Research Article

The Use of Biofloculant and Biofloculant-Producing *Bacillus mojavensis* Strain 32A to Synthesize Silver Nanoparticles

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It is preferable to use an organism to produce more than one product at the same time. So, the aim of this study was to investigate the ability of biofloculant-producing *Bacillus mojavensis* strain 32A as a nanosilver synthesizer beside biofloculant production. To achieve this target, three media, nutrient broth, biofloculant-producing medium, and pure biofloculant, were tested. Produced nanosilver was characterized by UV-vis, XRD, and TEM. In all cases, the results demonstrated that UV-vis showed a peak at ~420 nm corresponding to the plasmon absorbance of nanosilver. XRD spectrum exhibited 2θ values corresponding to the silver nanocrystal that is produced in hexagonal and cubic crystal configurations. TEM confirmed formation, size, shape, and morphologies of nanosilver particles. The results emphasized that purified biofloculant has the ability to produce anisotropic clusters of nanosilver ranging in size from 6 to 72 nm proving that the biofloculant functioned as reducing and stabilizing agent in nanosilver synthesis.

1. Introduction

Nanotechnology, appearing abruptly in the 20th century, is an area of science devoted to the manipulation of atoms and molecules in the nanometer size range 1–100 nm [1]. Like many future areas of scientific exploration, nanoscience and nanotechnology exist on the borders between disciplines including but not restricted to catalysis, electronic, medicine, biotechnology, and environmental remediation and agricultural research. Nanomaterials often show unique and considerably changed physical, chemical, thermodynamic, magnetic, and biological properties compared to their macroscaled ones [2, 3]. Out of all kinds of nanoparticles, silver nanoparticles (AgNPs) seem to have attracted the most interests in terms of their potential application. Indeed, the widespread use of this precious metal in nanosize form from household paints to artificial prosthetic devices has imparted significant effects on our daily lives [2, 4].

AgNPs can be prepared by two methods; the first one is a physical approach that utilizes several methods such as

evaporation/condensation and laser ablation. The second one is a chemical approach in which the metal ions in solution are reduced in conditions favoring the subsequent formation of small metal clusters or aggregates. Most of these techniques are extremely expensive and capital intensive; they also involve the use of toxic, hazardous chemicals which may pose potential environmental and biological risks as well as inefficiency in materials and energy use [5]. However, development of simple and ecofriendly synthetic method would help promoting further interest in the synthesis and applications of metallic nanoparticles [6, 7]. Recently, biosynthetic methods employing naturally occurring reducing agents such as polysaccharides, biological microorganisms such as bacteria and fungus, or plants extract, that is, green chemistry, have emerged as a simple and viable alternative to more complex chemical synthetic procedures to obtain AgNPs. It provides advancement over chemical and physical methods as it is of slower kinetics, offers better manipulation, is cost effective, is environmentally safe, and is easily scaled up for large scale synthesis; in addition, there is no need to use high pressure,

energy, temperature, and toxic chemicals to control crystal growth and its stabilization [2].

Many microorganisms including bacteria [8], fungi [9–11], actinomycetes [12, 13], and algae [14] can aggregate inorganic materials and form nanoparticles (NPs) intracellularly or extracellularly. Natural compounds such as microbial biopolymers are also one of the resources which could be used for green synthesis of AgNPs. Recently, Sathiyarayanan et al. [15] reported that polysaccharides from microbial origin such as biofloculants are a promising alternative for the synthesis and stabilization of nanoparticles. Indeed, this is an interesting area of research where both biosynthetic nanosilvers and biofloculants are economically important. Biofloculants are biodegradable, safe, and ecofriendly biopolymers secreted by microorganisms [16, 17]. They are used in the field of wastewater treatment for removing suspended solids and metal ions, at which colloids come out of suspension in the form of floc or flakes [18]. In this regard, recently in our lab *Bacillus mojavensis* strain 32A was isolated as an efficient biofloculant producer [19]. In the present study, synthesis of nanosilver particles using biofloculant-producing strain 32A and its extracted and purified biofloculant was investigated. Produced nanoparticles were characterized using transmission electron microscope (TEM) and X-ray diffraction (XRD) techniques.

2. Material and Methods

2.1. Chemicals, Strain, and Cultural Conditions. Silver nitrate (AgNO_3) required for synthesis of AgNPs was obtained from Sigma-Aldrich. Other required chemicals were purchased from Merck. *Bacillus mojavensis* strain 32A used in this study was previously identified as an efficient biofloculant producer by Elkady et al. [19]. Culturing, media, and production of biofloculant and/or synthesis of silver nanoparticles were performed as described elsewhere [5, 19]. The initial pH of all media was adjusted to 7.2 to 7.5 with NaOH (1 M) and HCl (0.5 M). All media were prepared with distilled water and sterilized at 121°C for 20 min.

2.2. Synthesis of AgNPs. The following three media were examined to synthesize AgNPs using the biofloculant and biofloculant-producing bacterial strain 32A.

2.2.1. In Nutrient Broth Medium. AgNPs were synthesized as described by Zaki et al. [20]. Strain 32A was precultured for overnight in LB medium at 30°C in a rotary shaker with 200 rpm. Subsequently, 10% of precultured LB were transferred into 100 mL nutrient broth medium (NB) containing (w/v) 1% peptone, 0.5% yeast extract, and 0.5% beef extract supplemented with 3.5 mM of AgNO_3 in 500 mL flask. The mixture was incubated in darkness for 7 days at 30°C with 200 rpm shaking. The extracellular synthesis of AgNPs was monitored by visual inspection of the change in medium color from a clear light yellow to brown. The control was maintained without addition of AgNO_3 with the experimental flask containing NB medium.

2.2.2. In Biofloculant-Producing Medium. About 10% of the precultured LB medium was used as a seeding medium of the biofloculant-producing medium (BP) as described by Elkady et al. [19]. The BP medium contained (per liter) L-glutamic acid 20 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, MgSO_4 0.5 g, FeCl_3 40 mg, CaCl_2 150 mg, and MnSO_4 140 mg supplemented with 3.5 mM of AgNO_3 , and 80 mL sterile glycerol was added just before the cultivation. Synthesis and monitoring of AgNPs occurred as described above. The control was maintained without addition of AgNO_3 with the experimental flask containing BP medium.

2.2.3. In Purified Biofloculant Solution. Purified biofloculant (PB) from strain 32A was obtained as described by Elkady et al. [19]. The cell-free supernatant was concentrated to 0.2 volumes with a rotary evaporator and dialyzed overnight at 4°C in deionized water. Three volumes of cold anhydrous ethanol (4°C) were added to the dialyzed broth. The precipitate obtained was redissolved in deionized water followed by the addition of 10% cetylpyridinium chloride (CPC) with stirring. After several hours, the resultant precipitate was collected by centrifugation (5000 rpm, 15 min) and dissolved in 0.5 M NaCl. Three volumes of cold anhydrous ethanol (4°C) were then added to obtain the precipitate, which was then washed with 75% ethanol three times and lyophilized to obtain purified biopolymer. The final concentration of 3 mM AgNO_3 was added into 200 mL of 10% biofloculant solution in 500 mL flask. The flasks were incubated in darkness for 2 days. The monitoring of AgNPs synthesis was performed as described above. The control was maintained without addition of AgNO_3 with the experimental flask containing purified biofloculant.

2.3. Characterization of AgNPs. The synthesized AgNPs were first characterized by UV-visible spectrophotometer (Labomed model UV-vis double beam spectrophotometer) in the range of 200–600 nm. Quartz cuvettes with optical path length of 10 mm were used in the measurements. One milliliter from each AgNPs production way was withdrawn and the absorbance after centrifugation was measured. The produced AgNPs were collected by centrifugation at 10,000 rpm for 10 min. The finely powdered samples were dried in vacuum oven at 60°C overnight. Subsequently, the finely powdered samples were analyzed using X-ray diffractometer (Schimadzu-7000, USA). Through packing the dried samples into a flat aluminum sample holder, where the X-ray source was a rotating anode operating at 30 kV and 30 mA with a copper target, data were collected between 10° and 80° in 2 θ . Transmission electron microscope (JEOL JEM-1230, Japan) was utilized to confirm and detect morphology of AgNPs under an accelerating voltage of 120 kV; samples were prepared by placing a drop of hydrophobic nanosilver colloid or its aqueous coordinate on carbon-coated copper grids and drying at room temperature.

3. Results and Discussion

3.1. Visual Examination. In all cases, appearance of a stable brown color indicates AgNPs formation in aqueous solution,

FIGURE 1: The optical photograph of three studied ways to produce AgNPs: (a) 3.5 mM AgNO_3 solution control without cells; (b) broth with 3.5 mM AgNO_3 solution and without culture; (c) culture broth without AgNO_3 as a negative control; (d), (e), and (f) are the results of AgNPs production in NB medium, BP bioflocculant-producing medium, and PB purified bioflocculant, respectively, after 7 days of incubation.

while there was no color change that could be observed in the negative controls (Figure 1). However, the light yellow color appearing with the control broth was due to the components of NB medium which include yeast and beef extracts. It is established that color change of solutions is due to excitation of surface plasmon resonances (SPR) in metal nanoparticles [21, 22].

3.2. UV-Vis Spectrophotometer Analysis. Previously, it has been proved that UV-vis analysis is a very useful and quite sensitive technique for the analysis of nanoparticles [23]. In the present study, AgNPs were characterized by UV-vis after 7 days of incubation as described by Zaki et al. [20]. As shown in Figure 2, it is clear that when we used NB and BP media to produce AgNPs, a typical, sharp, strong, and single plasmon peak at 400 nm was obtained. Shrivastava et al. [24] reported that the AgNO_3 solution showed at about 300 nm; however, in the present study gradually underwent red shift with appearance of a hump at 400 nm was observed (Figures 2(a) and 2(b)). These results were consistent with formation of small and narrow size distribution of the spherical nanoparticles as described by Kumar et al. [25]. However, observation of such ideal bell, sharp plasmon band which appeared to exhibit semisymmetric shape and the absence of tailing at higher wavelength confirm that the solution exhibits monodispersity and does not contain many aggregated particles.

As shown in Figure 2(c), the UV-vis analysis of the AgNPs produced in PB demonstrated that the optical absorption spectrums of metal nanoparticles are dominated by surface response plasmon (SRP) shifts to longer wavelengths with increasing particle size [21]. In addition, about 2 SRP bands were detected at 465 and 510 nm indicating the presence of anisotropic large particles. Also, absorbance intensity provided indication on the reduction, productivity, and amount of Ag^+ ion [21]. This may be due to the availability of more reducing biomolecules and concentrations of AgNPs. The absence of symmetric SPR bands and presence of tailing at high wavelength may be attributed to the agglomeration of AgNPs with different size distribution [26].

3.3. TEM. TEM has been employed to characterize the size, shape, and morphologies of the formed AgNPs and their producing biofactory. As an electron beam passes through the particles at a slower rate than through the carbon grid due to the difference in atomic electron density, having an increased chance of scattering, the electron sensor is able to identify the high-density area from the overall background by collecting the number of electrons. Particles with high density will appear darker in the TEM [27]. In the NB medium used to produce AgNPs, cells appeared to have normal characteristics, their cell walls and cytoplasmic membranes were intact, and the internal structure showed unanimous homogeneous electron density cytoplasm. This suggested that cells are in normal conditions without environmental disturbance and DNA molecules distribute randomly (Figure 3(a)). In addition, tiny biogenic nanoparticles less than 6 nm in size were deposited as seeds like on the cytoplasmic membrane which appeared as electron opaque. As illustrated in Figure 3(b), cell-free supernatant of NB medium indicates that biosynthesized AgNPs were spherical, small, and monodispersed.

However, in the bioflocculant-producing medium (BP) it seems that bacteria differ from the previous one due to the difference in incubation media, where the glutamic acid and ammonium chloride were usually used as carbon and nitrogen sources, respectively, which are considered being the precursor substrate for biopolymer production [17, 28], while glycerol enhanced the polymer synthesis as reported by [29]. So it exhibits enhancement for two types of biopolymers that were defined by their cellular location, as indicated in Figures 4(a) and 4(b). Firstly, intracellular inclusion bodies that can be distinguished in TEM as electron light granules reach more than 60% of cell volume and about 30 to 500 nm in diameter; such nanobiopolymers like lipid, polyhydroxyalkanoates (PHAs), triacylglycerols, wax esters, polyphosphates, and glycogen have been reported in many bacteria [8]. Secondly, extracellular polysaccharides (EPSs) which were secreted in the growth medium exhibiting flocculating properties cannot be distinguished easily with TEM due to processing rupture. Contrary to AgNPs produced in NB medium, small spherical silver nanoparticles were not detected on the cytoplasmic membrane of the cell but detected outside the cell in little agglomeration on ruptured extracellular bioflocculant.

To explain biosynthesis of AgNPs in NB and BP media, encoded proteins such as nitrate reductase appear to be the choice of *Bacillus mojavensis* 32A strain to detoxify silver ions by its reduction to less toxic oxidative state. It is known that nitrate reductase enzyme shuttles the electron to the silver ions in an aqueous solution and in the presence of NAD and H^+ ions that act as a reducing agent. Some of other peptides/proteins may be responsible for the subsequent stabilization of silver nanoparticles as well [30, 31].

The TEM observations of AgNPs produced in the purified bioflocculant solution (PB) show several shapes of AgNPs in varying sizes including nanospheres, hexagonal, polygonal, nanoprisms, and uneven shapes (Figure 5). These nanoparticles are polydisperse and aggregated, and their particle sizes were ranging from 6 to 72 nm; such variation in shape and size

FIGURE 2: ((a)–(c)) The UV-vis spectra of the silver nanoparticles obtained from all examined ways: (a) NB medium, (b) BP medium, and (c) PB solution, respectively.

FIGURE 3: TEM images recorded from the drop-coated film of the AgNPs synthesized by strain 32A cells cultured in NB medium (a) and cell-free supernatant of the same medium (b).

FIGURE 4: TEM images recorded from the drop-coated film of the AgNPs synthesized by strain 32A cells cultured in BP medium (a) and cell-free supernatant of the same medium (b).

FIGURE 5: TEM image recorded from the drop-coated film of the silver nanoparticles synthesized by the silver nitrate solution with purified bioflocculant (PB).

of nanoparticles synthesized by biological systems is common [32].

To date, most of the preparation methods published are based on using organic materials due to the hydrophobicity of the stabilizing agents used, such as natural polymers [33]. The purified bioflocculant was used as bioreductant for silver ion. As reported by Elkady et al. [19], the chemical analysis of the purified biopolymer demonstrates that it contains 1.6% (w/w) protein and 98.4% polysaccharide. However, its amino acid analysis indicates the presence of glutamic acid (38%), aspartic acid, and glycine, respectively. In addition, FTIR spectrum

was used to reveal the functional groups of the biopolymer which were hydroxyl, amino groups, aliphatic C–H, C=O, and CH₃ CH₂. Generally, the carboxyl groups in aspartic and/or glutamine residues and the hydroxyl group in tyrosine residues of the proteins were suggested to be responsible for the Ag⁺ ion reduction and stabilization [3]. Firstly, these silver ions oxidize the hydroxyl groups to carbonyl groups, during which the silver ions are reduced to elemental silver. Subsequently, the reducing end of polysaccharides can be used to introduce an amino functionality that is capable of complexation and stabilizing metallic nanoparticles carbohydrates with such amino groups binding tightly to the surface of the AgNPs giving them a hydrophilic surface [34].

Finally, both hydroxyl and carbonyl groups of bioflocculant are involved in the synthesis of AgNPs and both amino and carboxylate groups of it are involved in the capping and stabilizing of AgNPs and that is in agreement with [15]. These results are in concurrence with an earlier biosynthesis of AgNPs carried out with fungus *Trichoderma asperellum* and gum kondagogu. It is known that proteins can bind to nanoparticles either through free amino groups or by electrostatic interaction of negatively charged carboxylate groups. The gum tragacanth is known to contain protein, and the protein content was reported to be in the range of 1.0–3.6% [35], so *Bacillus mojavensis* 32A strain bioflocculant acts as reducing and stabilizing agent in AgNPs synthesis.

3.4. XRD. The X-ray diffraction (XRD) technique was used to establish the metallic nature of particles. X-rays are electromagnetic radiation with typical photon energies that can penetrate deep into the materials and provide information about the bulk structure. In all studied cases, XRD pattern showed intense Bragg's reflections that can be indexed on the basis of the fcc structure of silver (Figure 6). The 2θ values of the XRD pattern were ranging from 30° to 80° and three strong peaks were observed at 38.1°, 46.3°, and 77.5° and corresponded to the planes 111, 200, and 311 and exhibited that

FIGURE 6: XRD patterns for the AgNPs produced by strain 32A.

the synthesized AgNPs were crystalline in nature. The values agree well with those which are indexed to the face centered cubic structures of silver nanoparticles [20].

4. Conclusion

In this study, the biofloculant-producing *Bacillus mojavensis* strain 32A and its pure biofloculant were examined as nanosilver producers; such a technique is important from both the economic and the scientific points of view where that the knowledge of the way in which biofloculant contributes to convert silver nitrate to AgNPs still needs several successive researches. This understanding will help in the use of biopolymers in general and biofloculants, in particular in the synthesis of AgNPs. The results showed that both bacteria and their biofloculant were able to synthesize AgNPs. However, TEM studies show that there are differences in the shape and size of nanoparticles manufactured by bacteria from those manufactured by biopolymer. These results were confirmed by the analysis of synthesized AgNPs spectrum as well as XRD. In addition, it is speculated that *Bacillus mojavensis* strain 32A has the ability to produce extracellular biofloculant and intracellular nanobiopolymer and activate nitrate reductase to produce AgNPs, as well. The study emphasized that in addition to its ability in aggregation and precipitation of suspended particles, the biofloculant is acting as reducing and stabilizing agent in AgNPs biosynthesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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