for subsequent experiments. Four containers containing 100 mL silver nitrate of 0.001 M and 1 mL poly(vinyl pyrrolidone) (PVP) were prepared. Poly(vinyl pyrrolidone) with 20g/L concentration was added in order to stabilize nanoparticles and to prevent their accumulation.

Bacterium suspension is filtered by means of a 0.22 μm filter and the obtained filtrate was added to containers with amounts of 1.0, 2.5, 4.0 and 5.5 mL, respectively. After a few minutes, colourless solution of silver nitrate in all of the four containers turns into brown colour. This colour change indicates possibility of silver nanoparticles production. In order to prove existence of nanoparticles, product efficiency and their size, UV-VIS spectroscopy, atomic absorption spectroscopy, transmission electron microscopy and DLS analysis were used.

Effect of silver nitrate concentration on the formation of silver nanoparticles: Three containers containing 100 cc of 0.01 M silver nitrate, 100 mL silver nitrate of 0.005 M and 100 mL silver nitrate of 0.001 M together with 1 mL PVP were prepared. To each container 1 mL culture filtrate of bacterium was added and after a few minutes, colourless solutions of silver nitrate changed into brown colour. The obtained samples in order to prove presence of nanoparticles, their production performance and size were studied and analyzed using UV-VIS spectroscopy, atomic absorption spectroscopy, transmission electron microscopy (TEM) and DLS analysis.

Effect of temperature on the formation of silver nanoparticles: To study temperature effect, four containers containing 100 mL of 0.001 M silver nitrate together with 1 mL PVP were put at four different temperatures. For this purpose, each container of silver nitrate was put at temperatures of 5, 25, 40 and 90 ºC in order to reach the required temperatures after a few minutes. Next, 1 mL bacterium's culture filtrate was added to each container. After a few minutes, a colour change was achieved in the solution. The produced samples for study and analysis were again prepared by means of UV-VIS spectroscopy, atomic absorption spectroscopy, transmission electron microscopy and DLS analysis.

Effect of pH on the formation of silver nanoparticles: For study of pH effect three containers containing 100 mL silver nitrate of 0.001 M together with 1 mL poly(vinyl pyrrolidone) were put at three different pH of 5.5, 7.5 and 9.0. Then, 1 mL bacterium's culture filtrate was added to each container. After a few minutes a colour change was resulted in the colourless solution. The produced samples of silver nanoparticles were used for analysis and study using UV-VIS spectroscopy, atomic absorption spectroscopy, transmission electron microscopy and DLS analysis.

RESULTS AND DISCUSSION

Study of effect of silver nitrate concentration: The colour change was observed after adding bacterium's filtrate to containers containing silver nitrate with different concentrations indicates reaction of silver nitrate reduction (Fig. 1). After 1 h, the reaction has been completed. The silver particles

at two concentrations of 0.005 M and 0.01 M settled and were accumulated at bottom of the containers. This accumulation at 0.01 M concentration was far greater than at 0.005 M concentration. For analytical study of the prepared samples at three concentration, the amount of absorption within wavelength of 350-600 nm was observed by UV-VIS spectroscopy. This technique has proven to be very useful for analyzing nanoparticles[6,15]. As illustrated in Fig. 2 (a), a strong surface plasmon resonance was centered at *ca.* 418 nm. Observation of this strong but broad surface plasmon peak has been well documented for various metal nanoparticles, with sizes ranging widely from 2 to 100 nm[13,18].

Fig.1. Solutions of silver nitrate (0.001 M) before (right) and after (left) exposure to the culture filtrate of *E. coli*

By increasing concentration from 0.001 M to 0.005 M, wavelength of peak (λ_{max}) has shifted from 422 to 436 nm. While, at highest concentration (*i.e.* 0.01 M) no peak was observed. It seems at concentration of 0.01 M order, no nanoparticle is produced. Presence of large amount of silver in small volume of the solution creates high attraction between silver atoms and nanoparticles conglomeration and formation of particles with large size at micro scale. This presence of very large particles (larger than nanometer) was the reason of fast settlement of particles in the container.

At 0.005 M concentration, the band observed in the curve is wide. The peak wideness suggests particles distribution at different sizes. It seems at concentrations of about 0.005 M, nanoparticles are formed together with particles with larger size (at micro scale). Increase of λ_{max} from 422 to 436 nm indicates nanoparticles with bigger size. On the other side, at λ_{max} the amount of absorption decreases with increase of concentration. This suggests higher nanoparticles production at lower concentrations.

Thus, the less the concentration of silver nitrate is, the higher the production of silver nanoparticles and the narrower the distribution of nanoparticles' size become. From three concentration, 0.001 M concentration has the best performance in amount of production and in narrowness of range of nanoparticles' size.

Study of effect of mixing ratio: In order to study mixing ratio, four different concentrations were tested. The samples after colour change were measured by spectroscopy within wavelength of 350-600 nm. Results are shown in Fig. 3.

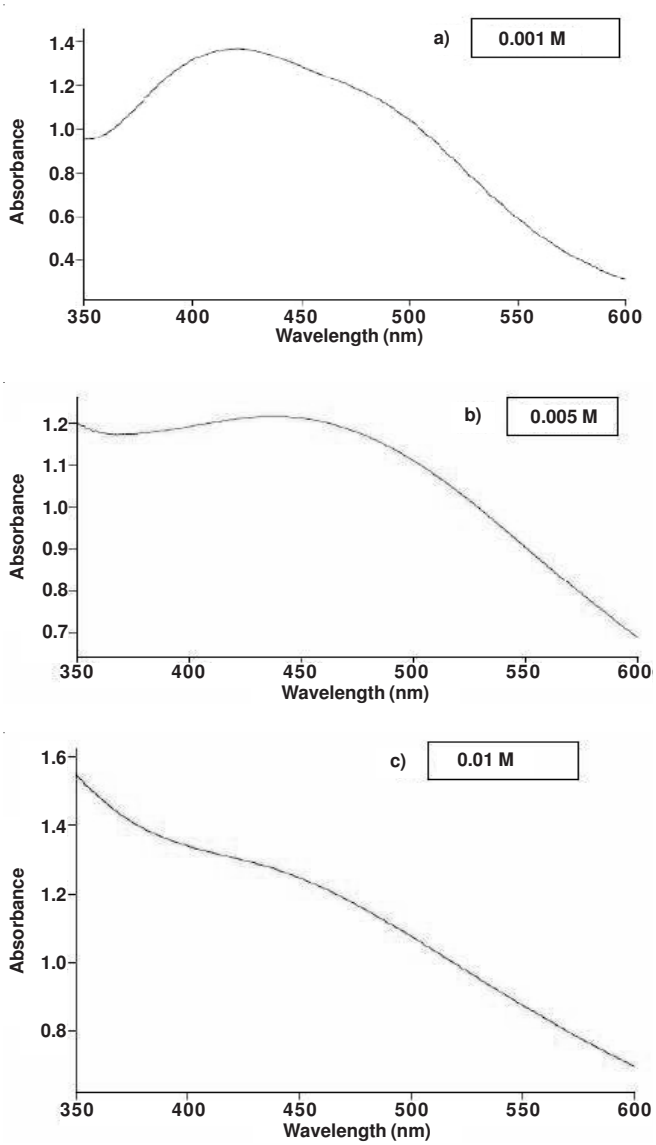
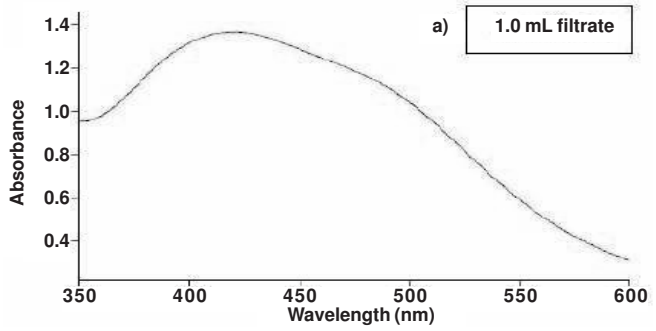


Fig. 2. UV-VIS spectra of Ag colloids. Spectra recorded after the addition of culture filtrate (1 mL) to 100 mL of silver nitrate solution with concentration a) 0.001 M, b) 0.005 M and c) 0.01 M.

As is seen in Fig. 3, by change of mixing ratio in similar environmental conditions, the observed wavelength of maximum peak (λ_{max}) does not change much and it is between wavelengths of 415-422 nm. However, by increase of filtrate amount, the peak wavelength has decreased from 422 to 415 nm. This decrease in the peak wavelength suggests reduction in nanoparticles size. It seems by increase of filtrate amount, nanoparticles size becomes smaller.



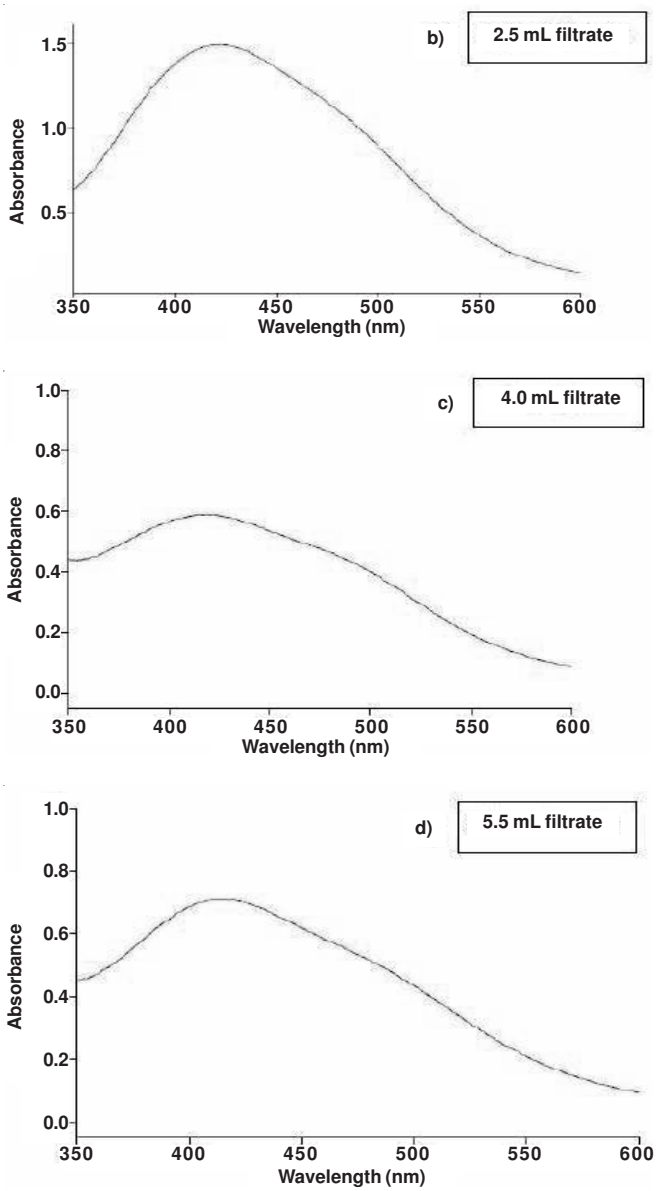


Fig. 3. UV-VIS spectra of Ag *colloids*. Spectra recorded after the addition of a) 1 mL, b) 2.5 mL, c) 4 mL and d) 5.5 mL of culture filtrate to 100 mL of silver nitrate solution (0.001 M)

Dynamic light scattering (DLS) analysis thoroughly confirms this finding. As is seen in Fig. 4, by increase of mixing ratio from 1 to 4 mL, average size of silver nanoparticles decreases from 129.3 to 104.3 nm.

In addition, in this experiment to study of kinetics of reaction, the amount of absorption of samples was measured after adding bacterium's filtrate to $AgNO_3$ solution at times 0, 1, 2 min, *etc.* by spectroscopy in wavelength of maximum peak (λ_{max}). Since the silver concentration is proportional to its absorptions amount in maximum wavelength (λ_{max}), so the achieved curve shows the kinetics of reaction. As illustrated in Fig. 5., by increase of mixing ratio, the time of reaction completion increase and the rate of reaction decrease. If the mixing ratio of silver nitrate to filtrate of bacterium is 1 to 100, after about 12 min, over 95 % of the reaction have been completed. This means that the residence time to design a bioreactor to satisfy these conditions should be about 12 min. If the mixing ratio increases, this time about 25 to 40 min will increase. It means that to achieve to the same conversion percentage is needed larger bioreactor.

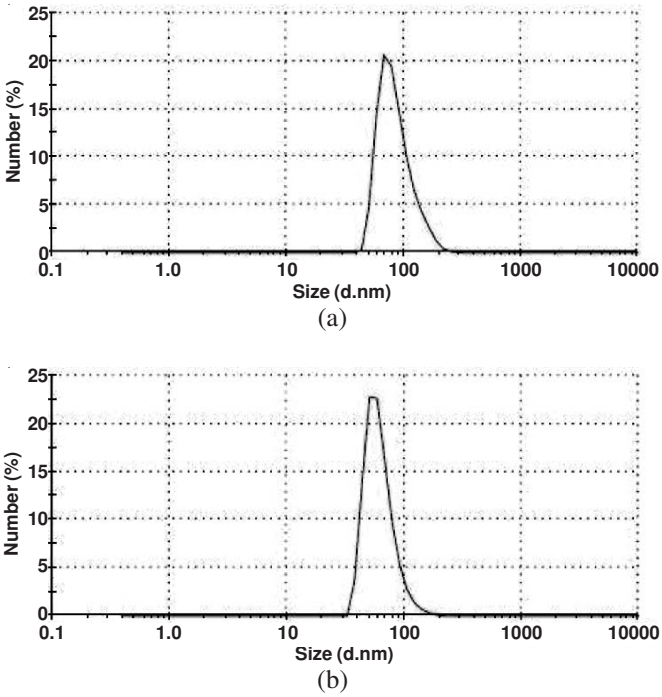


Fig. 4. The curve of size distribution by number, a) mixing ratio 1 to 100, b) mixing ratio 4 to 100

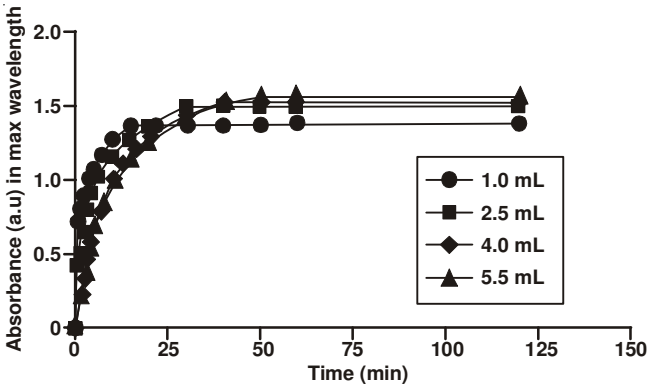


Fig. 5. The kinetics of silver nanoparticles formation in different mixing ratio in 100 mL of silver nitrate solution (0.001 M)

Samples after 12 min were measured by atomic absorption spectroscopy.The results showed that with increase of mixing ratio, the silver ion reduction and reaction rate have been decreased in the same conditions. So the less mixing ratio provides better conditions.

Study of temperature effect: In this experiment, the phenomenon of silver ion reduction at 5, 25, 40 and 90 ºC was examined. The noticeable point was that colour change at 5 ºC was completed quicker than other temperatures. On the other hand, at temperature of 90 ºC, the observed colour change relative to other temperature was different. To prove the existence or non-existence of nanoparticles, the provided four samples were undergone light spectrum within wavelengths of 350-600 nm. Fig. 6 shows the mentioned light spectrum.

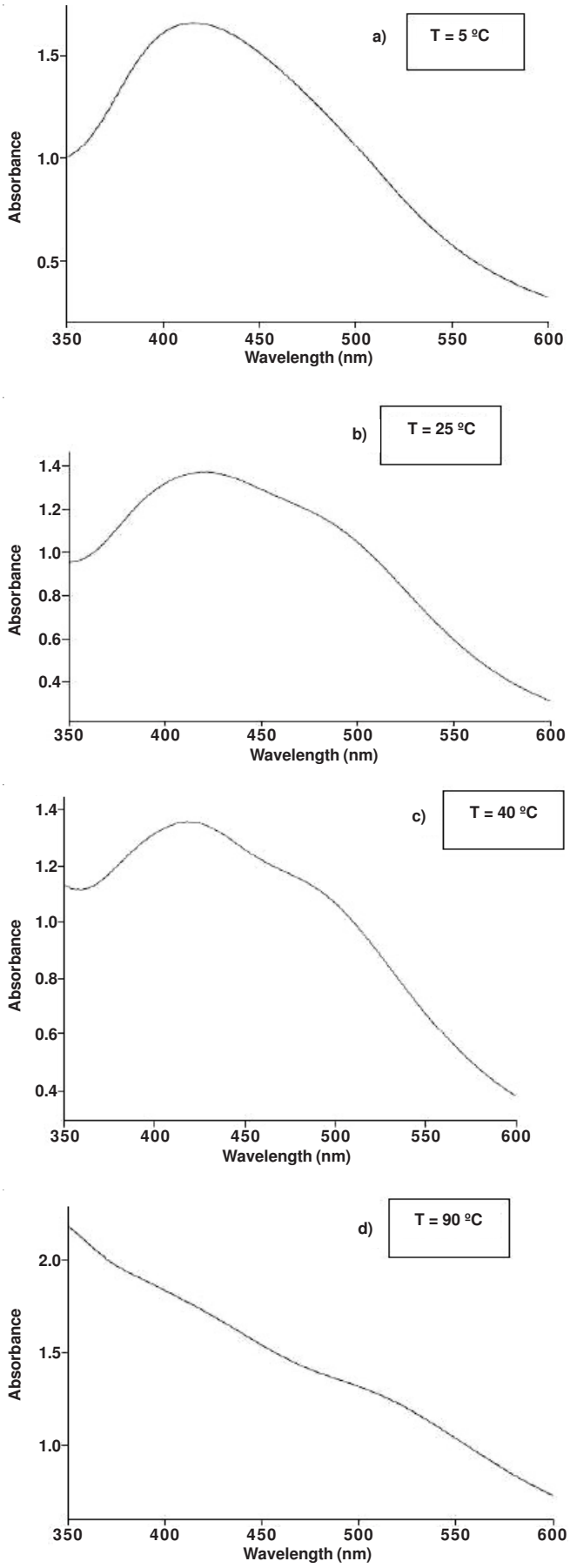


Fig. 6. UV-VIS spectra of Ag colloids. Spectra recorded after the addition of culture filtrate (1 mL) to 100 mL of silver nitrate solution (0.001 M) in temperatures a) 5 ºC, b) 25 ºC, c) 40 ºC and d) 90 ºC.

As is seen in the Fig. 6, no peak is observed at 90ºC. Therefore, at 90 ºC, silver nanoparticles are not produced. In addition, absorption amount at λ_{max} decreases by increase of temperature. Also, the band of peak curve at low temperatures is narrower indicating narrowness of dispersion distribution of nanoparticles. It means that range of particles size is small. Dynamic light scattering analysis confirms this instance. As is seen in Fig. 7 at 5 ºC, average size of nanoparticles is 121 nm and at 25 ºC, 72.11 nm and width of the peak curve is 26.59 nm, whereas at 25ºC, percentage majority of nanoparticles is at size of 87 nm with a curve width of 31 nm. Thus, by increase of temperature, size of particles becomes larger and their dispersion becomes wider.

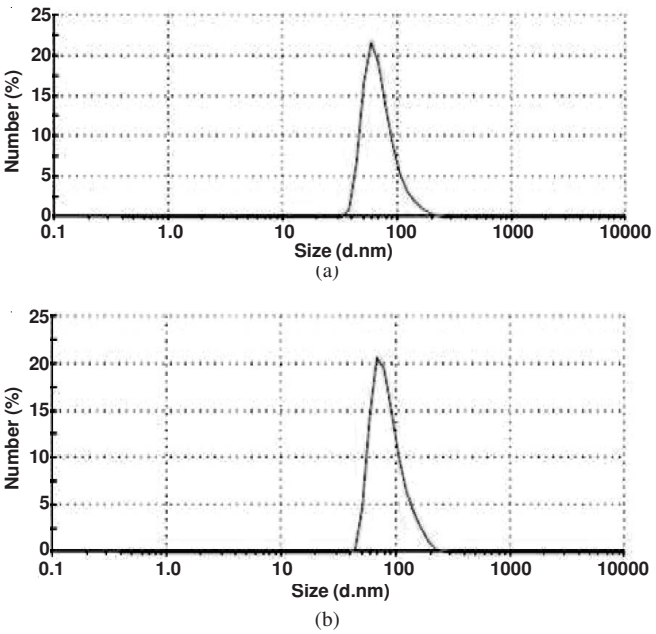


Fig. 7. The curve of size distribution by number, a) T=5 ºC, b) T=25 ºC.

Fig. 8 shows the kinetics of silver nanoparticles formed at different temperatures. As illustrated with change of temperature, the time of reaction completion is not much different, although the amount of nanoparticles is more at lower temperatures. Atomic absorption spectroscopy also shows by increase of temperature decrease the amount of silver ions reduction although silver ions reduction is not significant nanoparticles formation.

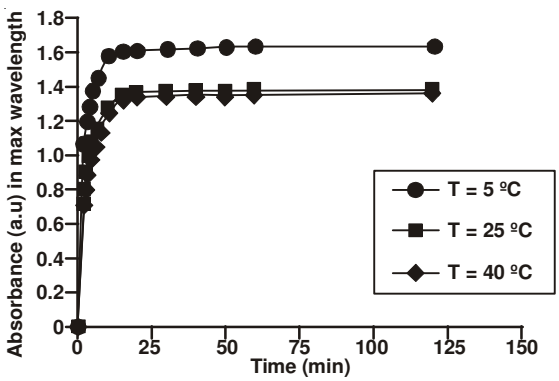


Fig. 8. Kinetics of silver nanoparticles formation in different temperatures in 100 mL of silver nitrate solution (0.001 M).

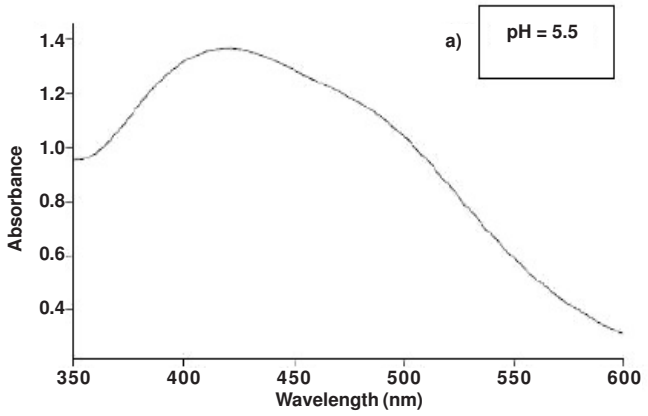
The above results suggest that among the four tested concentrations, silver nanoparticles production has the best performance and efficiency at 5 ºC temperature.

Study of pH effect: Fig. 9. shows the light spectrum at three pH levels. As is seen in this figure, the wavelength of maximum peak (λ_{max}) at pH of 5.5 and 7.5 is about 422 nm, which indicates the presence of silver nanoparticles with a size about 2-100 nm. On the other side, by increase of pH up to 9, it is seen that the wavelength of maximum peak (λ_{max}) has been shifted from 422 to 396 nm. In addition, another peak at *ca.* 510 nm is observed. It seems the peak shifting to 396 nm is the result of change in the solution's medium due to addition of sodium hydroxide. Since addition of sodium hydroxide at pH = 7.5 was slight, it didn't change much the place of the peak, although a slight shift from 422 to 421 nm has been observed. But the second peak at 510 nm at pH = 9 is due to the coupling of silver nanoparticles. In fact, from the interparticle dipole-dipole couplings of silver nanoparticles, a peak at wavelength of ~510 nm is observed. If conglomeration of nanoparticles is great, it act like silver nanoparticle film. In silver nanoparticle film, the peak is observed at about *ca.* 580 nm[22,23].

Fig. 10 demonstrates the kinetics of the formation of silver nanoparticles in pH 5.5 and 7.5. It Seem that change pH isn't effective on the time of reaction completion although it is effective on efficiency of production.

Dynamic light scattering analysis shows that by increase of pH, size of nanoparticles increases (Fig. 11). In addition, it is observed that at pHs of 7.5 and higher, multi-micron particles are formed as well. Thus, pH = 5.5 has the smallest size with the least dispersion and no particles with micrometer size is observed. Furthermore, by means of atomic absorption spectroscopy, it became known that the amount of silver ion reduction decreases by increase of pH. This result, accompany with the result obtained from DLS analysis shows that maximum performance in silver nanoparticles production is achieved at pH = 5.5.

Transmission electron microscopy of the silver nanoparticles: The silver nanoparticles synthesized by *E. coli* bacterium (DH5α) were studied by transmission electron microscopy (TEM) and images show and confirm silver nanoparticles production at nano-size. TEM images of the produced nanoparticles are shown in Fig. 12.



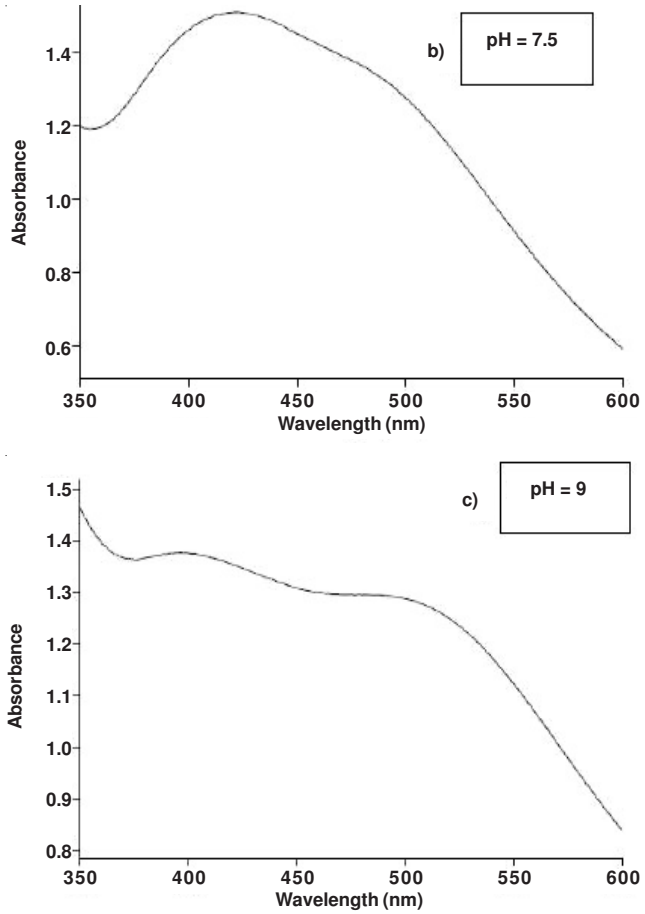


Fig. 9. UV-VIS spectra of Ag colloids. Spectra recorded after the addition of culture filtrate (1 mL) to 100 cc of silver nitrate solution (0.001 M) in a) pH = 5.5, b) pH = 7.5, c) pH = 9.

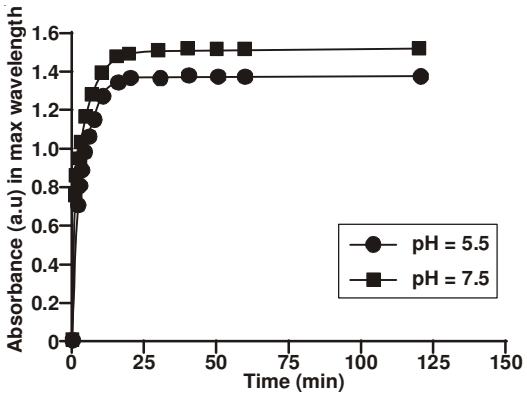
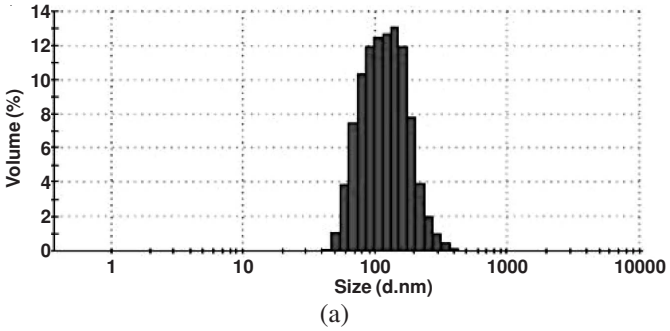


Fig. 10. Kinetics of silver nanoparticles formation in different pHs in 100 mL of silver nitrate solution (0.001 M).



(a)

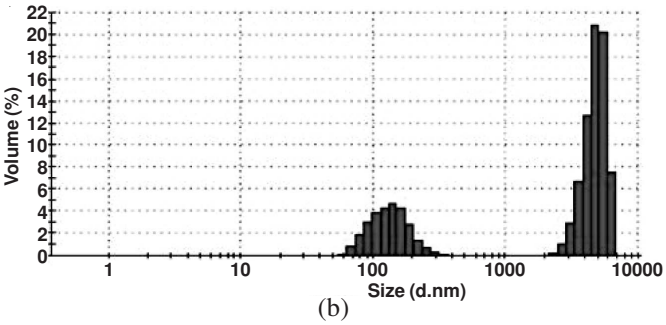


Fig. 11. Curve of size distribution by number (statistics graph), a) pH = 5.5, b) pH = 7.5

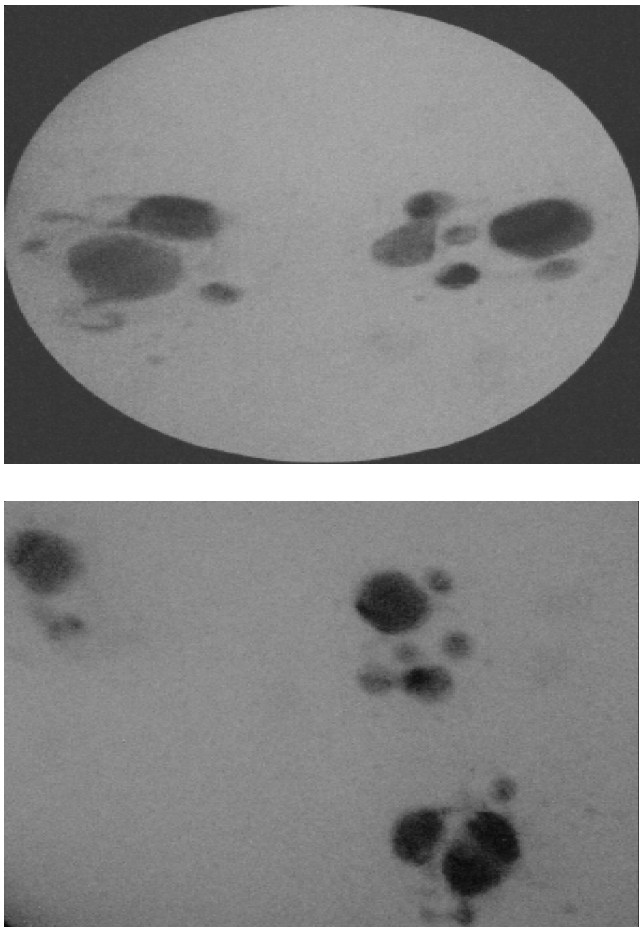


Fig. 12. TEM micrograph of silver particles synthesized by *E. coli* in (top) T = 25 ºC, pH = 5.5 and (bottom) T=5 ºC, pH=5.5 (scale bare: 100 nm)

Conclusion

In this research, production of silver nanoparticles using *E. coli* bacterium (DH5α) in different conditions was studied. Temperature, silver nitrate concentration, pH and mixing ratio of the bacterium filtrate to silver nitrate were studied in a time and specified environmental conditions. In addition, the kinetics of reaction of silver nanoparticles formation was considered in difference conditions. The reason for study of these quantities is achieving optimal and suitable conditions in order to produce silver nanoparticles at a scale larger than experimental scale. Obviously, completion time of reduction reaction of Ag^+ and consequently nanoparticles formation plays a key role in production of nanoparticles. One reason for advantage of chemical and physical methods compared to biological methods is the time of nanoparticles production. In many biological methods, required time period for silver nanoparticles formation takes more than 24 h. Therefore, use of these methods with all bio-environmental advantages and control of particles size and distribution does not seem much suitable, unless the time of nanoparticles production is shortened. One of microorganisms which are capable of such behaviour is *E. coli* bacterium. This bacterium is able in different environmental conditions to form nanoparticles in less than 15 min. Growth rate of this bacterium is high and has a high resistance against different conditions. Therefore, it is a suitable option for production of silver nanoparticles. In this research, it became apparent that at low temperatures the rate of nanoparticles production is higher and their size is smaller. Thus, temperature of 5 ºC was considered to be the optimum temperature. pH is also effective on nanoparticles size. It has been seen with increase of pH, the size of nanoparticles becomes larger and at pHs higher than 7.5, micro particles are produced. It seems that pH = 5.5 is the best conditions for production of silver nanoparticles. Silver nitrate concentration play also key role in formation of nanoparticles so that 0.001 M concentration has the best performance. In addition, with increase of concentration, particles with micro size are formed, by conglomeration of which a precipitation of silver accumulated at bottom of the container takes place. With increase of mixing ratio of bacterium filtrate to silver nitrate at environmental conditions, size of nanoparticles decreases. But microparticles formation and also decrease of reaction rate and increase of reaction completion time cause falling of production performance. Therefore, the best mixing ratio of bacterium filtrate is considered to be equal to 1 mL. With these optimum conditions, one can produce silver nanoparticles in 12 min and in larger scales. All the obtained results were examined using dynamic light scattering analysis, transmission electron microscopy, UV-VIS spectroscopy and atomic absorption spectroscopy.

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