Comparative study on silver nanoparticles properties produced by green methods

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Abstract
In the present study, properties of silver nanoparticles (AgNPs) such as average size, size distribution and morphology were investigated by Tollens, polysaccharide, modified polysaccharide and microbial methods. The synthesized AgNPs were characterized by UV-visible spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), dynamics light scattering (DLS) and energy dispersive X-ray (EDX) analyses. Analysis of reaction mixtures confirmed that Tollens, polysaccharide and modified polysaccharide methods generated smaller AgNPs with better size distribution as compared to that produced in microbial method. The average size of produced AgNPs by Tollens, polysaccharide and modified polysaccharide were 42, 30 and 20 nm respectively. Moreover, microbial method generated AgNPs with average size of 54 nm in the case of cell-free filtrate mediated synthesis and 84 nm in case of the supernatant mediated synthesis. Analysis of fungus-mediated synthesis of AgNPs showed that the size distribution of AgNPs produced by supernatant is narrower than that produced by filtrate. Also, cell-free filtrate resulted in the formation of smaller AgNPs with average size of 59 nm compared to the supernatant. The comparative analysis of produced AgNPs by the above mentioned methods confirmed that modified polysaccharide method led to the formation of AgNPs with smallest size and highest productivity.

Keywords: Green synthesis; Silver nanoparticles; Tollens method; Polysaccharide method; Microbial synthesis

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INTRODUCTION

Among the metal nanoparticles, silver nanoparticles (AgNPs) have become the focus of extensive research due to their wide ranges of applications (Le *et al*., 2010). AgNPs have several characteristics that make them useful in many different areas of science, medicine, agriculture and catalysis (Hebeish *et al*., 2011). These applications strongly depend on the properties of the produced AgNPs such as particle size and shape, size distribution and the surface charge (Soukupov *et al*., 2008). Therefore, the control of these properties has become a major challenge in research.

Conventionally, AgNPs are synthesized using chemical reducing agents in the presence of stabilizers (Yoksan and Chirachanchai 2010). Microbial applications of AgNPs, for example, in diagnostic devices require the use of biocompatible and non-toxic materials in the synthesis process (Singh *et al*., 2009). Among reducing and stabilizing agents, natural Polymers such as chitosan, soluble starch, carboxymethyl cellulose and secreted enzymes and proteins of fungi, are eco-friendly materials for the synthesis of AgNPs (Le *et al*., 2010; Hebeish *et al*., 2011).

These green methods are based on using such materials in nanoparticle synthesis protocol. Increasing the awareness towards green chemistry has led to the development of an eco-friendly approach for the synthesis of nanoparticles, that has several advantages such as simplicity, cost effectiveness, compatibility for biomedical and pharmaceutical application as well as for large-scale commercial production (Filippo *et al*., 2010).

For the first time, Wallen and co-workers reported the green synthesis of silver nanoparticles by using soluble starch and β-D-glucose as nontoxic reducing and stabilizing agents respectively (Chairam *et al*., 2009). Recently, starch has been used as a green capping agent (Vigneshwaran *et al*., 2006). In microbial synthesis, filamentous fungi are able to produce highly stable NPs, which prevent molecular aggregation even after prolonged storage. *Fusarium oxysporum* has been studied widely in many researches for AgNPs synthesis. In our previous research, various aspects of AgNPs synthesis have been studied (Khosravi *et al*., 2009; Mohammadian *et al*., 2007).

The ability of green methods in AgNPs synthesis has not been compared so far. In present work, AgNPs were generated by green methods of polysaccharide, modified polysaccharide, Tollens and microbial. In order to improve the properties of synthesized AgNPs such as particle size and size distribution, the abilities of these green methods in nanoparticles production were compared.

MATERIALS AND METHODS

Microorganism and materials: The fungus *Fusarium oxysporum strain* 24 was obtained from National Institute of Genetic Engineering and Biotechnology, Tehran, IR Iran. The fungal inoculates were prepared in potato dextrose agar (PDA) media at 28ºC in Petri plates and the plates stored at 4ºC. All chemicals including starch, β-D-glucose and silver nitrate were analytical grade and purchased from Sigma Aldrich.

Growth conditions of fungus: *Fusarium oxysporum* was grown aerobically in 250 ml flasks each containing 100 ml of the MGYP liquid medium consisted of (g/l): glucose 10; malt extract 6; yeast extract 6; peptone 5. The final pH was adjusted to 6.4-6.8 by addition of 1N HCl. The flasks were inoculated and then incubated on an orbital shaker at 28ºC and 200 rpm for 96 h.

Green synthesis of AgNPs: In all experiments, a carefully weighed quantity of $AgNO_3$ was added to flasks to obtain a final Ag^+ ion concentration of 1 mM in the aqueous solution. Furthermore, all reactions were carried out in the presence of light.

Microbial synthesis

Biosynthesis of AgNPs by using cell-free filtrate: The fungal biomass was harvested after 96 h of growth by filtration through Whatman filter paper no. 42 and washed extensively with sterilized distilled water. Then the biomass was resuspended in 25 ml sterilized distilled water and incubated at 28ºC, agitated at 200 rpm for 72 h. Then, cell-free filtrate was obtained by passing the solution through Whatman filter paper no. 42. The resultant filtrate was challenged with $AgNO_3$ and incubated at the same conditions mentioned above.

Biosynthesis of AgNPs using culture supernatant: After the incubation period, the fungal culture was centrifuged at 6000 rpm for 20 min, and the supernatant was collected. The fungal supernatant was reacted with the aqueous solution of silver nitrate. The mixture was incubated at 28ºC and 200 rpm shaking for 72 h.

Polysaccharide and modified polysaccharide synthesis: In this method, silver nitrate was reduced by β-D-glucose as a reducing agent. Starch solution (1% wt) was used as a stabilizer to prevent aggregation of nanoparticles. Initially, β-D-glucose solution (1 mM) was added to a mixture of silver nitrate and starch solution, with various β-D-glucose: silver nitrate volume ratios of 2:1, 3:1 and 1:1. The solutions were heated at 80ºC for 4 h. In modified polysaccharide method, AgNPs were synthesized by autoclaving a solution of $AgNO_3$ and starch at 15 psi and 121ºC for 15 minutes.

Tollens synthesis: Firstly, sodium hydroxide solution was mixed with silver nitrate. Then ammonium was added gradually until the precipitation eliminated. Finally, starch (1% w/v) and β-D-glucose (1 mM) were added as stabilizing and reducing agents respectively. The reaction occurred at room temperature (Soukupov *et al*., 2008). Various volume ratios of reducer (β-D-glucose) and silver nitrate were evaluated as mentioned in section 2.3.2.

Charecterization of AgNPs

UV-visible spectral analysis: Preliminary detection of

AgNPs synthesis was done by observation of the color changes of the mixtures. These samples subjected to optical measurements. Absorption measurements were recorded using Cary 100 double beam spectrophotometer (Varian) at the wavelength range of 200 to 800 nm.

Dynamic light scattering particle size analyzer (*DLS*): The size distribution profile of the nanoparticles along with its polydispersity was determined using a particle size analyzer. These measurements were carried out by means of Malvern UK instrument in the range between 0.1 nm and 10 μm.

Field Emission Scanning Electron Microscope (*FE-SEM*) *and Energy-dispersive X-ray spectroscope* (*EDX*): FESM (Hitachi S4160) equipped with EDX microanalyser was used to evaluate the structural and elemental composition of prepared AgNPs. Analysis of colloidal samples was performed by mounting nanoparticles on specimen stubs with double-sided adhesive tape and coated with gold in a sputter coater and examined under FE-SEM.

Transmission Electron Microscope (*TEM*): Zeiss EM900 transmission electron microscope (TEM) operated at 50 KV, was used for characterisation of synthesized AgNPs.

RESULTS

The properties of the synthesized silver nanoparticles, which were produced by polysaccharide, modified polysaccharide, Tollens and microbial methods, were evaluated by the following analysis methods:

UV-Vis spectrophotometer analysis: The appearance of a yellowish-brown color in all reaction flasks were the indication of silver nanoparticles formation. The color intensity increased as a function of time due to the reduction of Ag^+. The verification of AgNPs formation and their stability were monitored by UV-vis spectral analysis. In order to obtain maximum productivity of AgNPs by polysaccharide and Tollens methods, initially, different ratios of reducer and the silver nitrate (1:1, 2:1 and 3:1 v/v) were assessed. Then the optimum ratios were determined based on maximum absorption intensity of the reaction solutions. Figure 1 shows the UV-vis spectra of the AgNPs obtained by various ratios of reducing agent and the silver nitrate solutions. Results revealed that the optimum volume ratios of β-D-glucose: $AgNO_3$ for polysaccharide and Tollens methods were 2:1 and 3:1, respectively. Therefore, the optimum ratios were used in the further experiments.

The UV-vis spectra recorded for all of the samples of the AgNPs synthesized by various methods at different reactions times are presented in Figure 2. As shown in Figures 2B and 2C, the completion of the reactions for polysaccharide and Tollens methods occurred after 4 h. The reduction of Ag^+ in modified polysaccharide method was completed after 45 minutes (Fig. 2A). In microbial synthesis the maximum intensity for both supernatant and cell-free filtrate were observed after 72 h (Figs. 2D and 2E).

The stability of AgNPs in solution was investigated by measuring their absorbance intensities at related surface bands over a period of time at room temperature. Results are presented in insets of Figures 2 A-E. As it can be seen from the inset of Figure 2A, absence

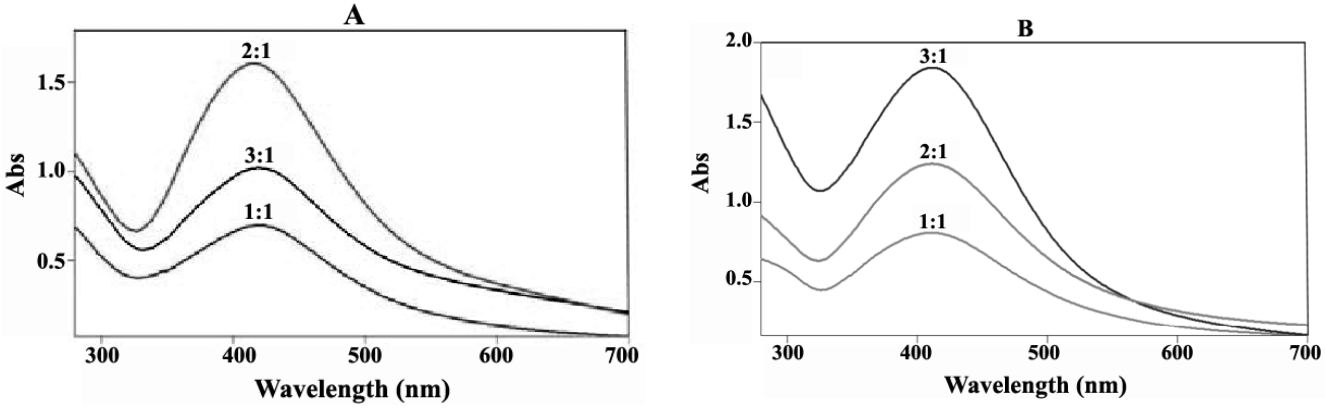

Figure 1. UV-vis spectra recorded for colloidal AgNPs solutions at different volume ratios of β-D-glucose: $AgNO_3$ (1:1, 2:1 and 3:1), A: polysaccharide; B: Tollens method.

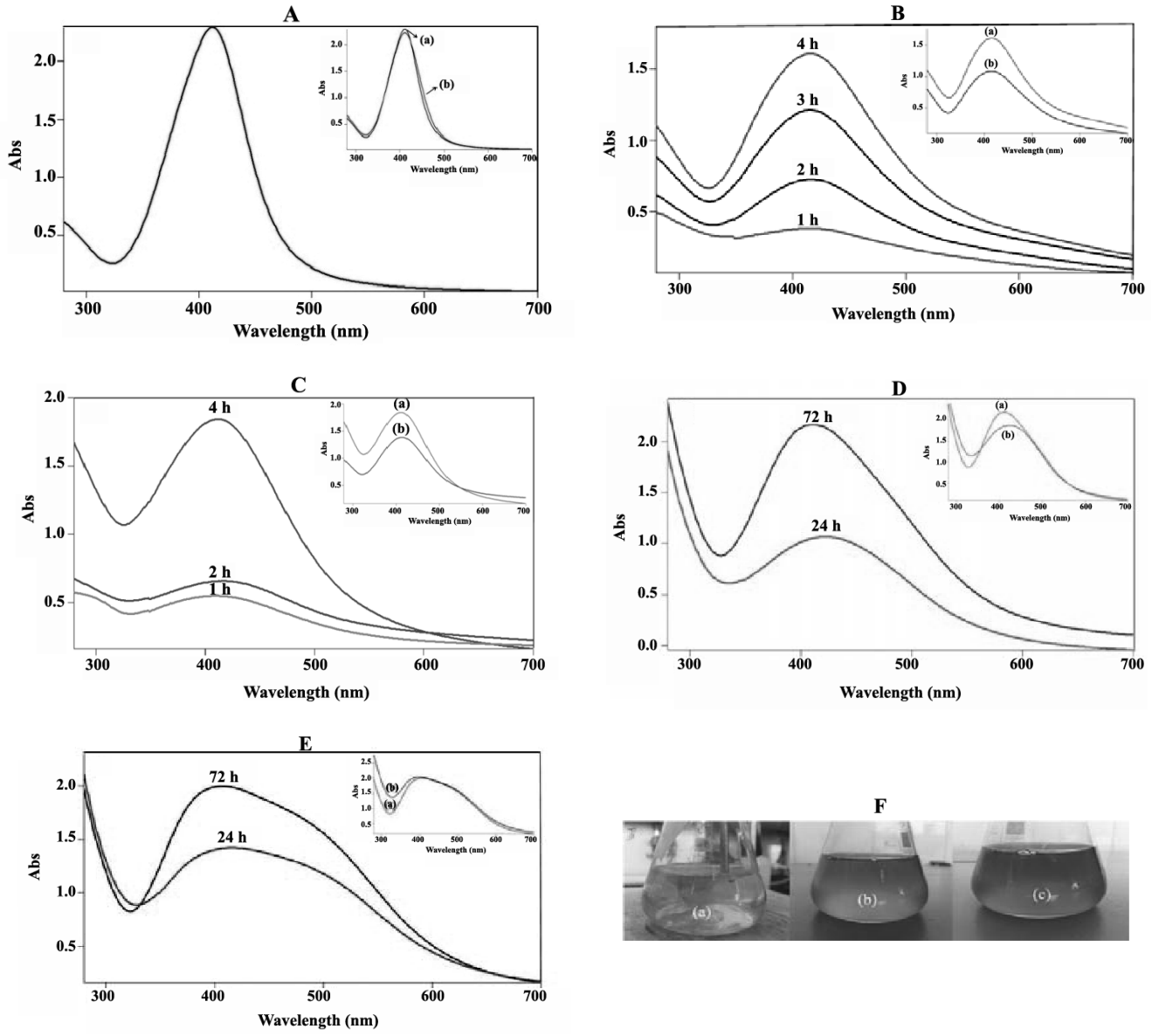

Figure 2. UV-vis absorption spectra recorded as a function of time in reaction solutions containing AgNPs for A: modified polysaccharide with β-D-glucose: $AgNO_3$ volume ratio of 2:1; B: polysaccharide with β-D-glucose: $AgNO_3$ volume ratio of 2:1; C: Tollens with β-D-glucose: $AgNO_3$ volume ratio of 3:1; D: Microbial (using cell-free filtrate of *F. oxyspoum*); E: Microbial (using culture supernatant of *F. oxyspoum*) methods. [Insets in Fig. 2 (A-E) illustrate the time intervals of stability for synthesized AgNPs in solutions (a) after complete reduction of Ag^+; (b) after the stability period of colloidal AgNPs]. F: Color changes of reaction mixtures containing colloidal AgNPs in polysaccharide method (a) at the start (b) after 2 h and (c) end of reaction.

of significant changes in the intensities and maximum absorbance band for the period of two months for modified polysaccharide method proved the stability of synthesized AgNPs. Inset of Figure 2B indicated that there is no any shift in maximum absorbance, which was obtained by polysaccharide method during one month. Otherwise, as shown, the intensity of absorbance decreased slightly after the mentioned period. The stability test for Tollens method during two weeks confirmed that the maximum absorbance band shifted to the larger wavelength (from 416 to 418 nm) which evidences the formation of larger AgNPs (Travan *et al*., 2011; Gao *et al*., 2011). The stability evaluation for microbial method was performed during one month. In cell-free filtrate-mediated synthesis, the absorbance intensity decreased. However, in culture supernatant-mediated synthesis, the maximum band shifted to larger wavelength indicating an increase of

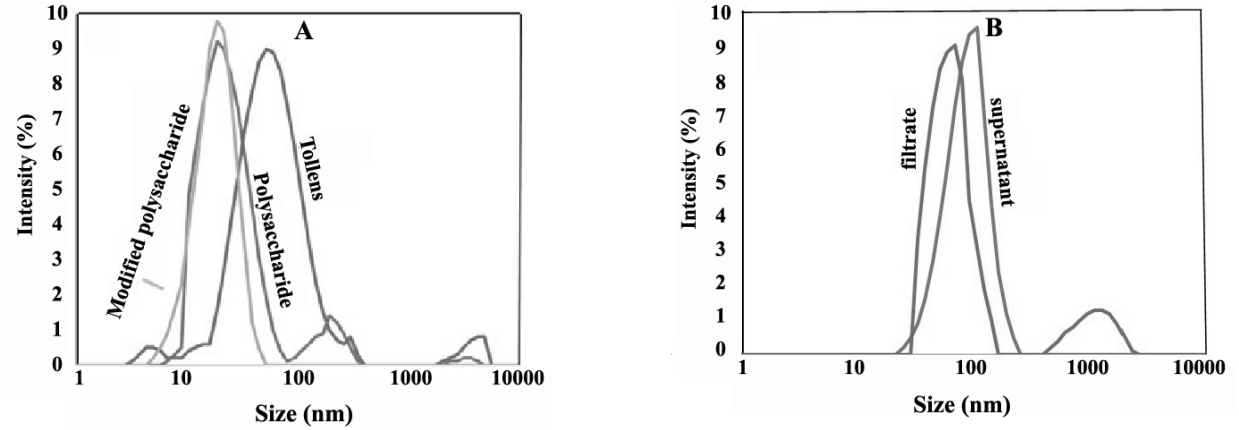

Figure 3. DLS analysis of colloidal AgNPs for A: polysaccharide, modified polysaccharide and Tollens methods, B: Microbial method (cell-free filtrate and culture supernatant of *F. oxysporum*).

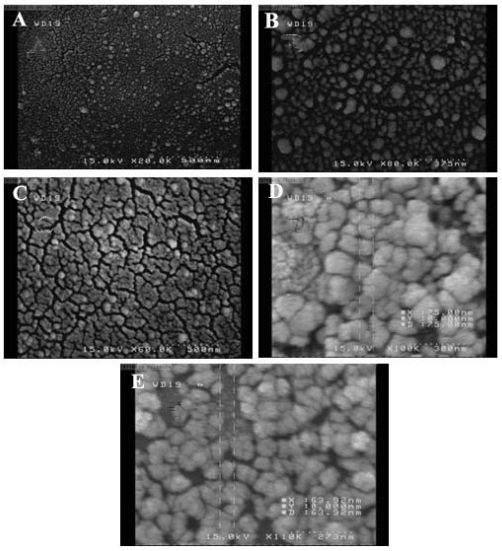

Figure 4. Scanning electron micrograph of AgNPs synthesized by green methods; A: modified polysaccharide; B: polysaccharide; C: Tollens; D: Microbial (cell-free filtrate of *F. oxysporum*; E: Microbial (culture supernatant of *F. oxysporum*) methods.

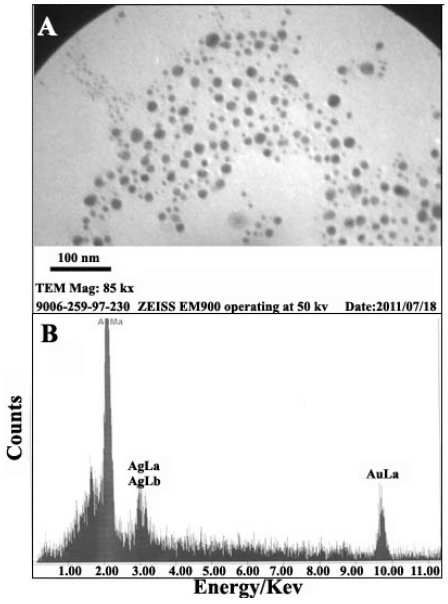

Figure 5. (A) TEM image; (B) EDX spectrum of AgNPs synthesized by modified polysaccharide method.

mean particles diameter.

Particle size analysis: Average particle size, size distribution and Polydispersity index of synthesized AgNPs in solutions were evaluated by DLS technique, which is shown in Figure 3. The average particle sizes of polysaccharide, modified polysaccharide and Tollens methods were 30, 20 and 42 nm, respectively. Average particle sizes of nanoparticles synthesized using fungal supernatant and cell-free filtrate were obtained as 84 and 59 nm, respectively. The comparison of average particle sizes revealed that the utilization of starch and β-D- glucose in synthesis of AgNPs, resulted in the generation of smaller particles compared to those obtained by the use of microbial extracts (Fig. 3). As it is presented in Figure 3B, the culture supernatant of fungus was able to form AgNPs with narrower size distribution than the cell-free filtrate. The overall evaluation of all selected methods in aspects of monodispersity and average size showed that the modified polysaccharide method among other methods used in this study produce smallest AgNPs with best size distribution.

SEM analysis: The size and shape of AgNPs were further characterized by SEM analysis. SEM images

show individual AgNPs as well as a number of aggregates. The morphology of the synthesized AgNPs by the methods was predominately spherical and aggregated into the larger irregular structure with no well-defined morphology (Fig. 4).

TEM analysis: The TEM and EDX images of synthesized AgNPs by modified polysaccharide method are presented in Figure5. The shape of AgNPs is prominently spherical (Fig. 5A). Furthermore, the AgNPs are well-dispersed and almost no aggregates of AgNPs are observed. EDX spectrum revealed the strong signal in the silver region and confirmed the formation of silver nanoparticles (Fig. 5B). The typical optical absorption band peaked nearly at 3 KeV confirming the metallic silver nanoparticles due to SPR.

DISCUSSION

The present study demonstrated the AgNPs characteristics that were synthesized by environmentally-friendly procedures including polysaccharide, modified polysaccharide, Tollens and microbial methods. In fungus-mediated synthesis of AgNPs, secreted enzymes and proteins act as reducer and stabilizer agents. In the case of Tollens, polysaccharide and modified polysaccharide methods, starch and β-D-glucose serve as stabilizer and reducer respectively. Therefore, the presence of different reducers and stabilizers resulted in production of AgNPs with different characteristics such as average size, size distribution and stability. The properties of synthesized AgNPs were compared by UV-vis, DLS, SEM, EDX and TEM analysis.

It is well known that color change of solutions is due to excitation of surface plasmon resonances (SPR) in metal nanoparticles (Natarajan *et al*., 2010). Colloidal silver nanoparticles are known to exhibit a UV–vis absorption maximum in the range of 390-420 nm (Vigneshwaran *et al*., 2006). It was observed that the SPR band of samples obtained by each method was different and steadily increased in intensity as a function of time without any shift in the peak wavelength. Therefore, the formation of AgNPs in various methods depends on the incubation period of the reaction mixtures. No change in intensity of the recorded spectra by UV-vis, revealed a complete reduction of Ag+. Comparison of the synthesis methods showed that the fastest method was the modified polysaccharide method.

The SPR is extremely affected by the shape and size of the nanoparticles (Mohammed *et al*., 2009). The absorption band of metal nanoparticles shifts to longer wavelengths with increasing particle size (Prathna *et al*., 2011). In addition, absorbance intensity provided insight into the reduction of Ag^+ and productivity of each method (Vahabi *et al*., 2011). The presence of symmetric SPR bands may be attributed to the formation of AgNPs with narrow size distribution (Gao *et al*., 2011). The long tail on the large wavelength may be due to the small amount of aggregated particles (Maliszewska *et al*., 2009). As described above, modified polysaccharide method produced the smallest nanoparticles with best size distribution. Utilization of high temperature and pressure in modified polysaccharide method result in formation of smaller nanoparticles with higher productivity than polysaccharide method.

The largest plasmon resonance peak was observed at 411 nm for modified polysaccharide synthesis protocol (Fig. 2A), which shows the highest productivity. Moreover, the existence of the long tail in Fig. 2A, verifies the small aggregation of synthesized AgNPs by the modified polysaccharide method. The absorbance intensity of the synthesized AgNPs by Tollens method was higher than polysaccharide method. However, the size of AgNPs sample was smaller in polysaccharide method. Furthermore, the size distribution of AgNPs by polysaccharide method was narrower than Tollens method. The observed plasmon band for culture supernatant-mediated synthesis in Fig. 2D was symmetric compared to cell-free filtrate-mediated synthesis. This can be attributed to narrower size distribution and less aggregation of particles. It is evident that electron shuttles or other reducing agents, e.g. extracellular enzymes and proteins, released by *F. oxysporum* are capable of reducing Ag^+ to AgNPs (Vahabi *et al*., 2011). The different amounts of secreted enzymes and proteins, which exist in the culture supernatant and cell-free filtrate solutions, in addition the presence of secondary metabolites in the culture supernatant, are probably the cause of AgNPs production with different properties.

SEM images showed that the nanoparticles are stable and not in direct contact. This may be attributed to the stabilization of the nanoparticles by secreted proteins (in microbial method) and starch (in polysaccharide, modified polysaccharide and Tollens methods) as capping agents.

The final comparison of the green methods elucidated that the modified polysaccharide is the most efficient method in AgNPs synthesis. The rate of AgNPs synthesis by modified polysaccharide was higher than

Table 1. Comparison of Tollens, polysaccharide, modified polysaccharide and microbial methods for AgNPs synthesis.

Methods	Reducer	Stabilizer	Stability	Average size (nm)	Reaction time	λ (nm)	Comments
modified polysaccharide	β- D-glucose	Starch	Two months	20	45 min	411	Easy, high productivity, rapid synthesis
Tollens	β- D-glucose	Starch	Two week	42	4 h	416	Easy, high productivity
polysaccharide	β- D-glucose	Starch	Two months	30	4 h	412	Easy, energy saving, cost-effective, high productivity
microbial	Cell-free filtrate of *F. oxysporum*	Cell-free filtrate of *F. oxysporum*	One month	59	72 h	410	Ease of downstream processing, High number of proteins and enzymes as a reducer and stabilizer
microbial	Culture supernatant of *F. oxysporum*	Culture supernatant of *F. oxysporum*	One month	84	72 h	420	Ease of downstream processing, High number of proteins and enzymes as a reducer and stabilizer

the other methods. In addition, this method showed high productivity and small aggregation of synthesized AgNPs. The abilities of these green methods in AgNPs synthesis were compared in Table 1.

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