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Original article

An-ecofriendly synthesis of silver nanoparticles using microalga *Desmodesmus protuberans* and evaluation of their antimicrobial activity

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Abstract

Due to the numerous uses for nanoparticles, nanoparticle production is currently a particularly fascinating topic of research. Since no harmful chemicals are employed during the biological process, it is thought to be the safest and cleanest method.

The strong ability of algae to absorb metals and decrease metal ions makes algal synthesis of Ag-NPs particularly intriguing.

The current work concentrated on the green synthesis of silver nanoparticles (AgNPs) using the microalgae *Desmodesmus protuberans*. The preparation of nanoparticles was confirmed by the observation of the color change of the mixture of silver nitrate, after the addition of the cell free algal extract, from bright green to reddish yellow. The biosynthesis of AgNPs was further confirmed by scanning electron microscopic and energy dispersive X-ray analysis. Furthermore, silver nanoparticles demonstrated a significant antimicrobial activity against the pathogenic microorganisms.

Keywords

silver nanoparticles, microalgae, Green synthesis, *Desmodesmus protuberans*, antimicrobial activity

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Introduction

The field of nanotechnology is either one of the foremost important and active areas of research in modern science.

Nanotechnology is an emerging field that includes synthesis, characterization, and development of various nanomaterials [1, 2] that have significant roles in daily life, providing valuable products that improve industrial production, agriculture, communication, and medicine) [3].

Nowadays, some metallic nanoparticles are crucial antibacterial agents. They are appropriate for additional applications such as carriers for medication administration, chemical sensing, cosmetics, antioxidants, and others due to their size, which fall within the range of 1-100nm [1, 2, 4, 5]. Synthesis of nanomaterials containing noble metal requires alternative strategies because of their hight cost.

Though several physicochemical approaches (photoinduced reduction, electrochemical deposition, microwave-assisted, laser ablation, high-energy irradiation, pyrolysis) may be used to create AgNPs, they are both costly and possibly hazardous to the environment.

The use of biological species to synthesize nanomaterials is gaining popularity since biological approaches are less costly, nontoxic, environmental friendly aligned to "green chemistry" principles and presents simple approach [6, 7, 8]

A wide range of materials, including plants and plant products, algae, fungi, yeast, bacteria, and viruses, can be used in the biological production of NPs. The initial steps in the synthesis of NPs involve combining biomaterials and precursors of noble metal salts [9]. Proteins, alkaloids, flavonoids, reducing sugars, polyphenols, and other substances found in the biomaterials function as reducing and capping agents for the production of NPs from their metal salt predecessors [10].

Microalgae are microscopic organisms found in fresh or sea water, but also in nonaquatic habitats. Also, microalgae can potentially be used in a large number of biotechnological areas, including cosmetics, pharmaceuticals, nutrition, food additives, aquaculture, and pollution control such as wastewater treatment [11]. For this reason, algae are commonly chosen for green synthesis because their structures are a rich source of biologically active compounds like phycocyanin and phycoerythrin, containing varying concentrations of carbohydrates, proteins, minerals, vitamins, fatty acids, antioxidants, and pigments. Nanoparticle synthesis depends on the amount of algae and the type of algae used. Nanoparticle characteristics, as well as process variables like pH, temperature, precursor concentration, and reaction time, are known to be influenced by the specific biological component used, such as the whole cell, extracted molecules, or the supernatant of the culture [12, 13, 14]. Common problems in nanoparticle synthesis include stabilization, crystal development, and particle aggregation. The ability to control the size and shape of metal nanoparticles obtained by green synthesis can only be possible by investigating the relevant biosynthesis mechanisms.

To date, several reports about the biosynthesis of silver nanoparticles thorough a diverse species of macro-and microalgae have been published [15,16] . Jayshree et al. [17] used the extract of economically important unicellular green alga *Chlorella vulgaris*, for the synthesis of silver nanoplates. During synthesis of Ag-NPs, chromatic changes in the reaction mixture act as a visual marker affirming the continuity of the process. Kannan et al. [18] observed an obvious change of brown to yellow colour after 48 h during reduction of $AgNO_3$ by the extract of *Codium capitatum* and a time-dependent increase in brown colour intensity at 422 nm. Other green algal species like *Chlorococcum humicola*, *Euglena gracilis*, *Caulerpa serrulata* etc. have been reported to synthesize Ag-NPs with variable shapes and applications [19, 20, 21]. Additionaly, limited research has been done on utilizing *Desmodesmus sp*. (*Scenedesmaceae*) to generate silver nanoparticles [22].

In most of the medicinal and industrial processes, silver has long been acknowledged as having inhibitory effects on microbes (Morones et al. 2005). Researchers who have successfully achieved green synthesis have simultaneously assesed the antibacterial and antifungal effects on a variety of microorganisms.

Present study developed an ideal, cost-effective and safe procedures for silver nanoparticles synthesis from fresh water green microalga *Desmodesmus protuberans.* The synthesized silver nanoparticles were characterized by UV-VIS spectroscopy and scanning electron microscopy (SEM) coupled with EDX (Energy Dispersive X-ray). Also, the antimicrobial effect on the human pathogen like, *Fusarium oxysporum, Aspergillus alterata, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* was investigated.

Materials and methods

Chemicals, Algae Culture and growth conditions

Silver nitrate ($AgNO_3$, Cat. No. 209139) was obtained from Sigma-Aldrich Co, St. Louis, MO, USA. The freshwater microalgae used in our study, *Desmodesmus protuberans AICB 141* was procured from the Collection of Algae and Cyanobacteria of the Institute of Biological Research, Cluj, Romania. The culture was grown on Bold's Basal Medium which is composed of (mg L^{-1}): $NaNO_3$, 250; K_2HPO_4,

100; KH_2PO_4; 150; $MgSO_4 \cdot 7H_2O$, 75; $CaCl_2$, 21.35; KOH, 31; 0.06; FE-EDTA, 0.05; 1mL; trace elements solution (g $L^{-}1$): H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$,0.08; MnO_3, 0.015, pH 7.0, at an ambient temperature of 28±2 °C. The microalgae were transferred to 250 mL Erlenmeyer flask and left to proliferate under sterile conditions.

Cultures were maintained under 3000 lx fluorescent light, 16:8 light–dark cycle, and 120 rpm for 15–20 days to be reproduced in suitable medium. When algae passed the log phase stage, cells were centrifuged at 1000 rpm and the biomass was obtained. All chemicals reagents were used as received, without any additional purification.

Biosynthesis of silver nanoparticles

Cell free extract was prepared from *Desmodesmus protuberans* for nanoparticle synthesis. Healthy microalgal cultures were harvested in the logarithmic phase by centrifugation at 5.000 rpm for 10 min (Heltich Universal 320R) at 4°C and washed 3 times with sterile distilled water. Pellets were dried at 80° C (Drying Oven Sanyo, Japan) to constant weight.

About 1 grame of algal power was added with 30 ml of double distilled water in 100 mL conical flask and boiled at 100 °C for 20 minutes. After cooling, the crude extract was centrifuged at 5.000 rpm for 10 min (Heltich Universal 320R) and stored at 4°C for exeprimental use.

Biosynthesis of AgNP was done by adding 10 mL of cell free extract in 90 mL of 1mM aqueous $AgNO_3$ (Sigma,St. Louis, MO) solution, pH 7 and incubated at room temperature for 24 hours under static condition. A color change of the solution was noted by visual inspection confirming the AgNP synthesis. As a control, fresh Bold's Basal Medium with addition of $AgNO_3$ was used. Dark conditions were provided by wrapping the flasks with aluminum foil.

This experiment was repeated twice and the obtained data (presence of absorbance pick) were consistent for the tested strain. Stability of the synthesized AgNPS was checked by exposing the samples to ambient conditions for several months.

Characterization of the Green-Synthesized AgNPs

UV–visible spectroscopy analysis

Biosynthesis of Ag-NPs was followed by the change of color of $AgNO_3$ solution. The bioreduction of $AgNO_3$ was confirmed by sampling the reaction mixture at regular intervals and the absorption maximum was scanned by UV–vis spectra, at the wavelength of 300–700 nm in Hanna HI839800 spectrophotometer.

For futher characterization studies the nanoparticles solution was centrifuged at 10.000 rpm for 15 minutes and the resulting suspension was washed in sterile double-distilled water to get free of any biological molecule present in algal extract (i.e, proteins/enzymes), which are not able to capping the silver nanopaticles.

SEM and EDX analysis

The size and exterior morphology of biosynthesized $AgNO_3$ particles were deliberated by SEM (Scanning Electron Microscopy) coupled with EDX (Energy Dispersive X-ray) method. The imagistic evaluation of nanoparticles was carried out with a Quanta 200 type SEM equipment (FEI, Netherlands), under the low-vacuum conditions, at „Dunarea de Jos" University of Galati, Romania. Prior the insertion into the microscope chamber, the samples were fixed on the metallic stub surface by their attachment on a carbon adhesive tape. The analyses were performed with increasing the samples' conductivity. Thus, the $AgNO_3$ nanoparticles were coated with a metallic layer of 7 nm as thickness by sputtering process, using a Sputter Coater system (SPI Supplies, USA). SEM micrographs were acquired at 2.000 and 5.000x100.000 magnifications, the most relevant images being analysed in this paper.

The microanalysis of biosynthesized $AgNO_3$ particles was performed by EDX spectroscopy method, having a sapphire detector connected to the SEM equipment. The electron accelerating voltage of 25 kV was enough to detect a secondary electron signal due to the surface-beam interaction, and to stimulate the specific atoms from the samples. EDX data of chemical components were processed by dedicated software and quantified using the ZAF matrix correction, where Z is the atomic number, A is the absorption, and F is the fluorescence. SEM-EDX results indicate a local character of this analysis, taking into consideration the scanned micro-area of about $100mm^2$ or μm^2.

Antimicrobial assay

The antimicrobial effects of synthesized AgNPs were examined against various types of microorganisms like: *Fusarium oxysporum, Aspergillus alterata, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans.* The antimicrobial activity of biosynthesized AgNPs was performed by agar well diffusion method. About 20 mL of sterile Nutrition broth agar media was poured into the sterile Petri plates. The solid medium was gently punctured with cork borer to make a well. Positive control, Cell free algal extract, silver nitrate, and silver nanoparticles were added into each well on the nutrition broth agar media at various concentrations (50 µl, 75 µl, and 100 µl) and incubated for 24–48 h at 37 °C. After incubation, the zone of inhibition was measured

Figure 1. Color intensity after the addition of algal cell-freee extract with 1 mM AgNO3, indicates formation of silver nanoparticles. a) initial color change, (b) 15 minutes, (c) 24 hrs.

and expressed as millimeter (mm) in diameter. The statistical analysis of standard error was calculated using triplicates of experiments (n=3).

Results and discussion

Visual examination and UV-Vis spectroscopy

The test organism *Desmodesmus protuberans* belonging to the class *Clorophyceae* is a water alga with an oval or shuttle-shaped body. The colonies most often have two or four cells arranged linearly and are occasionally unicelular. In the present study, when algal extract was exposed to silver ions, the reaction started within 15 minutes and the colour of the algal extract changed from bright green to reddish yellow, indicating biotransformation of Ag^{+} ion to Ag^{0}, compared with the control. Also, the color is changed into deep brown color after 24 hrs incubation time (Figure 1). The color change resulted from excitation from SPR (surface plasmon resonance) in the metal of surface [23]. Intensity of brown colour increased in direct proportion to the incubation period. Surface plasmon resonance (SPR) is the manifestation of a resonance effect due to the interaction of conduction electrons of metal nanoparticles with incident photons.

Also, after 24 hrs time incubation, there is no sifnificant color change, indicating the saturation of the reaction of silver nanoparticle formation.

UV-VIS spectroscopy is one of the important techiques to determine the formation and stability of metal nanoparticles in aqueous solution. The biosynthesized AgNPs were measured by UV–visible spectroscopy at different time intervals to study the change in light absorption and increase in intensity.

The absorption spectra of nanoparticles showed highly symmetric single band absorption with a definite peak at 453 nm at 15 minutes, and steadly increased in intensity at 24 hrs without any shift in the peak (Figure 2). Also, previous studies demonstrated that a usual silver nanoparticles SPR pattern is present in the range of 400-480 nm [23]. It was observed the band occurs at 420 nm and 450 nm for *Scenedesmus abudans* and *Spirulina sp*. [24].

SEM and EDX analysis

Scanning electron microscopy was carried out to analyze the synthesized nanoparticles for the morphology and their size. SEM image revealed spherical nanoparticles with high agglomeration with an average size ranging from 58 nm to 107 nm (Figure 3).

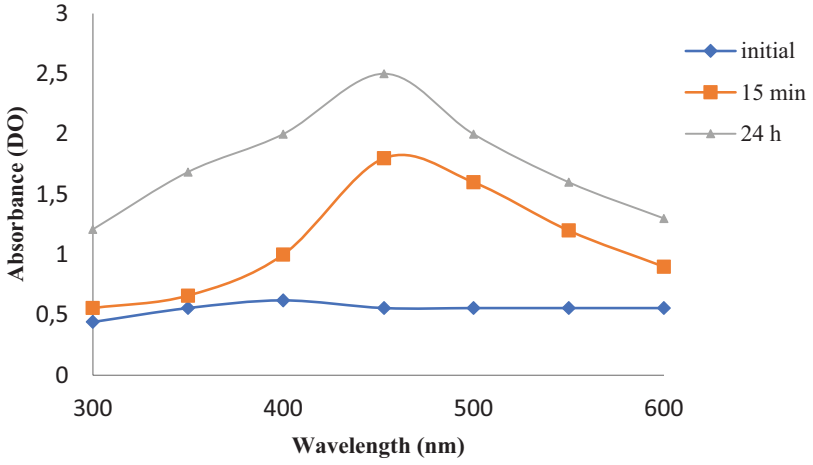

Figure 2. UV-Visible spectrum recorded the formation of nanoparticles in the reaction mixture of algal cell-free extract and $AgNO_3$ at different time intervals.

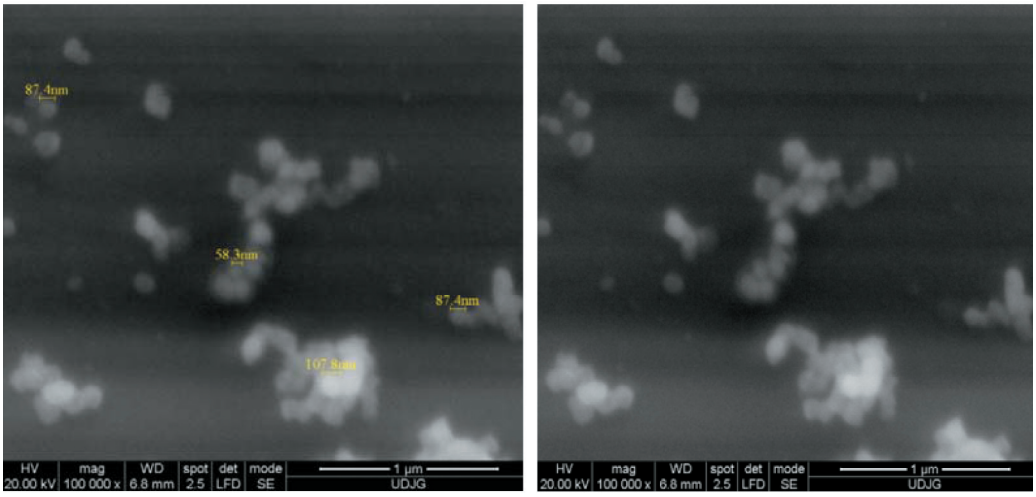

Figure 3. SEM image of silver nanoparticles synthesized from microalga Desmodesmus protuberans

The silver nanoparticles formed mostly spherical and cubical structure whith hight aggregation. Similarly, spherical, truncated and ellipsoidal nanoparticles biosynthesized by marine brown seaweed *Padina tetrastromatica* was reported [25]. Jayashree et al. [26] reported spherical nanoparticles synthesized by microlaga *Scenedesmus.*

Formation of elemental silver was confirmed throught EDX analysis. The optical absorption band peaks were found in the range of 3-4 keV. It is the best evidence to identify that formation of pure silver (Figure 4).

EDX spectrum clearly confirms the purity of the silver nanoprticles with the weight percentage of 76.33% along with the signals of O, C, K and Cl as the mixed components in the reaction medium. Similar results were reported by Manivasagan et al. [27].

Antimicrobial studies

Based on the recent reports of the World Health Organization, infectious diseases pose one of the greatest health challenges. The resistance of human pathogens to the commercially available antimicrobial agents and antibiotics has prompted the researchers to explore new innovative strategies for developing new antimicrobials. Also, antimicrobial activity of the silver ions, silver compounds has been thoroughly investigated, and surveys have revealed the remarkable antibacterial activity of SNPs. The antimicrobial activity of silver nanoparticles mainly depends on the size and shape of the nanoparticles.

The antimicrobial potential of the algal-synthesized silver nanoparticles showed the highest antimicrobial efficacy compared to pure cell algal extract against the various types of microorganisms, such as *Fusarium oxysporum, Aspergillus alterata, Staphylococcus aureus, Bacillus subtilis* and *Candida albicans* using agar well diffusion method. The inhibition zones formed against the pathogenic microorganisms at different concentrations ar presented in Table 1.

The gram-positive bacteria *Bacillus subtilis* showed the highest clear zone of inhibition of 29.30 mm. at a concentration of 100 µg/ml, and then *Staphylococcus aureus* showed an inhibition zone of 28.00 mm. The maximum zone formation of silver nanoparticles against *Candida albicans* was 20.30 mm, followed by *Fusarium oxysporm* and *Aspergillus alterata* with 18.30 mm and 18.10 mm respectively.

Also, the biosynthesized AgNPs from algal aqueous cell-free extract were moderately susceptible to fungal pathogen

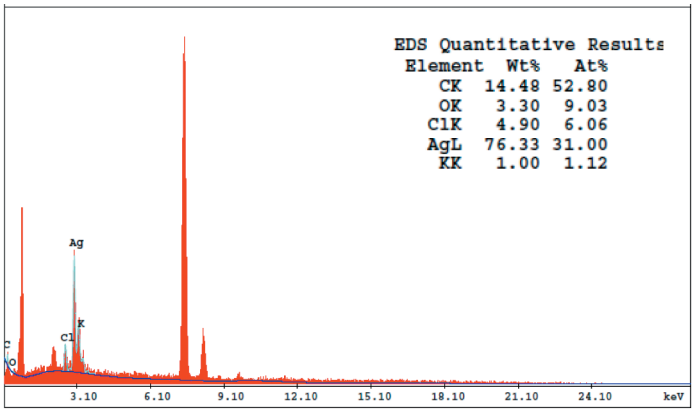

Figure 4. EDX spectra of silver nanoparticles synthesized from microalga *Desmodesmus protuberans*.

Table 1. The antimicrobial activities of biosynthesized AgNPS

Microorganisms strain	Inhibition zone (mm in diameter)			
	Concentrations	Silver nitrate	Cell free algal extract	AgNPS
Fusarium oxysporum	50µl	09.50	0	13.50
	75µl	11.30		16.00
	100µl	12.60		18.30
Cyclohexaminide (100µl)		17.10		
Aspergillus alterata	50µl	08.20	0	13.30
	75µl	11.10		15.20
	100µl	12.10		18.10
Staphylococcus aureus	50µl	12.50	0	20.20
	75µl	12.30		24.60
	100µl	14.00		28.00
Streptomycin (100µl)		27.60		
Bacillus subtilis	50µl	08.80	0	19.60
	75µl	10.50		25.60
	100µl	11.10		29.30
Streptomycin (100µl)		28.30		
Candida albicans	50µl	10.20	0	14.60
	75µl	11.00		18.00
	100µl	12.30		20.30

like, *Candida albicans*, suggesting that the increase in dose may tend to be efficiently toxic [28, 29]. However, the strains *Bacillus subtilis* and *Staphylococcus aureus* were found to be more susceptible bacteria against the synthesized AgNPs. A similar result was reported by Anita et al. [30], who observed a zone of inhibition in Ag nanoparticles generated using the aqueous algal extract of *Sargassum tenerrimum* in Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Thus, one can explore the medicinal properties of AgNPs synthesized from the cell free algal extract.

Conclusions

Nanoparticles have been employed because they are easy to process, environmentally benign, free of pollutants, nontoxic, low-cost, and have an excellent atom economy. Compared to other conventional procedures including physical and chemical methods, green synthesis methods offer a clean, non-toxic, and environmentally friendly approach to the synthesis of metal NPs. Similar to other biological species including fungi, yeast, and bacteria, microalgae significantly influence the creation of nanoparticles.

In this study, we have demonstrated a simple and reproducible way for the synthesis of silver nanoparticles using the fresh water microalgae *Desmodesmus protuberans.*

Characterization of synthesized silver nanoparticles was carried out by UV-vis spectroscopy and SEM equipped with EDX. Silver nanoparticles exhibited a single absorbance band at 453 nm at 15 minutes and steadily increased in intensity at 24 hrs without any shift in the peak. SEM image of silver nanoparticles synthesized from *Desmodesmus* sp. showed highly agglomerated spherical nanoparticles at different magnifications. AgNPs shows an average size ranging from 58-107nm. EDX spectrum clearly confirms the purity of the silver nanoparticles with the weight percentage of 76.33%.

The biogenic AgNPs showed significant antimicrobial effects against all studied species particularly, *Staphylococcus aureus* and *Bacillus subtilis.* Thus, biosynthesized AgNPs can find immense application in the field of biomedical and biotechnological applications.

Undoubtedly, it is necessary to conduct further research on the toxicity Of silver nanoparticles in relation to living organisms.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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