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ORIGINAL RESEARCH PAPER

PRODUCTION OF SILVER NANOPARTICLE FROM *CHLORELLA VULGARIS* AND *DUNALIELLA SALINA*

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Abstract: Green microalgae have advantages as they are easily available, grow rapidly and produce various secondary metabolites including carotenoids, fatty acids, proteins and lipids. Synthesis of nanosilver microalgae *C. vulgaris* and *D. salina* offers environmental antimicrobial and biomedical agent. The objective of this study was to produce silver nanoparticle (SNP) microalgae using *C. vulgaris* and *D. salina* as an eco-friendly antimicrobial agent. This research method was conducted by synthesizing silver nanoparticle microalgae using *C. vulgaris* and *D. salina* along with characterization under UV–visible spectroscopy, transmission electron microscopy (TEM), and scanning electron microscope (SEM) and Energy-dispersive X-ray spectroscopy (EDX). The results showed that SNP of *C. vulgaris* and *D. salina* microalgae could be produced using agitation treatments. The synthesized AgNPs *C. vulgaris* and *D. salina* exhibited maximum absorption at 398 nm, and EDX analysis had determined that the abundance of chemical elements presented in the sample is carbon (53 %) and silver (32 %). TEM analysis revealed that they had spherical form with a size of 9.3 nm. The spot of EDX analysis shows the presence of silver atoms in both microalgae. SEM analysis shows a spherical shape with an average size of 50 nm with several silver particles in the cell. These results indicate that the formation of SNP using *C. vulgaris* and *D. salina* microalgae has been successfully obtained under agitation treatment.

Keywords: *EDX, microalgae, SEM, SNP, TEM*

INTRODUCTION

Bionanotechnology dealing with metal nanoparticles and living organisms has increased due to its wide applications ranges in almost every field of science and technology such as materials and manufacturing, nanoelectronics, information technology, medicine and health care, energy, biotechnology, food storage, household products, disinfectants, biomonitoring and environmental remediation [1 – 3]. Eco-friendly nanoparticles have advantages especially in compatibility with pharmaceuticals over physical, chemical and microbial synthesis. High cost, inefficient maintenance, contamination of toxic chemicals were leading to several effects when silver nanoparticles are being applied in medical and pharmaceutical applications [2, 4]. The development of bionanotechnology in microalgae has shown that integration of microalgae with nano silver to produce SNP had spurred a great interest in the potential of microalgae as an antifungal, antimicrobial, and anticancer accomplishing with good electrical conductivity, chemical stability, and catalytic activity [2, 4 – 12]. This development also makes nano silver microalgae become an acceptable product because it is environmentally safe. *Chlorella* is widely used as a health food and feed supplement, as well as in the pharmaceutical and cosmetics industries. *C. vulgaris* contains 17 amino acids from both essential amino acids and non-essential amino acids. *C. vulgaris* also contains 34 lipid acids, Omega 3, Omega 6, Omega 9, AA, DHA and PUFA. Its carotenoid content is α-carotene as much as 0.24 $mg \cdot g^{-1}$ and β-carotene as much as 0.86 $mg \cdot g^{-1}$. *D. salina* is a microalgae which produces high 9-cis-β-carotene pigment which reaches more than 100 $g \cdot kg^{-1}$ for each gram of dry cell weight. *Dunaliella* produces antioxidant β-carotene carotenoids which can prevent vision loss [13 – 14]. Although the synthesis and characterization of silver nanoparticles in microalgae *C. vulgaris* and *D. salina* has been carried out [15 – 16], there have been no reports of synthesis of SNP in *C. vulgaris* and *D. salina* in higher SNP concentration using agitation treatments, their effect on cells and how much SNP concentration is in microalgae cells.

MATERIALS AND METHODS

Microalgae material

C. vulgaris and *D. salina* microalgae were obtained from Brackishwater Aquaculture Development Centre (BBPBAP) in Jepara Indonesia. They are held in seawater tanks, recirculated and aerated, with temperatures set at 25 °C to 28 °C and salinity at 30 ‰ to 32 ‰. The tanks are cleaned daily. Microalgae are cultivated using seawater enriched with Walne media.

Microalgae Media

Walne media for microalgae growth and cultivation consisted of H_3BO_3 3.36 $g \cdot L^{-1}$, $NaNO_3$ 10 $g \cdot L^{-1}$, $FeCl_3$ 0.15 $g \cdot L^{-1}$, $MnCl_2 \cdot 4H_2O$ 0.36 $g \cdot L^{-1}$, Na_2EDTA 45 $mg \cdot L^{-1}$, NaH_2PO_4 20 $g \cdot L^{-1}$, trace metal solution 1 $mL \cdot L^{-1}$, and distilled water. The trace metal solution consists of H_3BO_3 2.86 $g \cdot L^{-1}$; $MnCl_2.4H_2O$ 1.81 $g \cdot L^{-1}$; $ZnSO_4.7H_2O$ 0.222 $g \cdot L^{-1}$; $NaMoO_4.5H_2O$ 0.39 $g \cdot L^{-1}$; $CuSO_4 \cdot 5H_2O$ 0.079 $g \cdot L^{-1}$; and $Co(NO_3)_2.6H_2O$

0.0494 $g \cdot L^{-1}$; *p*H solution is 6.8. The ingredients are dissolved in 200 mL of distilled water. The solution was boiled for 10 min while adjusting the *p*H to 7.6 with HCl or NaOH, filtered and bring to 1 L. Sterilization was done by autoclaving at 15 $lb \cdot in^{-2}$ (103 kPa and 120 ^{o}C). The medium was using by adding 0.1 mL solution to every 10 mL of seawater [17 – 18]. All the reagents used are pure analytics.

Preparation of 2 mM $AgNO_3$ solution

The solution of 2 mM $AgNO_3$ is prepared by dissolving 0.338 g $AgNO_3$ in 1000 mL double distilled water and stored in Amber colored bottle to avoid auto oxidation of silver.

Biosynthesis of microalgae SNP

100 mL microalgae extract was added to 250 mL $AgNO_3$ 2 mM solution, and then the mixing solution was stirred for 6 hours using a magnetic stirrer to carry out agitation treatment on the sample. Color change indicates the formation of SNP.

UV –Visible spectra analysis

SNP synthesis results were analyzed by taking 3 ml aliquot samples then the absorbance spectrum was measured using a UV–Visible spectrophotometer at a wavelength of 300-700 nm using a Spectroquant Pharo 300 Spectrophotometer.

SEM-EDX analysis of SNP

SEM-EDX (Energy-dispersive X-ray spectroscopy) analysis was performed using SEM Jeol JSM 6510 LA model. Dry material samples from an aqueous solution of SNP were prepared by centrifugation at 8,000 rpm for 5 minutes. Pellets are dried. SEM micrographs have been produced with magnifications 3000, 5000, 10000 and 20000 x (diameter). SEM is equipped with capability for X-ray analysis. Thus topographic, crystallographic, and compositional information can be obtained rapidly, efficiently, and simultaneously from the same area. The X-ray excitation technique is used for element analysis or chemical characterization of samples.

RESULTS AND DISCUSSION

UV –Visible spectra analysis

Marine microalgae such as *C. vulgaris* and *D. salina* contain a number of biodynamic compounds of therapeutic value. This compound provides valuable ideas for the development of new drugs against microbial infections and contamination [19]. The present study was conducted to use the marine microalgae, *C. vulgaris* and *D. salina* for the synthesis of nanosilver microalgae. Microalgae extract may act as a reducing and capping agent in the biosynthesis of SNP. Reduction of silver ions into nanosilver microalgae during exposure to extracts of *C. vulgaris* and *D. salina* can be followed by

changes in color. Adding silver nitrate solution to microalgae solution will convert the reaction mixture to white and brown (Figure1), due to excitation of plasma vibrations surface, indicating the formation of SNP.

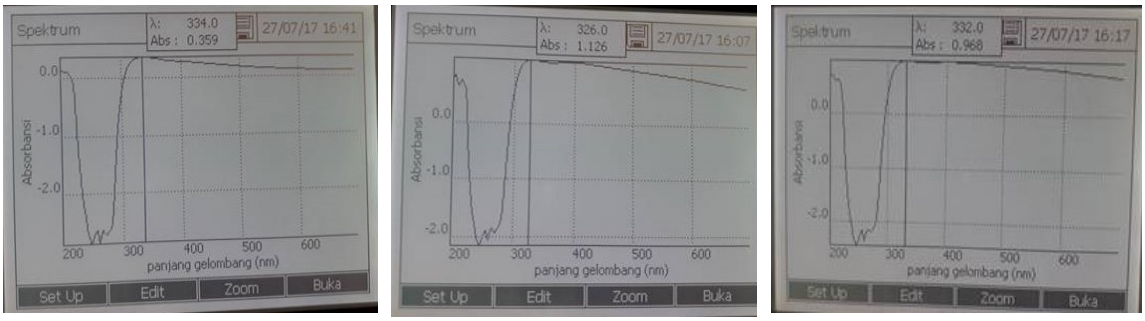
Figure 1. *Color of D. salina (left) and C. vulgaris (right) SNP*

Characterization of nanosilver microalgae cried out using UV-Visible spectroscopy has proven to be a very useful technique for the analysis of these nanoparticles. The peak was observed at 343 nm for *C. vulgaris* as control, and 312-398 nm for SNP of *C. vulgaris*. This spot correlates with plasmon excitation of nanosilver microalgae as illustrated in Figure 2. Other researchers were in agreement with these results, in finding that the absorption of *C. vulgaris* SNP is about 400 nm [15]. However, results with *D. salina* exhibited peak at 334 nm as a control and 322-326 nm for *D. salina* SNP after being monitored using UV-Vis spectroscopy, while other researchers had at 430 nm (Figure 3). These results also show differences in UV-Vis absorbance values between *C. vulgaris* and *D. salina*.

a b c

Figure 2. *UV-Visible absorption spectrum of microalgae: (a) C. vulgaris, (b) SNP of C. vulgaris with agitation, (c) SNP of C. vulgaris without agitation*

Research based on the absorbance and wavelength values also shows that the synthesis of SNP with agitation provides stability of microalgae SNP in accelerating the formation of silver nanoparticles. In *C. vulgaris*, absorbance values tend to increases with increasing contact reaction time while values in *D. salina* tend to be the same.



a b c

Figure 3. *UV-Visible absorption spectrum of microalgae: (a) D. salina, (b) SNP of D. salina with agitation, (c) SNP of D. salina without agotation*

The result of this study were also supported by changes in the color of the solution in SNP in *D. salina* which tended to be light brown while the solution of *C. vulgaris* was dark brown. As the microalgae suspension is mixed with the aqueous solution of the silver ion complex, it changes from green to light or dark brown color. This is due to the excitation of plasma vibrations surface, which indicates the formation of nanosilver microalgae. UV-Visible Spectrograph of nanosilver microalgae has been recorded as a function of time.

Studies have indicated that biomolecules such as proteins, carbohydrates, lipids and phenols not only play an important role in reducing ions to nano size, but also in capping of nanoparticles. Biomolecules found in extracts such as enzymes, vitamins, proteins, amino acids, and polysaccharides play a vital role in reducing Ag^+ ions. Strong absorption due to collective oscillation of electrons conduction show the formation of *C. vulgaris* and *D. salina* nanosilver microalgae which are monitored by UV-Vis spectroscopy after appropriate excitation by suitable radiation. This phenomenon is confirmed as local surface plasmon resonance, which is highly dependent on the size and shape of the nanoparticles.

SEM analysis

SEM analysis showed changes in the ultrastructure of cellular morphology of *C. vulgaris* and *D. salina* cells after 160 hours of exposure with AgNPs which also accomplished by differences in surface topography when electron beam sweeps the specimen. As shown in Figure 4, the morphology of *C. vulgaris* and *D. salina* cell with silver addition retains a smooth exterior, a round and spherical shape with size 9.3 µm for *C. vulgaris* while *D. salina* has size 10.5 µm. It also shows that agitation treatment do not cause a greater effect on cell structure and morphology by intense contact among AgNP particles and cell surfaces. The AgNP microalgae also revealed spherical and cuboidal nanoparticles. This result was in contrast with another researcher which was proven that nanoparticles can cause change in morphology and dimensions of green algae *Chlamydomonas reinhardtii* and *D. salina* [2, 15].

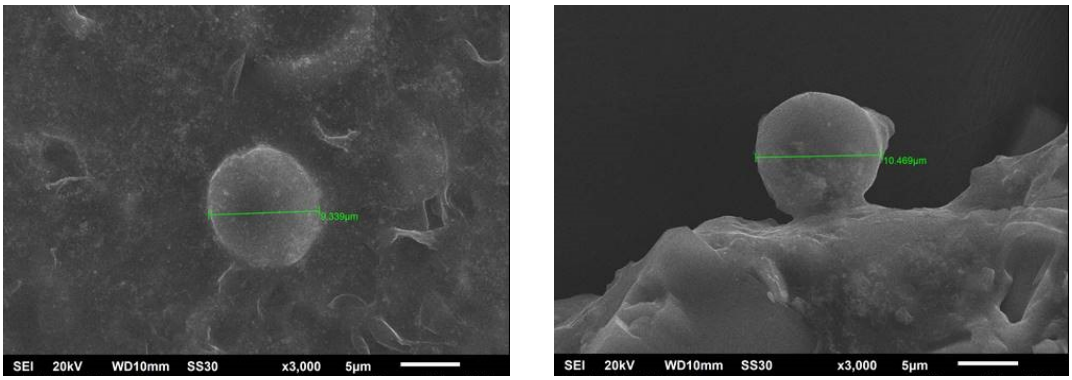


Figure 4. *SEM image of microalgae SNP formed by C. vulgaris and D. salina*

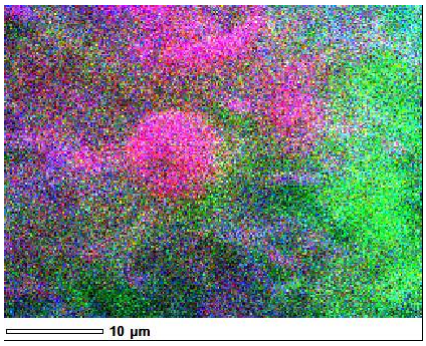
EDX analysis

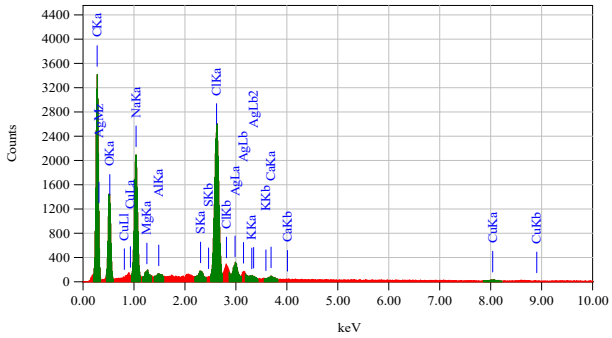
Characterization of chemical composition and the location of AgNPs on cell surface was carried out using a combination of SEM and X-ray (EDX). EDX analysis was performed to confirm formation of *C. vulgaris* SNP. Figure 5 shows the evidence of EDX analysis in the spot profile mode for each treatment. The n and location of AgNPs on the cell surface [8].

The ZAF factor for *C. vulgaris* has calculated that carbon and silver are the dominant composition of the sample. Several other chemical compounds are also found in $AgNO_3$ solution in very small quantities consisting of Chromium (Cr), Iron (Fe), Copper (Cu), Chloride (Cl) and Aluminium (Al). Although carbon, oxygen, sodium, kalium, magnesium and chloride are used as standard in samples but their concentration is low in the microalgae cell. The EDX analysis shows the higher concentration of Ag in surface cell indicating the formation process of microalgae nanoparticle of *C. vulgaris*. These results support the hypothesis that silver metal is attached to the cell wall of *C. vulgaris* (Figure 5).

The ZAF factor for *D. salina* has calculated that carbon, sodium and chloride are the dominant compositions of the sample as illustrated in Figure 6. Some other chemical compounds in $AgNO_3$ solution in very small quantities. *D. salina* was shown several dominant composition consisting of magnesium (Mg), Sulphur (S), and Potasium (K). Nevertheless, carbon, oxygen, sodium, kalium, magnesium and chloride which used as standard based showed high concentration in *D. salina* cell. The EDX analysis also showed low concentration of Ag indicating most of the silver was entering in the cell as indicated by dominant green color inside of the cell (Figure 6). These results shows the difference between *C. vulgaris* and *D. salina* which shows that silver particle enter more easily in *D. salina* compared to *C. vulgaris*.

The chemical composition of $AgNO_3$ is illustrated with the EDX analysis on Figure 5 and Figure 6. The cell surface of *C. vulgaris* containing 31.9 % Ag concentration was indicated by the peak appearance in XRD images which showed by green color. $AgNO_3$ concentration on the surface of *D. salina* cell is about 2.13 % as illustrated by EDX analysis in Figure 5.



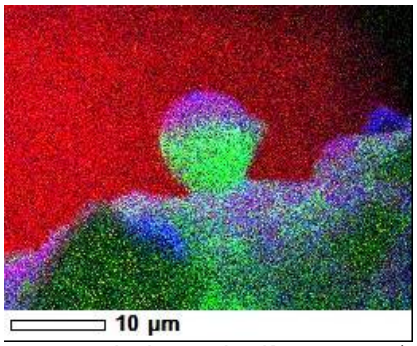


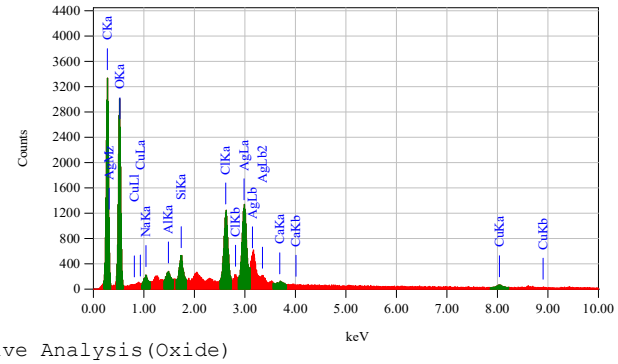
ZAF Method Standardless Quantitative Analysis(Oxide)
Fitting Coefficient : 0.0269
Total Oxide : 24.0

Element	(keV)	Mass%	Sigma	Mol%	Compound	Mass%	Cation	K
C K	0.277	81.46	0.36	95.10	C	81.46	0.00	61.0550
O		2.93						
Na K	1.041	5.81	0.09	1.77	Na2O	7.84	33.13	12.5416
Mg K	1.253	0.23	0.03	0.13	MgO	0.38	1.23	0.3755
Al K	1.486	0.08	0.02	0.02	Al2O3	0.15	0.37	0.1494
S K	2.307	0.25	0.03	0.11	SO3	0.63	1.03	0.6604
Cl K	2.621	6.54	0.06	2.59	Cl	6.54	0.00	19.1020
K K	3.312	0.12	0.02	0.02	K2O	0.14	0.39	0.2891
Ca K	3.690	0.13	0.02	0.04	CaO	0.18	0.42	0.3257
Cu K	8.040	0.32	0.05	0.07	CuO	0.40	0.65	0.6935
Ag L	2.983	2.13	0.06	0.14	Ag2O	2.29	2.59	4.8079
Total		100.00		100.00		100.00	39.81	

Figure 5. *EDX analysis of SNP formed by C. vulgaris*

The X-rays pattern of *C. vulgaris* and *D. salina* on the XRD spectrum shows the pure crystal structure of silver on both microalgae. Energy Dispersive Analysis of X-ray (EDAX) gives the qualitative and quantitative status of elements that may be involved in the formation of AgNPs.





ZAF Method Standardless Quantitative Analysis(Oxide)
Fitting Coefficient : 0.0371
Total Oxide : 24.0

Element	(keV)	Mass%	Sigma	Mol%	Compound	Mass%	Cation	K
C K	0.277	52.96	0.07	91.12	C	52.96	0.00	28.1921
O		3.79						
Al K	1.486	0.71	0.05	0.27	Al2O3	1.35	2.68	0.8382
Cl K	2.621	8.07	0.04	4.71	Cl	8.07	0.00	16.6706
Cr K	5.411	0.45	0.05	0.09	Cr2O3	0.66	0.88	0.7148
Fe K	6.398	1.37	0.05	0.51	FeO	1.76	2.48	2.2236
Cu K	8.040	0.75	0.07	0.25	CuO	0.94	1.20	1.2061
Ag L	2.983	31.89	0.15	3.06	Ag2O	34.26	29.95	50.1545
Total		100.00		100.00		100.00	37.19	

Figure 6. *EDX analysis of SNP formed by D. salina*

TEM analysis

TEM analysis has shown detailed information of the three-dimensional structure of microalgae SNP from planar serial sections for both microalgae *D. salina* and *C. vulgaris*. Figure 7 and Figure 8 have shown the development of microalgae cell image through a series of focal images formed in scanning optical confocal microscopes. It also shows that in agitation treatment, silver crystal that enter the cell do not cause the disruption to the cell wall or making lysis of the cell.

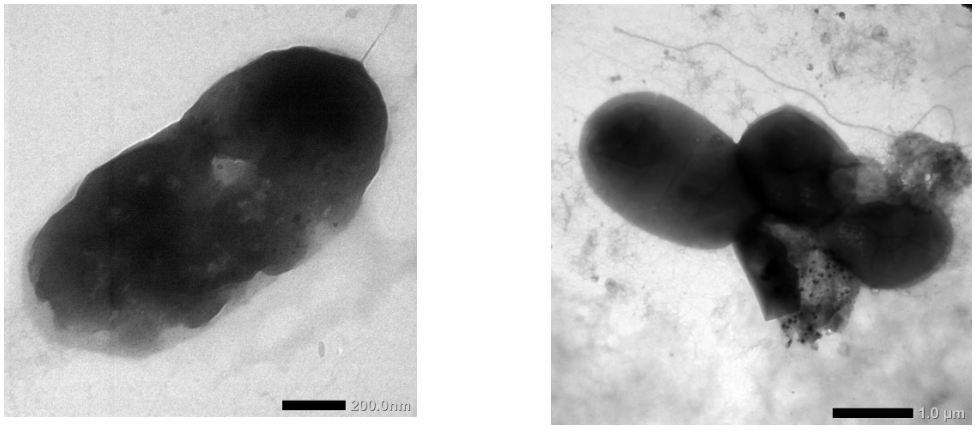


Figure 7. *TEM analysis of microalgae SNP of D. salina without agitation (left) and with agitation (right)*

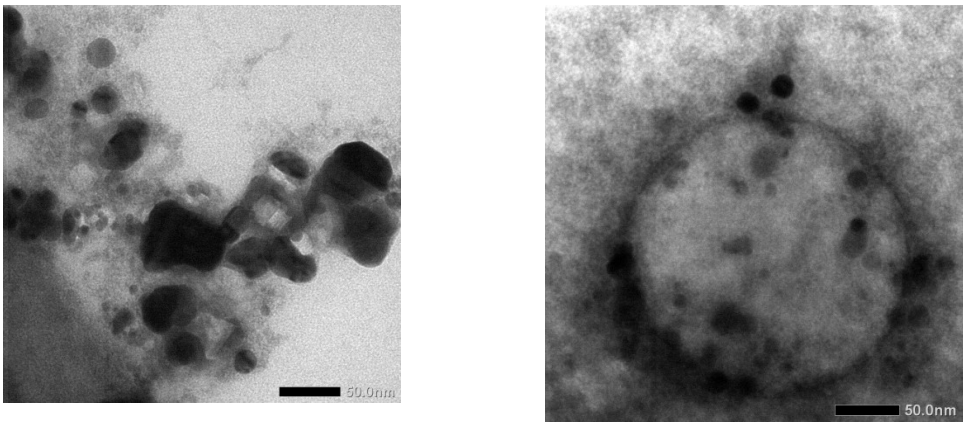


Figure 8. *TEM analysis of microalgae SNP of C. vulgaris without agitation (left) and with agitation (right)*

These results also illustrate that the silver metals is attached to the cell wall. Without agitation treatment silver particle will accumulate on the cell surface and cause cell lysis. The result of the study also showed the characterization of particle structure and confirmed the presence of the nanoparticles in cells. Silver ions released from AgNPs can penetrate into the cell membranes that interact with compounds containing sulfur and phosphorus such as proteins and DNA [20].

The results also showed that production of microalgae SNP using *C. vulgaris* and *D. salina* could be carried out at a concentration of 2 mM of silver with agitation treatment.

The study was in contrast with other researcher in having cell lysis in 1 mM of silver concentration [15]. This study also showed that cells still retain the stability of their structures while containing high concentrations of silver in cells. These results indicate the research implications that agitation treatment supports cell stability in dispersing the silver material around cells. The novelty of the study shows that the accumulation of silver particles on the cell surface will cause lysis cell of microalgae, although this indication still need further exploration.

CONCLUSION

This study revealed that microalgae *C. vulgaris* and *D. salina* are good sources for the synthesis of silver nanoparticles with high silver concentration. The formation of silver nanoparticle has been confirmed by characterization using UV-Vis, SEM, EDX and TEM techniques. The microalgae silver nanoparticle formed are quite stable in solution. Agitation treatment acts as stabilizers of surface active molecules and cell structure for the synthesis of silver nanoparticles. This method is also fast and environmentally friendly.

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