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Nanosilver microalgae biosynthesis: cell appearance based on SEM and EDX methods

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Abstract. Microbial contamination has caused public health problems in the world population. This problem has spurred the development of methods to overcome and prevent microbial invasion. The extensive use of antibiotics has facilitated the continued emergence and spread of resistant organisms. Synthesized of silver nanoparticle (AgNPs) on microalgae *Chlorella pyrenoidosa* offer environmentally safe antimicrobial agent. The present study is focused on the biosynthesis of AgNPs using microalgae *C. pyrenoidosa*. The research methods was conducted by insertion of nanosilver particle into microalgae cells with and without agitation to speed up the process of formation nanosilver microalgae. The formation of microalgae SNP was analyzes by UV-Vis spectrophotometer, Scanning Electron Micrograph (SEM) and Energy-dispersive X-ray spectroscopy (EDX) methods. The research result showed that nanosilver microalgae biosynthesis using the agitation treatment was exhibited better performance in particle insertion and cell stability, comparing with no agitation treatment. However, synthesis of nanosilver microalgae tend to reduce the cell size.

1. Introduction

The development of bionanotechnology in microalgae has shown that integration of microalgae with nano silver to produce AgNPs had increased the potency of microalgae as antifungal, antimicrobial, and anticancer accomplishing with their advantages characters in electrical conductivity, stability, and activity of catalysis [1-9]. Natural nanoparticles have advantages especially in compatibility with pharmaceuticals over physical, chemical and microbial synthesis. High cost, inefficient treatment, contamination of toxic chemicals were leading to several effects when silver nanoparticles was us for medical and pharmaceutical purposes [5,10]. This organic silver nanoparticles also proven as an alternative way to develop new antimicrobial agents in overcoming the problem of resistance [3]. Moreover, *Chlorella* as one of a primary producer on aquatic environment is commonly us for natural supplement on pharmaceutical and cosmetics attempt [7-12]. This microalgae and its extracts have produced an enormous amount of interest for the pharmaceutical industry as a bioactive compounds with immense medicinal potential. Although synthesis and characterization of Silver nanoparticles on

microalgae *C. vulgaris* had been conducted [13], there are no reports concerning synthesis of AgNPs on *C. pyrenoidosa* in higher concentration of silver using agitation treatment, their effect to the cell and how much concentration of silver in cell of microalgae.

2. Materials and Methods

2.1. Microalgae material

C. pyrenoidosa microalgae were obtained from Brackishwater Aquaculture Development Centre (BBPBAP) on Jepara Indonesia. The microalgae were cultivated using sea water enriched with Walne media in Oceanography Laboratory on Diponegoro University.

2.2. Microalgae Media

The microalgae was grown and cultivated on Walne media. The media were dissolved in 200 mL of distilled water and bring to 1 L on the pH 7.6. The medium was using by adding 0.1 mL steril solution to each 10 mL of seawater [14,15].

2.3. Preparation of 1 mM $AgNO_3$ solution

The solution of 1 mM $AgNO_3$ was prepared by dissolving 0.169 mg $AgNO_3$ (169.87 g/mol) in 1000 ml distilled water and keep from auto oxidation of Silver.

2.4. Biosynthesis of microalgae Silver nanoparticles

The 100 mL microalgae extract was added to 250 mL $AgNO_3$ 2 mM solution. The half of reacting solutions were agitated for 6 hours with a stirrer at 120 rpm at room temperature, while the other was not. The colour change, UV-Vis absorption spectra, SEM and EDX performance indicate the formation of Silver nanoparticles.

2.5. Characterization of SNP microalgae

The optical properties of the microalgae silver nanoparticles and the optical density of microalgae *C. pyrenoidosa* cultures in solution supplemented with the particles were evaluated in 10 mm optical path length quartz cuvettes using a Spectroquant Pharo 300 UV–Vis spectrophotometer. Characterization of AgNPs was started by taking small aliquot of sample in to UV–Visible spectrophotometer absorption spectra at 200-600 nm using UV-Vis Spectrophotometer. The size and the morphology of the silver nanoparticles were observed by transmission electron microscopy (TEM). The sizing of the samples was measured on transmission electron micrographs using the software Image Tool for Windows (Version 2.0). Data analysis was conducted using the software Microcal Origin 6.0. The size and morphology of the microlgae AgNPs were examined by scanning electron microscopy (SEM) Jeol JSM 6510 LA model. Samples of the dray material of the silver nanoparticles (AgNPs) were done by centrifugation at 8,000 rpm for 5 min using Eppendorf microsentrifuge 5424. The SEM micrographs have been produced with magnifications 3000, 5000, 10000 and 20000 x (diameters). SEMs are equipped with x ray analytical capabilities to obtain topographic, cristallographic, and compositional information simultaneously from the same area. The EDX using X-ray excitation technique was used for analysis the element or chemical characterization.

3. Result and Discussion

3.1. UV-Visible spectra analysis

UV–Vis spectroscopy was performed to observe the formation of the microalgae silver nanoparticles. Confirmation of AgNPs formation in the aqueus solution of microalgae was monitored by UV-Vis absorption spectrum in the range of 200–600 nm.The plasmon band of *C. pyrenoidosa*-Ag colloid was observed at 400–411 nm (Figure 1) which is in agreement range with other experiment on different organisms [7]. Appropriate excitation by suitable radiation would made by nano-sized silver showed a strong absorption caused by the collective oscillation of the conduction electrons which was known as localized surface plasmon resonance. The fact that the surface plasmon absorption maximum was found

with the wavelength around 410 nm confirmed the nanoessence of the manufactured silver particles. This process was depent dominantly on the size and shape of the nanoparticles [7]. Some studies have indicated that nutrient in microalgae not only supported on the capping of the nanoparticles, but also decreased the ions into the nano size [16,17] . The addition of silver nitrate solution into microalgae solution was changed the reaction mixture into brown, caused by the excitation of the surface plasma vibrations, was one of the indicator of AgNPs formation.

Characterization of nanosilver microalgae were primarily performed by UV-Visible spectroscopy, which is proved to be a very useful technique for the analysis of these nanoparticles. The UV-Vis absorption spectra are known to be quite sensitive to the formation of nanosilver microalgae. Thus the presence of nanosilver microalgae characterized by using a UV-Vis spectrum showed that they presented a maximum absorption at 410-411 nm. A single broad peak was observed at 410 nm for *C. pyrenoidosa* as a control 410 nm for *C. pyrenoidosa* with agitation and 411 for *C. pyrenoidosa* without agitation. This peak was corresponds to plasmon excitation of the nanosilver microalgae as illustrated on Figure 1. Several investigators have observed absorption maxima of colloidal silver solution between 410 to 440 nm, which is assigned to surface plasmon of various metal nanoparticles [13,18,19].

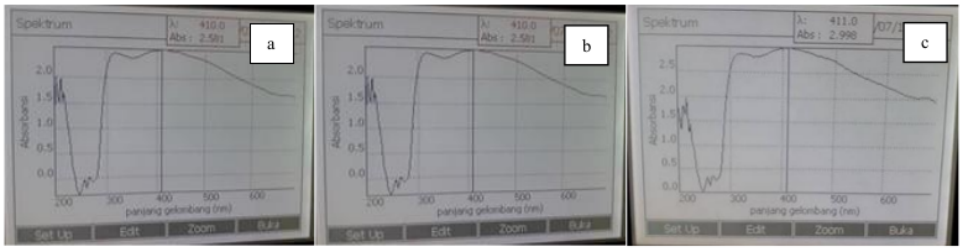


Figure 1. The spectrum of UV-Visible absorption on nanosilver microalgae : (a) *C. pyrenoidosa*, (b) nanosilver *C. pyrenoidosa* with agitation, (c) nanosilver *C. pyrenoidosa* without agitation

The results of the process of microalgae nanosilver formation the research based on the absorbance and wavelength values also show the synthesis of silver nanoparticles with agitation provide better stability comparing to the treatment without agitation. The agitation accelerates the process of forming silver nanoparticles. The absorbance value increases with the increasing contact reaction time. As the microalgae suspension was combined and homogenized with the aqueous solution of the silver ion complex it was changed from green to brown colour. This is due to the excitation of the surface plasma vibrations, which indicates the formation of the nanosilver microalgae. UV-Visible Spectrograph of nanosilver microalgae has been recorded as a function of time by using quartz cuvette with distilled water as the reference.

Formation of the nanosilver microalgae of *C. pyrenoidosa* monitored by UV–Vis spectroscopy showed a robust absorption due to the collective oscillation of the conduction electrons, after adequate excitation by sufficient radiation. This phenomenon is regarded as localized surface plasmon resonance, which is highly depend on the size and shape of the AgNPs.

3.2. SEM analysis

The SEM analysis showed morphological, cellular ultrastructural changes of *C. pyrenoidosa* cells after 160 hours of exposure with AgNPs which also accomplished by the differences in surface topography as the electron beam sweeps across the specimen. As showed in Figure 2-4, the morphology of *C. pyrenoidosa* cell without silver addition as a control unit maintained a smooth exterior, round and spherical shape with size 2.40-7.55 µm.

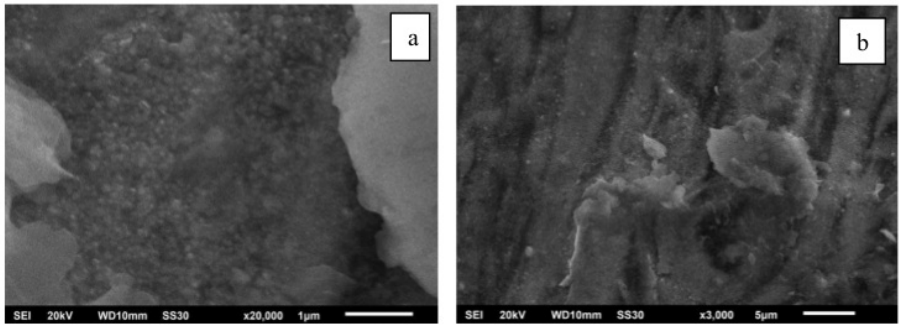


Figure 2. The image of SEM on AgNPs (a) and the size of AgNPs (b)

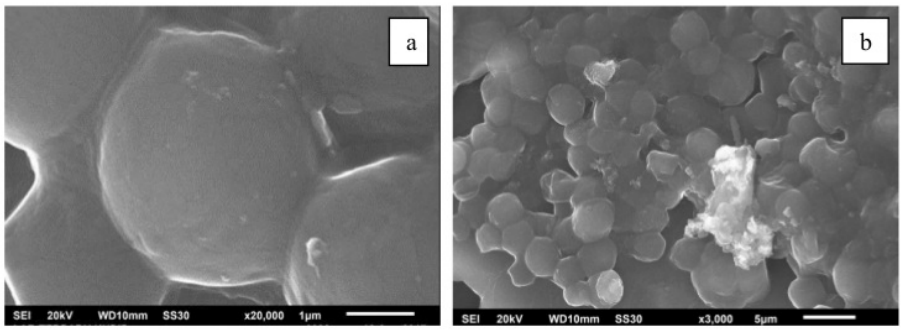


Figure 3. The silver nanoparticle on SEM image (a) and the size of microalgae *C. pyrenoidosa*

SEM microscopy was used to evaluate the surface morphology of both the agitated and non agitated microalgae AgNPs. The observation of the cell structure of *C. pyrenoidosa* exhibited that the cell was turned into distorted, shrunk and diminish cell after 160 h exposure with AgNPs. The size of the cell became 0.40-0.53 µm with agitation and 0.38-0.95 µm without agitation treatment respectively. Its also showed that agitation treatmen will caused greater effect on cell damaged caused by intense contact among AgNP particles and cells surface. This result was also supported by another researcher which was proven that nanoparticles can caused change in morphology and dimensions of green algae *Chlamydomonas reinhardtii* and *Dunaliella salina* [20]. Application of AgNPs on *Microcystis aeruginosa* showed inhibition on cell density and growth which is the inhibition reaches more than 95% [7]. In the *C. vulgaris*, the proteins of microalgae instead of caused Ag^{+} ion reduction, they also act as shape controlled synthesis of AgNPs [21].

The AgNP microalgae also revealed spherical and cuboidal nanoparticles with and without agitation treatment. The cells was forming clusters in specific area which was very difficult to found. The treatment also showed inhibition of cell growth that reduced the cell density. Images of SEM indicating toxicity of silver nanoparticles toward *C. pyrenoidosa* using 2 $mg.l^{-1}$ concentration. This result was in accordance with *M. aeruginosa* cell which is showed a shrunk and damaged cell wall indicating toxicity of silver nanoparticles in a lower concentration [7]. SEM microscopy also exhibited macroscopic aggregates composed of nanosized silver particles and dead microalgae cells. The other experiment with bacteria had reported that bacterial membrane undeilver nanoparticle treatment exhibits a significant increase in permeability, causing cells incapability of cells in proper transport regulationg through the plasma membrane followed by cell death [22].

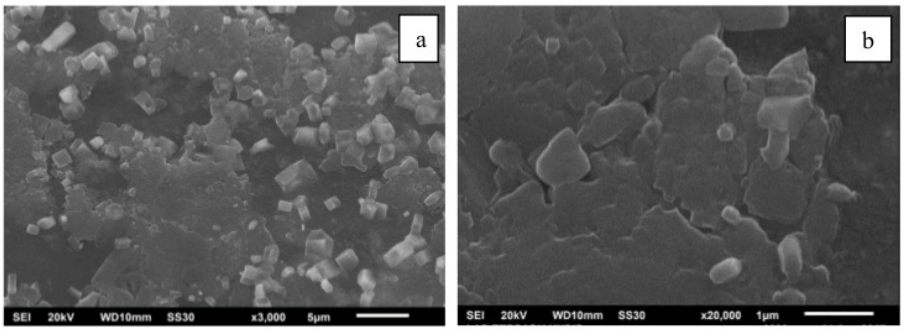


Figure 4. SEM image of nanosilver of *C. pyrenoidosa* with agitation (a) and the size of microalgae

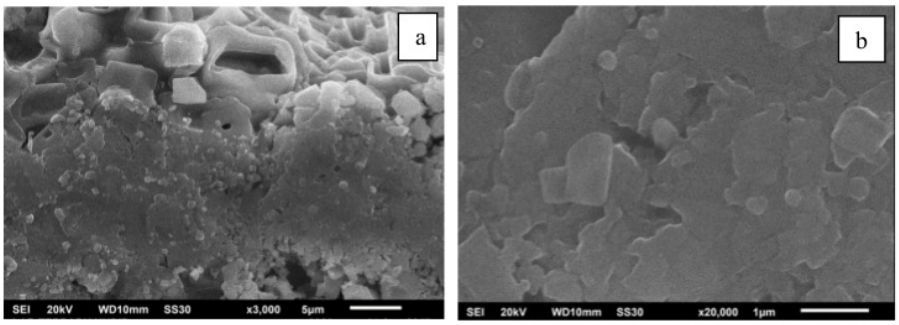
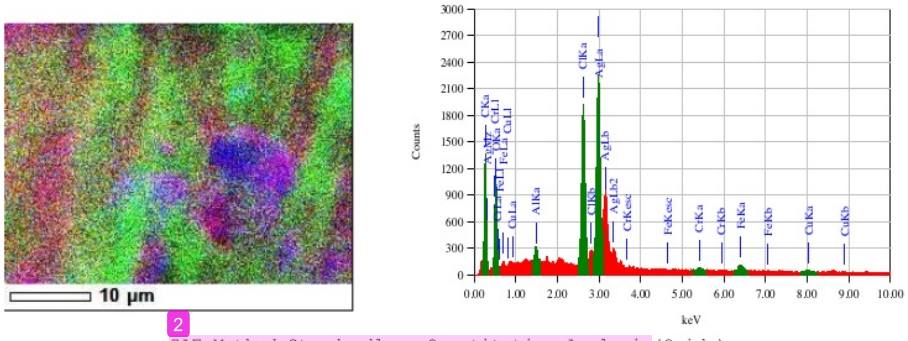


Figure 5. SEM image of nanosilver microalgae formed by *C. pyrenoidosa* without agitation (a) and the size of microalgae

3.3. *EDX analysis*

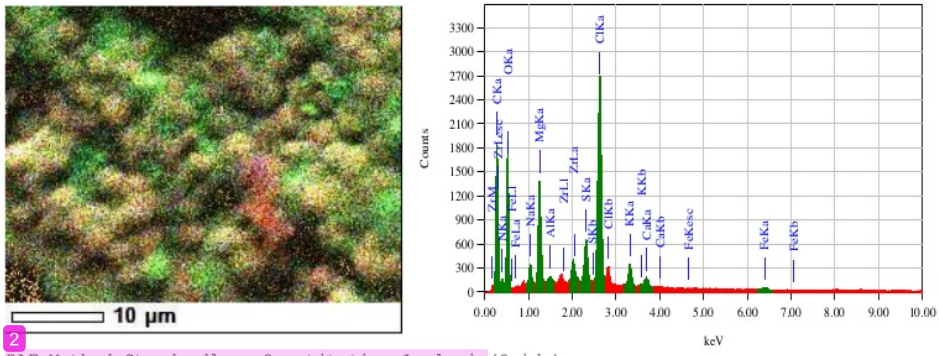
Characterization the chemical composition and the location of AgNPs on cell surface was analysis using the combination of SEM accomplished with X-ray (EDX).The EDX analysis of the microalgae AgNPs samples showed that silver nanoparticles were incorporated into the membrane of the treated microalgae cells. The EDX analysis was performed for the confirmation of *C. pyrenoidosa* silver nanoparticles. Figure 6-9 showed the evidence of EDX analysis in the spot profile mode for control, with agitation and without agitation treatment. The chemical composition of $AgNO_3$ as illustrated the EDX analysis on Figure 6 was contained Ag dominantly characterized by the highest and sharp peak appearance in the XRD image that clearly confirmed the main raw material marked by green colour. The sharp diffraction patterns of the XRD spectra indicates a pure crystalline silver structure which is in good agreement with the earlier report [23]. The observation analysis using EDAX confirmed the incorporation of silver nanoparticles into the membrane structure of microalgae.



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	

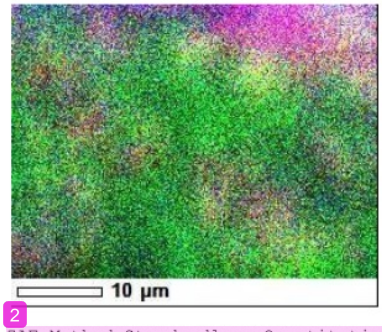
Figure 6. EDX analysis of $AgNO_3$

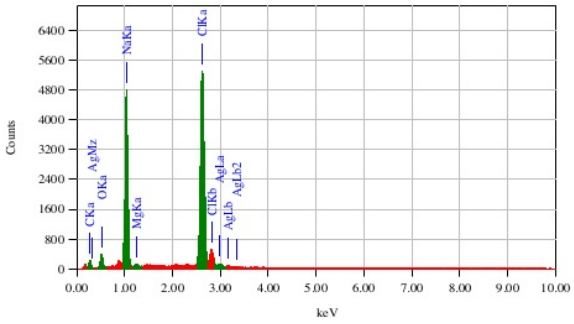


ZAF Method Standardless Quantitative Analysis (Oxide)
Fitting Coefficient : 0.0387; Total Oxide : 24.0

Element	(keV)	Mass%	Sigma	Mol%	Compound	Mass%	Cation	K
C K	0.277	60.92	0.08	74.86	C	60.92	0.00	40.9434
N K	0.392	17.50	0.27	18.44	N	17.50	0.00	14.6366
O		5.45						
Na K	1.041	0.82	0.03	0.26	Na2O	1.10	2.50	1.8270
Mg K	1.253	3.11	0.04	1.89	MgO	5.16	9.02	6.0237
Al K	1.486	0.14	0.02	0.04	Al2O3	0.26	0.36	0.2979
S K	2.307	1.42	0.04	0.65	SO3	3.54	3.11	4.0693
Cl K	2.621	7.73	0.03	3.22	Cl	7.73	0.00	24.3977
K K	3.312	1.04	0.02	0.20	K2O	1.25	1.87	2.8146
Ca K	3.690	0.48	0.02	0.18	CaO	0.67	0.84	1.3358
Fe K	6.398	0.37	0.02	0.10	FeO	0.48	0.47	0.9572
Zr L	2.042	1.04	0.06	0.17	ZrO2	1.40	0.80	2.6967
Total		100.00		100.00		100.00	18.97	

Figure 7. EDX analysis of *C.pyrenoidosa*



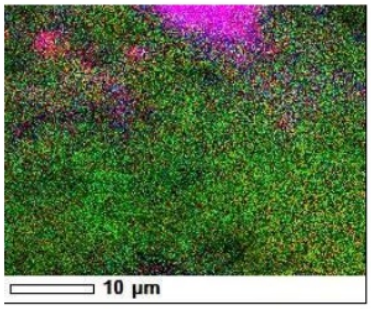


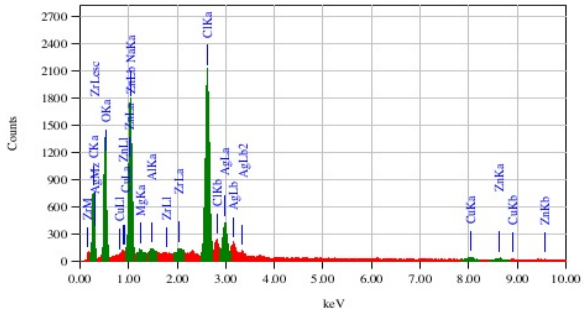
2

ZAF Method Standardless Quantitative Analysis (Oxide)
Fitting Coefficient : 0.0410
Total Oxide : 24.0

Element	(keV)	Mass%	Sigma	Mol%	Compound	Mass%	Cation	K
C K	0.277	62.59	0.59	89.07	C	62.59	0.00	26.9555
O		5.30						
Na K	1.041	10.80	0.20	4.01	Na2O	14.55	34.04	21.0010
Mg K	1.253	0.22	0.05	0.15	MgO	0.36	0.65	0.2976
Al K	1.486	0.24	0.05	0.08	Al2O3	0.45	0.64	0.3998
Cl K	2.621	11.54	0.12	5.56	Cl	11.54	0.00	31.7873
Cu K	8.040	0.91	0.11	0.24	CuO	1.14	1.04	1.9707
Zn K	8.630	0.76	0.11	0.20	ZnO	0.94	0.84	1.6324
Zr L	2.042	0.74	0.08	0.14	ZrO2	0.99	0.58	1.5767
Ag L	2.983	6.92	0.15	0.55	Ag2O	7.44	4.65	14.3790
Total		100.00		100.00		100.00	42.44	

Figure 8. EDX analysis of *C.pyrenoidosa* with agitation





2

ZAF Method Standardless Quantitative Analysis (Oxide)
Fitting Coefficient : 0.0233
Total Oxide : 24.0

Element	(keV)	Mass%	Sigma	Mol%	Compound	Mass%	Cation	K
C K	0.277	36.48	0.69	69.32	C	36.48	0.00	4.4427
O		8.80						
Na K	1.041	24.30	0.25	12.06	Na2O	32.76	46.12	38.8531
Mg K	1.253	0.30	0.05	0.28	MgO	0.50	0.54	0.2671
Cl K	2.621	28.15	0.17	18.12	Cl	28.15	0.00	53.8711
26 L	2.983	1.97	0.09	0.21	Ag2O	2.11	0.80	2.5660
Total		100.00		100.00		100.00	47.46	

Figure 9. EDX analysis of *C.pyrenoidosa* without agitation

Some other chemical compounds are also found in $AgNO_3$ solution in very small quantities consisting of Chromium (Cr), Ferum (Fe), Cuprum (Cu) and Aluminium (Al). The result of spectral processing of nanosilver microalgae as shown on Figure. 6. had calculated the number of X-ray counts in the peak of $AgNO_3$ compared with the number of X-ray counts in $AgNO_3$ standard with concentration 31,8 % of the element of interest, and from this derive the mass fraction of the element in the sample. The spectra obtained during EDX analysis were used for conducted the quantitative analysis by application of SEMQuant software and the ZAF procedure. Quantitative analysis proved lower silver contents in the examined samples comparing with 52% of control. This result was also in accordance with other report [24].

The number of X-ray counted in the peak of *C. pyrenoidosa* microalgae on Figure 7. showed zero concentration of $AgNO_3$ in the sample with 60.92% of carbon (C) mass concentration and 17.50% of nitrogen (N) mass concentration, respectively. This X-rays of *C. pyrenoidosa* were scattered by diffraction owing to the unique crystalline structure of the material analyzed. In the all standard EDX spectrum recorded on the examined sample were clearly several sharp peaks located between 0 kV and 3 kV. Those maxima are directly related to the silver characteristic lines K and L. The other way to obtain this type of detailed information would be more comprehensive using planar serial sections observation in the transmission electron microscope which will be improve in the next experiment.

4. Conclusion

The present study reveals that the microalga *C. pyrenoidosa* is good source for the synthesis of silver nanoparticles at a low silver concentration. The formation of silver nanoparticles was confirmed by characterization using UV-Vis, SEM, EDX and TEM techniques. The microalgae silver nanoparticles formed were quite stable in the solution. The agitation treatment act as the surface active stabilizing molecules and cell structure for the synthesis of silver nanoparticles. The method was fast and eco-friendly.

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