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Single cell level microalgal green synthesis of silver nanoparticles: Confocal microscopy and digital image analysis

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Abstract: Nanoparticles are attracting increasing attention due to their unusual and fascinating properties, which are strongly influenced by their size, morphology and structure. Among the developed nanoparticles, silver (Ag) nanoparticles are pertaining to have a wide range of application in the fields of physical, chemical and biological science. Physical and chemical methods are used to synthesize such nanomaterials, among the various known synthesis methods, biosynthesis of silver nanoparticles is preferred as it is environmentally safe, low cost and less toxic. In particular, the synthesis of nanoparticles in the cell can be achieved in a standard size and shape. In the present work, the coccoid green algae *Chodatodesmus mucronulatus* was used as a reducing agent for the synthesis of intracellular nanostructure silver particles (Ag-NPs). Algae are with autofluorescence characteristics. These properties are known to be due to chlorophyll pigments. In this context, a confocal laser scanning microscopy (CLSM) based method to assess to show that the amount of chlorophyll decreases at microalgae is reported.). During this process, changes in the amount of chlorophyll *a*, *b* and carotenoid of the *Chodatodesmus mucronulatus* were examined at 24 hours using UV-Vis spectrophotometer for 3 days. As a result, the amount of carotenoid, especially with the onset of the reaction, decreased markedly. After 72 hours of reaction, the amount of carotenoid decreased from 6,54 µg ml^{-1} to 0,00 µg / ml, chlorophyll *a* decreased 24,46 µg ml^{-1} to 0,06 µg ml^{-1},chlorophyll *b* decreased from 11,33 µg ml^{-1} to 4,15 µg /ml. This change (pigment amount in cells) was also observed with a confocal microscope every 24 hours. Using this technique, the effect of in-use concentrations of chlorophyll autofluorescence was defined. Determination of mean fluorescence intensity (MFI) per cell by collecting auto-fluorescence from single cells in x, y and z dimensions permitted evaluation at single-cell level. According to the results, there is a decrease in the amount of pigment in the cell. This suggests that the pigments may be capping agents and trigger nanoparticle synthesis.

Keywords: Microalgae, Chlorophyll, Confocal microscopy, Nanoparticle, Silver

1. Introduction

Bionanotechnology has emerged as the integration between biotechnology and nanotechnology for the synthesis of nanomaterials. Nanoparticles are the leading nanomaterials. It has attracted further attention of researchers, especially due to the adjustable hydrophilic-hydrophobic balance and target-specific localization properties of nanoparticles (Salem et al., 2016; Shankar et al., 2016). Especially, biologically synthesized nanoparticles have attracted great interest in biology and medicine because of their unique particle size and shape-dependent physical, chemical and biological properties. (Sun et al., 2008; Ko et al., 2007). Elemental silver and silver salts have the potential to kill pathogens and have also been used as a disinfectant in health care, even before the emergence of synthetically produced organic drugs such as penicillin. (Williams et al., 1999). In recent years, silver nanoparticles (AgNPs), synthesized as nanomaterials, have received great attention due to the beneficial properties of antimicrobial, antioxidant and antitumor activities that help in the development of pharmaceutical drugs. (Ramkumar et al., 2017). AgNPs have been successfully applied in the food industry, textile industry and agriculture (Gomaa, 2017). The synthesis of AgNPs is carried out by quite different methods. For example, chemical reduction, photoduction, thermal decomposition, ultrasound, microwave irradiation and electrochemical methods are used (Venugopal et al., 2017). However, in recent years, the green synthesis method has been used to prevent the toxicity of the process and increase the yield. Various organisms (bacteria, fungi, algae and plants) can be used for these processes. Stabilization and shape of nanoparticles are organic materials found in organisms. Among the biological systems, microalgae have very high potential for the production of nanoparticles by taking metal ions during the detoxification process. Compared to plants, microalgae can replicate their biomass with minimum requirements for sunlight, atmospheric

carbon dioxide and mineral salts. Microalgae performs synthesis of nanoparticles in less tim (Agarwal et al., 2019). At the same time, both live and dry algae biomass can be used in biosynthesis. However, there are deficiencies in the literature about which mechanism or organic material takes part in the synthesis of these nanoparticles. The aim of this study was to synthesize green intracellular (intracellular) AgNPs and characterize them with TEM and UV-Vis. In addition to describing the mechanism of production of macromolecules (such as pigments), which are responsible for the biosynthesis of nanoparticles, to prove the production mechanism of possible nanoparticles in biological model systems. Therefore, especially in our study, changes in the amount of pigment were observed by extraction. The pigments were also monitored by UV-Vis spectrophotometric analysis. In addition, with Confocal laser scanning microscopy (CLSM) using fluorescence characteristics, mean fluorescence intensity (MFI) analysis was followed and supported by quantitative results using Image J.

2. Materials and Method

2.1. Organism

The organism we used in our study is *Chodatodesmus mucronulatus* (Chodat) Hegewald, Bock & Krienitz which belongs to the group of Chlorophyta which is coccoid green algae. These algae were grown in BG-11 medium for 15-20 days under 3000 lux light at 28 ° C. When algae passed the log phase phase, the cells were centrifuged at 4000 rpm and biomass was obtained. All chemicals used in this study were analytical quality.

2.2. Intracellular AgNP synthesis

Silver nitrate was used for the stock solution. This solution was adjusted to 5 mM in BG-11 medium. The algal suspension was incubated at 28 ° C for 72 hours. Thus, the synthesis of intracellular AgNPs from living algae cells was completed. After the reaction, the biomass was separated by centrifugation and stored at -20 ° C until characterization.

2.3. Spectroscopic characterization of silver nanoparticles

The reduction of silver ions was monitored by measuring the absorbance scan at 190-1100 nm at selected time intervals by UV-Vis spectrophotometer. Color change was observed in silver nitrate solution incubated with *Chodatodesmus mucronulatus* cell-free extract. Silver nanoparticles dispersed in water were kept at room temperature.

2.4. Transmission Electron Microscopy (TEM) characterization of silver nanoparticles

The TEM sample of silver nanoparticles synthesized using coccoid green algae were prepared by placing drop of the reaction mixture over carbon coated copper grids and allowing the to evaporate. TEM micrographs of the sample were taken using JEOL JEM 1220 (Tokyo, Japan) operated at an accelerating voltage of 100 kV .

2.5. Spectroscopic characterization of pigment amount

The UV-Vis spectrum was also used for the quantity analysis of the pigments. Pigments were measured by methanol extraction every 24 hours (Jiang et al. 2015). After exposure to chlorophyll and carotenoid extraction $AgNO_3$, the cells were centrifuged at 4000 rpm for 20 minutes at 4000 rpm. 3 mL of methanol was added to the resulting biomass and mixed well. The tube was then incubated for 24 hours in the dark and at 45 ° C. The solution was then centrifuged at 9000 x g for 10 minutes and the absorbance of the supernatant was measured at a UV Visible spectrophotometer (Double Beam UV-Spectrophotometer in Spectrophotometer, China) at 470 nm, 652.4 nm and 665.2 nm wavelengths. Pigment amounts were calculated according to report of Yu (Yu et al., 2018).

2.6. Confocal microscopy analysis

Chodatodesmus mucronulatus was cultured in silver nitrate solution for 72 hours. The algae were then washed twice with ddH 2 O in the dark. The fluorescence scan was performed between 400 and 700 nm per 50 nm emission. Images were obtained by confocal laser scanning microscopy LSM 800 (ZEISS, Germany).

2.7. Digital Image Analysis

Digital image analysis was performed using Image 1.29 (http://rsb.info.nih.gov/ij). For mean fluorescence intensity (MFI) analysis, all images were obtained using the same CLSM settings. All data were obtained from xy confocal slices of the image to determine MFI in a two-dimensional image. Confocal segments were obtained using Zeiss confocal software for MFI. These data were used to determine the MFI per cell of Chodatodesmus mucronulatus using Image J.

3. Results

Various approaches have been applied to achieve a better synthesis of silver nanoparticles such as physical, chemical and biological methods. Recently, the synthesis of silver nanoparticles using the living thing directly (intracellular) has become more popular because it is environmentally friendly (Li et al., 2007; Song et al., 2009). The colorless solution of $AgNO_3$ turned dark brownish yellow, indicating the formation of silver nanoparticles. The formation of silver nanoparticles was monitored by UV-Vis absorption spectra at 190 to 1100 nm; where a dense band was clearly detected at 430 nm (Figure 1). This band was defined as the “surface plasmon resonance band ve and was connected to the excitation of free electrons in the nanoparticles. The shape of the band was symmetrical, suggesting a homogeneous distribution of spherically shaped nanoparticles (Travan et al., 2009).

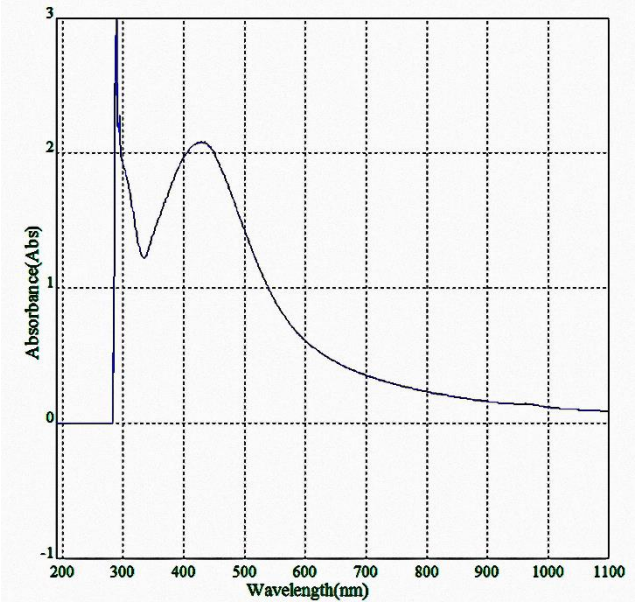

Figure 1. UV-Vis absorption spectrum of silver nanoparticles synthesized by treating 5mM aqueous $AgNO_3$ solution with *Chodatodesmus mucronulatus*

Silver nanoparticle size, morphology and distribution were analyzed by TEM. The nanoparticle size is about 20-40 nm Figure 2. A similar result Öztürk (2019), was obtained as a result of intracellular synthesis of *Desmodesmus* sp. cells. In Barwal et al.,2011 Intracellular silver nanoparticle was synthesized with *Chlamydomonas reinhardtii* intracellular nanoparticle of 5-35 nm.

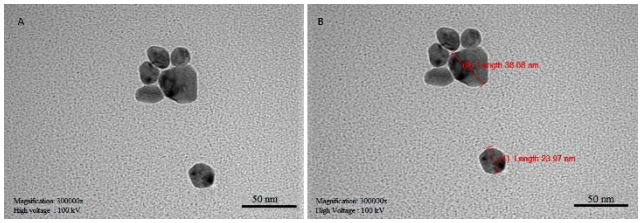

Figure 2. Morphological characterization of the silver nanoparticles. A, B TEM Image of *Chodatodesmus mucronulatus* cell mediated synthesized silver nanoparticles.

3.1. Spectroscopic characterization of pigment amount

Cells were observed for 72 hours by exposure to silver nitrate. Extraction was performed every 24 hours to measure the amount of pigments. The solutions obtained by extraction were read at 470 nm, 652.4 nm and 665.2 UV-Vis. After 72 hours of reaction, the amount of carotenoid decreased from 6,54 µg ml^{-1} to 0,00 µg / ml, chlorophyll *a* decreased 24,46 µg ml^{-1} to 0,06 µg ml^{-1},chlorophyll *b* decreased from 11,33 µg ml^{-1} to 4,15 µg /ml (Figure 3). According to these results, the most affected group was carotenoid. Carotenoids had been depleted as soon as they were exposed to silver nitrate. The next group was chlorophyll a. According to these results, the formation and formation of silver nanoparticles were first thought to be effective carotenoids.

3.2. Confocal microscopy analysis and Digital Image Analysis

Phototrophic organisms are automatic fluorescence relative to their pigments and allow non-invasive in situ analysis without external fluorescence labeling or staining (Neu and Lawrence, 1997). Recently, cellular fluorescence collection techniques from single-cell phototrophic organisms for phylogenetic identification have been reported (Roldan et al., 2004). Confocal laser scanning microscopy (CLSM) allows the quantitative assessment of the fluorescence properties of algal cells in situ, as well as the advantage that visualization and quantization are possible at the single-cell level (Neu and Lawrence, 1997). In our study, laser scanning confocal microscopy was used to make the pigment change of autofluorescent algae cells visible. So, during intracellular synthesis of silver nanoparticle from *Chodatodesmus mucronulatus*, pigment changes were observed by laser scanning confocal microscopy that *Chodatodesmus mucronulatus* pigments due to its autofluorescent property. The pigment change in this microscope is understood by reducing the fluorescence property. We performed a wide wavelength scan during the procedure. The initiation of silver nanoparticle synthesis within the cell and continued in parallel with pigment change was also visualized by confocal microscopy (Figure 4).

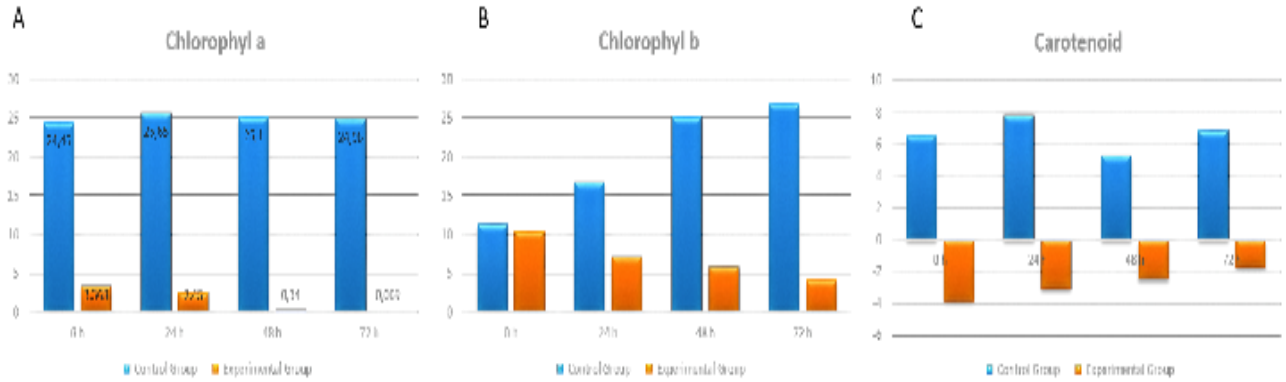

Figure 3. Amount of pigments (Chl-a, Chl-b, Carotenoids) during synthesis of intracellular silver nanoparticles in *Chodatodesmus mucronulatus*

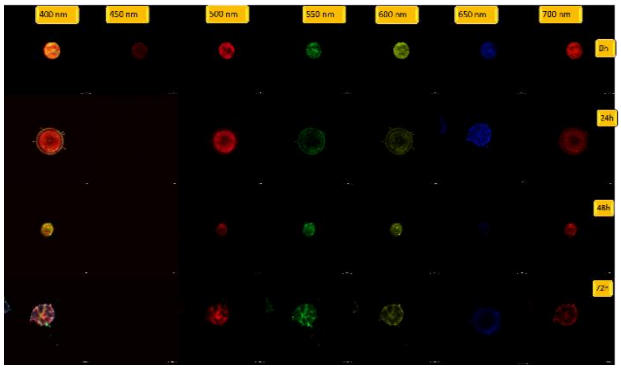

Figure 4. Laser scanning confocal microscopy image for pigment changes of *Chodatodesmus mucronulatus*

During nanoparticle formation, changes in autofluorescence radiation of living algae cells were visualized and digital image analysis was performed. In addition, in order to determine the chlorophyll autofluorescence, the regions of maximum intensity on the XY slides were observed as two-dimensional images and the mean fluorescence intensity (MFI) value was determined. Our results showed a decrease in fluorescence intensity caused by the synthesis of AgNPs. The mean MFI calculated from individual cells decreased over time and quantitative analysis is shown in Figure 5. The data clearly show that the decrease in chlorophyll fluorescence is dependent on the $AgNO_3$ exposure time.

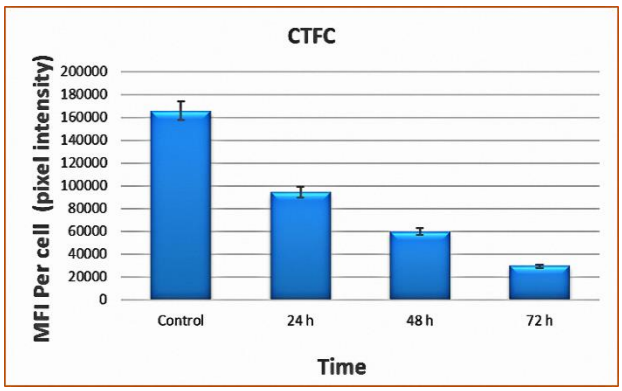

Figure 5. Mean fluorescence intensity after 0h, 24h, 48h, and 72h of contact time and subsequent transfer to fresh medium (error bars represent (±SD). *CTCF: Corrected total cell fluorescence

4. Discussion

It is an alternative method developed to synthesize metal nanoparticles by using green synthesis method which living organisms or using extracts from organisms. This method, unlike other methods (especially chemical method), is an environmentally friendly process. Algae, bacteria, fungi and plants can be used in this process and nanoparticles can be synthesized without the need for additional reducing and stabilizing agents. Among these groups of organisms, microalgae are thought to have very high potential for the production of nanoparticles due to their reduction properties of metals (Jena et al., 2014; Khatami et al., 2019). Synthesis of nanoparticles can be achieved in a short time with the use of algae. On the other hand, nanoparticle synthesis can be performed using both live and dry algae biomass (Dağlıoğlu and Öztürk, 2019; Öztürk, 2019).

It is generally accepted that UV-Vis spectroscopy can be used to examine the size and shape of nanoparticles in aqueous suspensions (Dubey et al., 2009). In this spectroscopic analysis, peaks around 420 indicate the formation of the Surface Plasmon Resonance (SPR) band. This band was clearly seen in our study and it was found to be compatible with the other studies reported. For example, Khatami and Pourseyedi (2015) are reported, silver nanoparticles were obtained by using *Phoenix dactylifera* (date palm) pit aqueous extract and SPR was formed at 428 nm wavelength. Jegadeeswaran et al., (2012) are reported, the absorption spectra of AgNPs obtained from Padina tetrastromatica were found to have absorbance peaks at 426 nm.

Phototrophic organisms are autofluorescent due to their pigments and can realize irradiate without further fluorescence labeling (Neu and Lawrence 1997). In this study, cell contents were determined using fluorescent emission with microalgae natural autofluorescent compounds. Therefore, microscopic fluorescence images of Chla are also useful for understanding the photochemical functions and thylacoid membrane structures of algae cells and chloroplasts (Harter et al. 2012; Sarafis 1998). CLSM is widely used in biomedical and bioscience research (Chandler and Volz 2004; Halbhuber and König 2003). CLSM allows rapid and multiple parametric analyzes of microalgal chlorophyll autofluorescence, and MFI analysis supports quantitative results using Image J (Nancharaiah et al. 2007). Possible participation of various macromolecules in Barwal et al., (2011) study the reduction of silver ion to silver nanoparticles has not yet been reported. The identification of macromolecules responsible for the biosynthesis of SNPs reported that it would describe the production mechanism of possible SNPs in biological model systems. Based on this idea, the mechanism of action during the synthesis of silver nanoparticles in microalgae was thought to be pigments and the results of this study were compared with UV-Vis and Confocal Microscopy and it was found to support this idea. *Desmodesmus* sp. algae were used in a similar study and similar results were reported (Dağlıoğlu and Öztürk, 2019). With the green synthesis method, the effect of macromolecules which are effective during the synthesis of silver nanoparticles should be investigated in more depth.

5. Conclusions

In conclusion, this study describes the intracellular synthesis of silver nanoparticles using microalgae. The formation of silver nanoparticles was confirmed by UV-spectroscopy and TEM. Spherical shape was observed using TEM. The resulting nanoparticles were very small in size (20 to 40 nm). The autofluorescence properties of intracellular silver nanoparticles were monitored by laser scanning confocal microscopy and it was observed that the resulting nanoparticles had a significant effect of pigments as reducing agents.

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Authors' contributions: All authors studied each analysis in this research paper. B.Y.Ö and D.Ö did the literature review and the D.Ö made the arrangements of the figure. B.Y.Ö wrote the entire article in English.

Conflict of interest disclosure:

Authors declare that there is no conflict of interest.

References

Agarwal P, Gupta R, Agarwal N 2019. Advances in Synthesis and Applications of Microalgal Nanoparticles for Wastewater Treatment. J Nanotechnol, 2019:1-9.

Barwal I, Ranjan P, Kateriya S, Yadav SC 2011. Cellular oxido-reductive proteins of Chlamydomonas reinhardtii control the biosynthesis of silver nanoparticles. J nanobiotechnology, 9(1): 56.

Chandler GT, Volz DC 2004. Semiquantitative confocal laser scanning microscopy applied to marine invertebrate ecotoxicology. Mar Biotechnol, 6:128–137.

Dağlıoğlu Y, Öztürk BY 2019. A novel intracellular synthesis of silver nanoparticles using *Desmodesmus* sp.(Scenedesmaceae): different methods of pigment change. Rend. Lincei Sci. Fis. Nat., 1-11.

Dubey M, Bhadauria S, Kushwah B 2009. Green synthesis of nanosilver particles from extract of *Eucalyptus hybrida* (safeda) leaf. Dig J Nanomater Biostruct, 4:537–543.

Gomaa EZ 2017. Antimicrobial, antioxidant and antitumor activities of silver nanoparticles synthesized by Allium cepa extract: a green approach. J Genet Eng Biotechnol, 15(1): 49-57.

Halbhuber K-J, König K 2003. Modern laser scanning microscopy in biology, biotechnology and medicine. Ann Anat Anat Anz, 185:1–20.

Harter K, Meixner AJ, Schleifenbaum F 2012. Spectro-microscopy of living plant cells. Mol Plant, 5:14–26.

Jegadeeswaran P, Shivaraj R, Venckatesh R 2012. Green synthesis of silver nanoparticles from extract of *Padina tetrastromatica* leaf. Dig J Nanomater Bios, 7(3): 991-998.

Jena J, Pradhan N, Nayak RR, Dash BP, Sukla LB, Panda PK, Mishra BK 2014. Microalga Scenedesmus sp.: a potential low-cost green machine for silver nanoparticle synthesis. J Microbiol Biotechnol, 24:522–533.

Khatami M, Pourseyedi S 2015. *Phoenix dactylifera* (date palm) pit aqueous extract mediated novel route for synthesis high stable silver nanoparticles with high antifungal and antibacterial activity. IET Nanobiotechnol., 9(4): 184-190.

Khatami M, Varma RS, Heydari M, Peydayesh M, Sedighi A, Agha Askari H, Rohani M, Baniasadi M, Arkia S, Seyedi F, Khatami, S. 2019. Copper Oxide Nanoparticles Greener Synthesis Using Tea and its Antifungal Efficiency on *Fusarium solani*. Geomicrobiol. J., 1-5.

Ko SH, Park I, Pan H, Grigoropoulos CP, Pisano AP, Luscombe CK, Frechet JMJ 2007. Direct nanoimprinting of metal nanoparticles for nanoscale electronics fabrication. Nano Lett, 7: 1869-1877.

Li S, Shen Y, Xie A, Yu X, Qiu L, Zhang L, Zhang Q 2007. Green synthesis of silver nanoparticles using *Capsicum annuum* L. extract. Green Chem., 9: 852-858.

Nancharaiah YV, Rajadurai M, Venugopalan VP (2007) Single cell level microalgal ecotoxicity assessment by confocal microscopy and digital image analysis. Environ Sci Technol, 41:2617–2621.

Neu TR, Lawrence JR 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. FEMS Microbiol Ecol, 24: 11-25.

Öztürk BY 2019. Intracellular and extracellular green synthesis of silver nanoparticles using Desmodesmus sp.: their Antibacterial and antifungal effects. Caryologia. International Journal of Cytology, Cytosystematics and Cytogenetics, 72(1): 29-43.

Ramkumar VS, Pugazhendhi A, Gopalakrishnan K, Sivagurunathan P, Saratale GD, Dung TNB, Kannapiran E 2017. Biofabrication and characterization of silver nanoparticles using aqueous extract of seaweed *Enteromorpha compressa* and its biomedical properties. Biotechnol Rep, 14: 1-7.

Roldan M, Thomas F, Castel S, Quesada A, Hernandez- Marine M 2004. Noninvasive pigment identification in single cells from living phototrophic biofilms by confocal imaging spectrofluorometry. Appl. Environ. Microbiol., 70: 3745-3750.

Salem JK, El-Nahhal IM, Najri BA, Hammad TM, Kodeh F 2016. Effect of anionic surfactants on the surface plasmon resonance band of silver nanoparticles: determination of critical micelle concentration. J. Mol. Liq., 223: 771-774.

Sarafis V 1998. Chloroplasts: a structural approach. J Plant Physiol, 152:248–264.

Shankar PD, Shobana S, Karuppusamy I, Pugazhendhi A, Ramkumar VS, Arvindnarayan S, Kumar G 2016. A review on the biosynthesis of metallic nanoparticles (gold and silver) using bio-components of microalgae: Formation mechanism and applications. Enzyme Microb. Technol., 95: 28-44.

Song KC, Lee SM, Park TS, Lee BS 2009. Preparation of colloidal silver nanoparticles by chemical reduction method. Korean J Chem Eng, 26: 153-155.

Sun C, Lee JSH, Zhang MQ 2008. Magnetic nanoparticles in MR imaging and drug delivery. Adv Drug Delivery Rev., 60: 1252-1265.

Travan A, Pelillo C, Donati I, Marsich E, Benincasa M, Scarpa T, Semeraro S, Turco G, Gennaro R, Paoletti S 2009. Non-cytotoxic silver nanoparticle-polysaccharide nanocomposites with antimicrobial activity. Biomacromolecules, 10: 1429-1435.

Venugopal K, Rather HA, Rajagopal K, Shanthi MP, Sheriff K, Illiyas M, RathEr RA, Manikandan E, Uvarajan S, Bhaskar M, Maaza M 2017. Synthesis of silver nanoparticles (Ag NPs) for anticancer activities (MCF 7 breast and A549 lung cell lines) of the crude extract of *Syzygium aromaticum*. J. Photochem. Photobiol., 167: 282-289.

Williams, DH, Bardsley, B 1999. The vancomycin group of antibiotics and the fight against resistant bacteria. Angew Chem Int Ed, 38(9): 1172-1193.

Yu Z, Pei H, Jiang L, Hou Q, Nie C, Zhang L 2018. Phytohormone addition coupled with nitrogen depletion almost tripled the lipid productivities in two algae. Biores Technol, 247:904–914.