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Chlorella vulgaris-Mediated Nanosilver Synthesis with Chitosan Capping Agent

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Abstract: Nanoparticles are widely used for their simplicity, nontoxic, low-production cost, pollutants free, and eco-friendly process. Green synthesis of nanosilver using microalgae is an environmentally benign and emerging technique. The current study aims to synthesize the nanosilver with a chitosan capping agent of the microalgae *Chlorella vulgaris*. The algal *C. vulgaris* extract was prepared with a variation of 3.0 g/L, 5.0 g/L, and 6.0 g/L. As a comparison, we use nanosilver synthesis using NaBH₄. Extract algal treated with silver nitrate solution to synthesize nanosilver with a chitosan capping agent. Biosynthesized nanosilver was characterized with UV-Visual spectroscopy, particle size analyzer (PSA) analyzer, SEM, and EDS. Results show that nanosilver is successfully synthesized with UV-Visual Spectroscopy results at a wavelength of 400 nm. EDS analysis indicates the presence of silver metal on synthesis results at 3 kV. The PSA confirms that nanosilver produced by adding *C. vulgaris* of 3.0, 5.0, and 6.0 g/L results in particle size distribution from 57.8 nm – 462 nm, 17.2 nm – 97.2 nm, and 28.9 nm – 81.8 nm, respectively. It is higher when compared with synthesis nanosilver using NaBH₄, resulting in particle size distribution from 2.26 nm to 171.9 nm. It is confirmed that adding *C. vulgaris* of 5.0 and 6.0 g/L has successfully made a nanosilver.

Keywords: *Chlorella vulgaris*, EDS, Microalgae, Nanosilver, UV-Vis

1. Introduction

Nanomaterials have become a great resource with enormous potential for broad areas of production fields¹⁾. Nanotechnology is increasingly getting much attention in the nanosilver synthesis of green and environmentally friendly processes. Researchers have been interested in nanosilver particles because of their unique properties in technology, science, and medicine, such as antibacterial²⁾, anticancer drug³⁾, biomedical⁴⁾, food industry⁵⁾, and textile industry⁶⁾.

Nanosilver can be made by conventional methods such as physical and chemical methods. The most common and principal physical methods are laser ablation and evaporation-condensation methods. In the chemical synthesis method, nanosilver is produced from the reaction of three reactant components, including a metal precursor of silver nitrate in an aqueous solution, a reducing agent, and a stabilizer⁷⁾. The synthesis commonly used following reducing agents: NaBH₄, ascorbate, sodium citrate, poly (ethylene glycol) block copolymers, N, N-dimethylformamide (DMF), and polyol

process⁸⁾. However, several studies have reported many drawbacks of nanosilver chemical and physical synthesis. The chemical synthesis of metallic nanoparticles requires a reductor from a chemical reagent to alter metallic ions into metallic nanoparticles, which involves using hazardous and aggressive chemicals¹⁰⁾. The synthesis process has high costs, requires specific equipment, embraces the risk of flammable and toxic substances, and has a problematic scaled-up process⁹⁾. Through these effective yet problematic physicochemical methods. Thus, the green synthesis using biological extract has arisen.

Green synthesis is inexpensive, and environmentally friendly. Living organisms, such as fungi, microbes, algae, and plants, even animals can be used in nanosilver synthesis. Synthesis of nanosilver has been reported using biological materials such as fungi¹⁹⁾, plants²⁰⁾, and algae⁸⁾ have been reported. The biological material used for biosynthesis includes algae, plants, fungi, bacteria, and other microorganisms¹⁴⁾. Their biomolecules constituent and some enzymes help reduce Ag⁺ ions to form nanosilver⁷⁾.

In contrast to chemical synthesis, green synthesis uses

environmentally friendly reagents as reductor and stabilizer agents instead of hazardous and toxic chemicals¹¹⁾. The synthesis has minimal impact on ecosystems¹²⁾, which is recognized as an environmentally friendly solvent, an eco-friendly reductor, and a capping agent for stabilizing nanoparticle¹³⁾.

The nanosilver synthesis has been developed for an anticancer agent and highly antimicrobial activity¹⁵⁾. Antibacterial efficacy of nanosilver increases exponentially with the size decrease¹⁶⁾ due to their unique optical properties. The exciting characteristic of metal nanoparticles' unique optical properties is surface plasmon resonance, making them very useful and efficient in medicine and electronics¹⁷⁾. Pure silver has high electrical and thermal conductivity and a low contact resistance¹⁸⁾. However, the eco-friendly synthesis of nanosilver offers chemically toxic free.

The plant-extract-based biosynthesis of nanosilver involves only the plant extracts and the Ag⁺ ions solution because they can accumulate metals, reduce, and detoxify. Plant extracts contain many compounds like polymers, enzymes, alkaloids, flavonoids, polysaccharides, and proteins so that they can act as reductor and capping agents⁸⁾. The reductor gets silver metal from silver ions and agglomerates it to form clusters, which capping agents can block it. The agent has multiple roles: increase stability, reduce toxicity, and prevent agglomeration²¹⁾. Common capping agents are polymers non-ionic, and ionic surfactants. The commonly capping agents used in nanosilver synthesis are polyethylene glycol (PEG), trisodium citrate, polyvinyl alcohol (PVA), polyvinyl alcohol (PVA), cetyltrimethylammonium bromide (CTAB), and polyvinylpyrrolidone (PVP)²²⁾.

This study promotes chitosan as a capping agent. Chitosan is an environmentally friendly polymer containing amino and hydroxyl groups, derived from chitin, which are biodegradable, biocompatible, antimicrobial, highly selective, and non-toxic²³⁾. Thus the use of chitosan as a capping agent for nanosilver synthesis is promising. The proposed capping mechanism in nanoparticle synthesis includes the interaction between platinum nanoparticles and chitosan has been proposed through secondary hydroxyl and amino groups²⁴⁾. Furthermore, an electrostatic interaction occurs between a metal precursor and a charged amino group, followed by a reduction process²⁵⁾. However, the interaction between any metallic nanoparticle and chitosan is still a discussion matter.

Microalgae are an aquatic microorganisms group that can adsorb carbon dioxide and sunlight energy and absorb it from the environment for photosynthesis²⁶⁾. The different marine and freshwater algae extract like *Padina* sp²⁷⁾, *Sargassum longifolium*²⁸⁾, *Spirulina*²⁹⁾, *Caulerpa racemosa*³⁰⁾, *Oedogonium* sp. and *Hydrodictyon* sp.³¹⁾ are used to synthesize nanosilver. The nanosilver size produced from the extracts depends on the extract concentration or the silver nitrate source³²⁾. The

development of environmentally friendly synthesis methods without the use of toxic chemicals in the protocol is an excellent concern for nanoparticle synthesis. Nanosilver biosynthesis has increased the ability of microalgae as antimicrobial, antifungal, and anticancer. Microalgae can be the ideal platform for nanoparticle synthesis because they overgrow and produce large biomass at a lower cost. However, the synthesis of nanoparticles using microalgae is less informative. This study aims to observe the characteristic of the synthesis product of nanosilver using microalgae *C. vulgaris* with a capping agent of chitosan. The result could be a base for fundamental and applied nanosilver synthesis.

2. Materials and Methods

2.1 Materials

C. vulgaris microalgae collected from cultivation ponds (Situbondo, Indonesia). The collected microalgae were put in plastic containers and brought to the laboratory. The samples were rinsed with dH₂O to remove impurity materials and dried in the oven at 50°C for four days. After drying, the sample was blended to get a fine powder, then saved for future use.

2.2 Methods

2.2.1 Nanosilver Synthesis

The nanosilver synthesis was carried out according to previously published methods³³⁾. Fine powdered *C. vulgaris* of 5.0 g was put into a 100.0 mL flask containing 50.0 mL of sterilized dH₂O, and then the solution was boiled for 10 to 15 minutes at 60°C. The extract was then filtered using a filter paper (Whatman no.1). The filtrate was treated with a precursor of AgNO₃ (Sinka, Indonesia) solution (1.0 mM silver nitrate in ddH₂O of 90.0 mL) and incubated at ambient temperature under static conditions. The nanosilver solution was centrifuged two times, and each process used 12,000 rpm for 15 min. The supernatant was discarded, and the pellet was dissolved in dH₂O. Nanosilver synthesis was conducted for algae concentrations of 3.0, 5.0, and 6.0 g/L. For comparison, we do a nanosilver synthesis using silver nitrate and NaBH₄ (Central Drug House Ltd, India) solution as a reductor. Silver nitrate solution of 10.0 mL 1 mM was added dropwise into NaBH₄ solution with a concentration of 2.0 mM and volume of 30.0 mL and then stirred for 3 min. The centrifugation process (Thermo Fisher Scientific, USA) was then conducted on the solution at 12,000 rpm for 15 min. and filtered. Before the nanosilver was analyzed, the obtained colloid was saved for 24 hours at room temperature.

2.2.2 Nanosilver with Chitosan Capping Agent

The nanosilver was stabilized by chitosan (Himedia,

India) by adding 5.0 mL of nanosilver colloid solution containing 3.0 mL of 1.0% chitosan. The solution was then treated by an ultrasonic homogenizer (Ningbo Lawson, China) at 400 watts, 20 kHz for 10 min.

2.2.3 Characterization of Nanosilver

UV-Visible Spectroscopy Analysis. Color change of the solution containing the silver nitrate incubated with microalgae *C.vulgaris* was visually observed. The reduction of precursor silver ions was observed by aliquot sampling (1 ml) at different concentrations of *C.vulgaris*. Wavelength absorption was scanned using a UV Visual spectrophotometer (Shimadzu, Japan) at a resolution of 1 nm and wavelength from 300 – 800 nm.

Scanning Electron Microscopy (SEM). For SEM observation, a solution containing synthesized nanosilver was put on the carbon film on a specimen holder and dried using warm air. Before loading them onto SEM, the sample was coated with a gold layer using a sputter-coater (Emitech SC7-620). Micrographs were taken on SEM, FEI, Inspect S-50 (Japan), at a voltage of 20 kV.

EDS Analysis. Elemental analysis on nanoparticles was carried out using energy dispersive spectroscopy (EDS). EDS attachment equipped with SEM, and the samples were prepared on the carbon film on a specimen holder.

Particle Size and Zeta Potential Analysis. The particle size and zeta potential measurement of nanosilver were monitored using dynamic light scattering nanoscale measurements conducted with a particle size analyzer (Nanotrac Flex, Microtac, USA). The particle sizes were measured thrice for each treatment group. Data were analyzed using one-way ANOVA and Tukey Least Significant Difference (LSD) with a significant level of 95% using Software Origin version 9.0.

3. Results and Discussion

3.1 Synthesis of Nanosilver

The results of the synthesis are shown in Figure 1. The nanosilver was formed through the process of reduction of silver nitrate compound by microalgae solution. The reactant's addition in reverse order will form silver nitrate immediately. The first indicator of Ag ions bioreduction to nanosilver is the change in color of the algae suspension. The chemical reaction between silver nitrate as a precursor and microalgae indicate by the change in the color solution, which means a successful reaction to produce silver particles. The results of synthesis using microalgae *C.vulgaris* with the amount of 3.0, 5.0, and 6.0 g/L produced different solution colors, such as bright brown, brown, and dark brown, respectively, as shown in Figure 1. The color change of resulted solution occurs due to the plasmon vibrations excitation on the nanosilver surface³⁴. This color is similar to the results of nanosilver synthesis using fruit extract³⁵. The color of a colloid solution containing nanosilver depends on the *C.vulgaris* concentration. The color change indicates a difference in

the large size of the nanosilver due to the aggregation process. The larger size of nanosilver was marked by the change of the solution color, for example, from bright yellow to dark yellow, then green to gray³⁶.

Microalgae function as reducers of silver ions because of their metabolites, such as phenols, alkaloids, flavonoids, carbohydrates, and amino acids^{37/38}. They not only influence the reduction process but also act as stabilizing and capping agents to prevent the aggregation of nanosilver. The biological extract affects the synthesis process in many routes depending on its chemical content³⁵. Some mechanisms have been proposed to describe biosynthesis to form nanoparticles using microalgae. The most likely mechanism is the secretion of microalgal cells that produce cellular reductases into the growth medium. Reductase enzyme can effectively reduce silver ions into nanosilver. In addition, metal ions can be trapped by carboxylic groups on the surface of microalgae cells. The reductase enzyme reduces the trapped ions, resulting in the formation of nanoparticles³⁹.

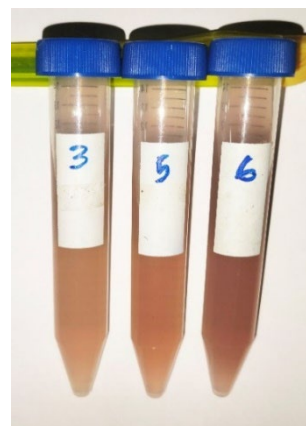


Fig 1: Nanosilver Synthesis Using Microalgae *C. vulgaris* of 3.0, 5.0 and 6.0 g/L

The popular analysis technique to determine the presence of nanosilver and its properties is UV-visible spectroscopy. At a different concentration of microalgae, the change in light absorption profile and increase in the intensity of the solution was observed. Silver particle has a peak absorption maximum of surface plasmon resonant at a wavelength of about 400 nm (Figure 2). The absorption energy hinge on the degree of plasmon resonance, i.e., the peak can shift to either side of this value depending on the ratio of silver ions and zero valences silver⁴⁰. The increasing silver nitrate concentration and the increasing intensity of the maximum plasmon resonant and nanosilver have resulted. The higher concentration of nanosilver obtains at synthesis using *C.vulgaris* of 6.0 g/L.

The stabilization of nanosilver using chitosan was carried out to prevent nanosilver aggregation. Chitosan has amine groups that have a positive charge⁴¹, and the nanosilver interacts with chitosan through amine groups (-NH₂) to make a steric stabilization on the surface of the

nanosilver³⁴). The stability of the particles or the colloidal solution can be predicted using the zeta potential value. The potential is the electric potential at the boundary of the double layer. The higher value of colloidal, the higher colloidal stability⁴²).

Zeta potential measurement of the colloid shows that synthesis routes using NaBH_4 , *C.vulgaris* with a concentration of 3.0 g/L, 5.0 g/L, and 6.0 g/L results average zeta potential of 18 ± 2.77 nm, 19.2 ± 6.51 nm, 94.8 ± 4.95 nm, and 110.4 ± 13.29 nm, respectively. The one-way ANOVA analysis indicates that the concentration of microalgae in nanosilver synthesis has a significant difference in nanosilver colloid stability at a significant level of 0.05 ($n = 3$, P-value = 0.0). After analysis using Tukey LSD, comparison by each membrane indicate that the *C.vulgaris* at a concentration of 3.0 g/L has no significantly different effect with nanosilver synthesis using NaBH_4 . A higher concentration of *C.vulgaris* with a chitosan capping agent in this synthesis results in higher nanosilver stability.

The stability behavior of the colloid based on zeta potential value indicate that synthesis routes using NaBH_4 and *C.vulgaris* with a concentration of 3.0 g/L had a category of incipient instability and synthesis routes using *C.vulgaris* with a concentration of 5.0 g/L and 6.0 g/L had a category of Incipient instability excellent stability⁴²). A low zeta potential value in suspension dispersion promotes agglomeration caused by van der Waals inter-molecular attractions. The zeta potential represents the electrical charge on the surface of the nanoparticles, which causes repulsive solid forces among the particles that prevent agglomeration⁴³).

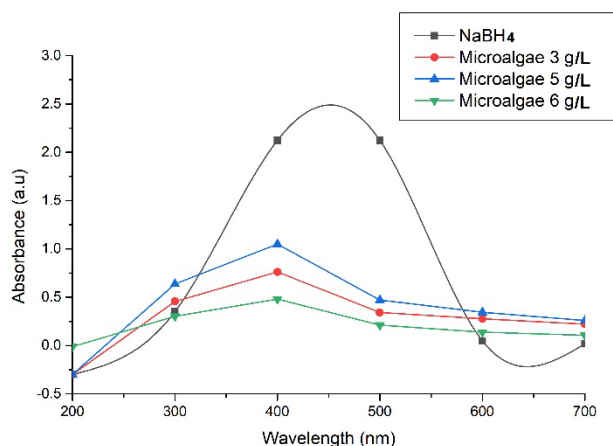


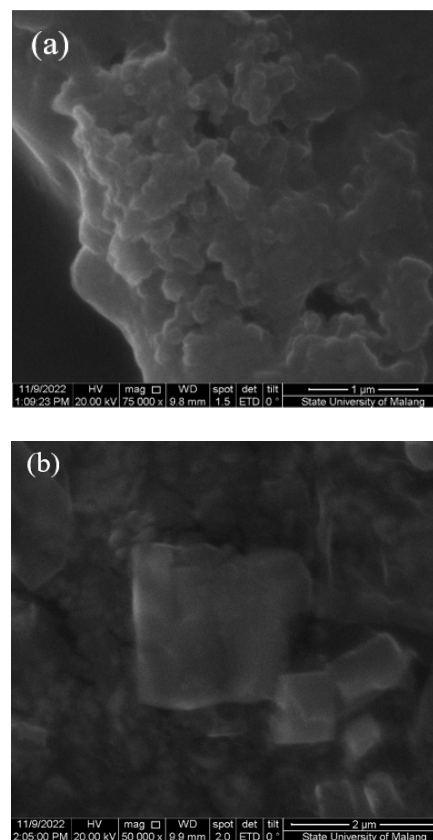
Fig 2: UV-Vis Spectrophotometer Results for Eco-Friendly Synthesis of Nanosilver Using Microalgae *C. vulgaris*

3.2 Morphology of Nanosilver

SEM images were observed for each sample to investigate the morphology of the synthesized nanosilver. The presence of nanosilver in the suspension of *C. vulgaris* was further confirmed by SEM images, EDS graph, and a particle size analyzer. SEM analyzed the morphology of nanosilver. Synthesized nanosilver has

formed a spherical shape, as shown in Figure 3. Figure 1A shows the agglomeration of nanosilver produced from the reduction of silver nitrate using NaBH_4 solution. Agglomeration of nanosilver is due to adhesion of among other nanoparticles by intermolecular forces leading to (sub) micro-sized entities. However, it is clearly observed that the sample consists of highly agglomerated nanoparticles. Figure 3B, 3C, and 3D show the product of biosynthesis nanosilver using *C. vulgaris*. Formation cubic among nanosilver is the salt from marine microalgae as an impurity inside the process. Figure 3B and 3D indicate the agglomeration of nanosilver, but Figure 3C clearly shows the presence of nanosilver with a spherical form. It indicates the chitosan's capping agent wrapping nanosilver effectively to inhibit nanosilver's agglomeration efficiently. The chitosan function to stabilize the nanoparticles by both electrostatic and steric stabilizations²⁵).

Another biosynthesis process using plant extract shows that the morphology of nanosilver was spherically shaped⁴⁴). Enzyme-based synthesis shows an overall spherical shape with an enlarged surface with nanoflowers or desert roses morphology⁴⁵). The reactant ratios and capping agent concentrations in synthesis resulted in nanosilver in bent wires, nanorods, triangular plates, and mainly in spherical morphologies⁴⁶). The morphologies of synthesized nanosilver depend on the reactant type and also by controlling kinetic and thermodynamic parameters.



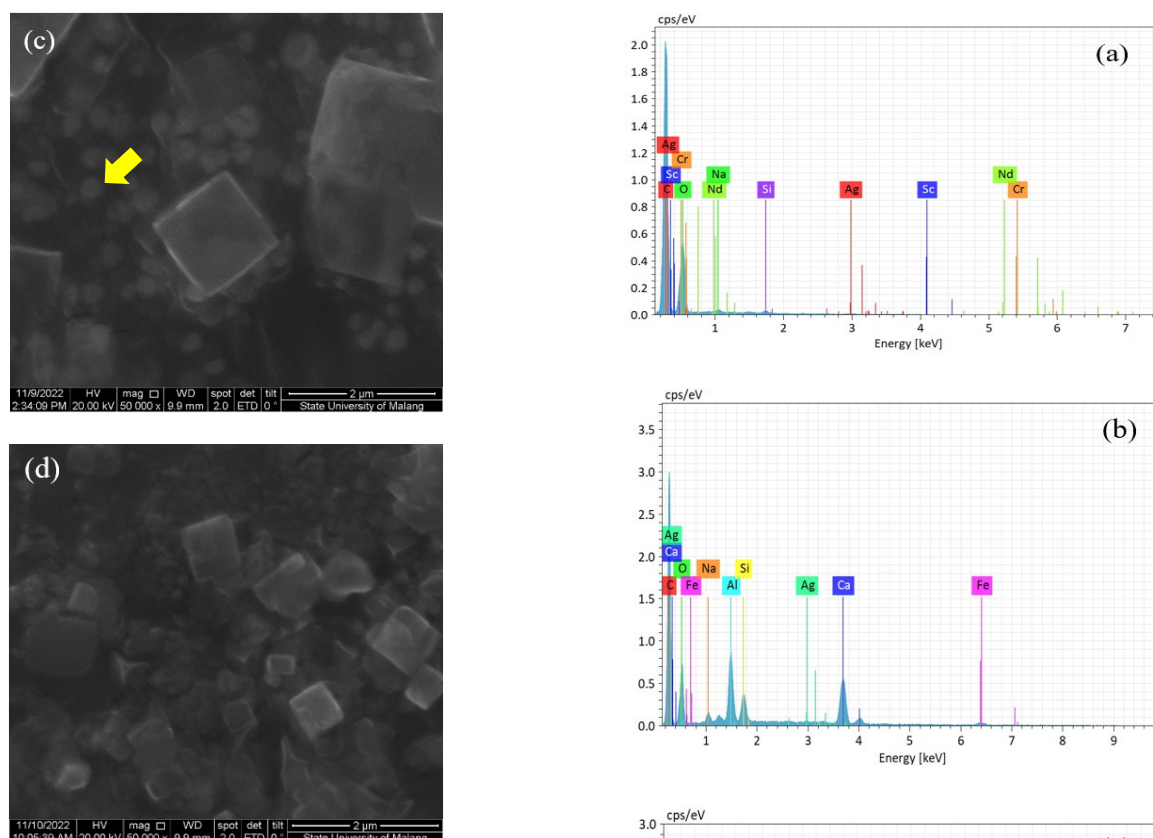


Fig 3: Results of Biosynthesis of Nanosilver Using; (a) NaBH_4 , (b) 3.0 g/L of Microalgae, (c) 5.0 g/L of Microalgae, and (d) 6.0 g/L of Microalgae

3.3 Chemical Composition of the Nanosilver

The chemical composition of synthesized nanosilver was analyzed using the EDS microanalysis during SEM observation. Figure 4a – 4d shows an overview EDS image of an aggregated nanosilver on a substrate.

An absorption peak at 3 keV in EDS analysis confirmed the presence of nanosilver in the solution. The carbon peak in the low-energy part of the spectrum results from conductive carbon adhesive. Furthermore, an oxygen peak is detected in the low-energy part of the EDS spectrum. Element Na and Cl indicated that the substrate still contains the salt as a contaminant from the marine microalgae.

Elemental analysis of biosynthesis nanosilver using NaBH_4 route, *C.vulgaris* with a concentration of 3.0, 5.0, and 6.0 g/L results in silver content with the mass percentage of 11.02%, 32.77%, 51.61%, and 53.69%, respectively. The increase in the concentration of *C.vulgaris*, the enhancement in silver particles product. The EDS spectra of synthesis using *C.vulgaris* with a concentration of 6.0 g/L result 2 peaks at 3 kV and 3.2 kV correspond to the binding energies of silver $\text{L}\alpha$ and $\text{L}\beta$, respectively⁴⁷. These profile EDS were typical for nanosilver absorption due to surface plasmon resonance. In an other study, spherical nanosilver indicated absorption peaks ranging from 2.5 to 4.0 keV⁴⁸. This silver structure is also similar to the synthesis of nanosilver using enzymatic reduction⁴⁵.

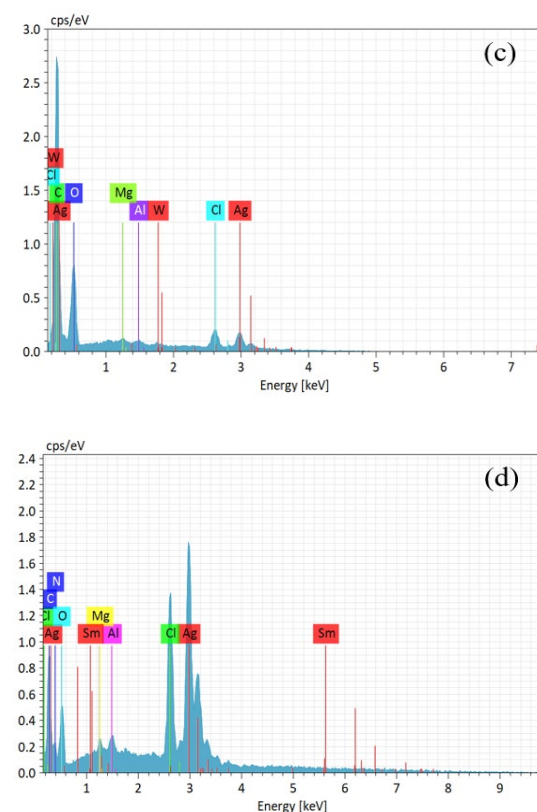


Fig 4: Metal Content Analysis Using EDS for the Results of Nanosilver Synthesis Using; (a) NaBH_4 , (b) 3.0 g/L of Microalgae, (c) 5.0 g/L of Microalgae, and (d) 6.0 g/L of Microalgae

3.4 Particle Size Distribution Analysis

Particle size distribution measurement of nanosilver is

presented in Figure 5. Each treatment results in different nanosilver sizes. Particle size distribution of nanosilver resulted from synthesis routes using NaBH_4 , *C.vulgaris* with a concentration of 3.0 g/L, 5.0 g/L, and 6.0 g/L are in the range of 2.26 nm – 171.9 nm, 57.8 nm – 462 nm, 17.2 nm – 97.2 nm, and 28.9 nm – 81.8 nm with an average diameter of 41.4 ± 4.05 nm, 111.1 ± 12.95 nm, 53.4 ± 8.68 nm, and 51.6 ± 7.45 nm, respectively.

Synthesis using *C.vulgaris* with a concentration of 3.0 g/L has a wide range of particle size distribution, and the best distribution is obtained at synthesis using *C.vulgaris* with a concentration of 5.0 g/L. Synthesis of nanosilver using a reductor of NaBH_4 results in the smallest average size of nanosilver. But, nanosilver still has wide particle size distribution. The greater degree of particles interaction results in increased levels of agglomeration and potential for particle deposition.

At higher microalgae concentrations, nanosilver particle size increases due to the Ag ions being reduced fast to facilitate further nanoparticle growth. The microalgae composition significantly affects the nanosilver morphology due to higher microalgae concentrations containing different biochemical reductor⁴⁹). It is fathomed that the bonding type of the nanosilver with the phytochemicals of microalgae is formed by a coordinating bond and the capping agent adsorption selectively on the nanosilver surface.

Results of one-way ANOVA analysis indicate that the concentration of microalgae in nanosilver synthesis has a significant difference in nanosilver size at a significant level of 0.05 ($n = 3$, $P\text{-value} = 0.0$). After analysis using Tukey LSD, comparison by each membrane indicate that the *C.vulgaris* at a concentration of 3.0 g/L has a significantly different effect on nanosilver size. It indicates that a lower concentration of *C.vulgaris* did not have the same results with chemical synthesis using NaBH_4 .

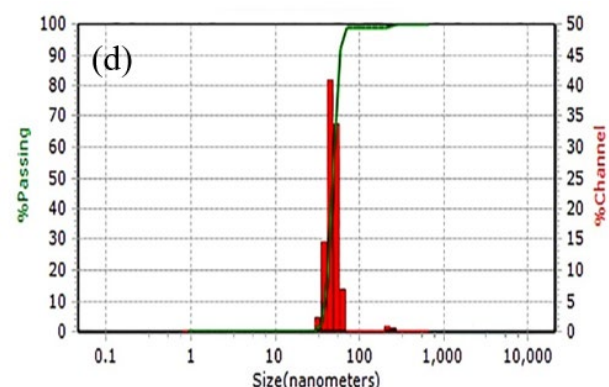
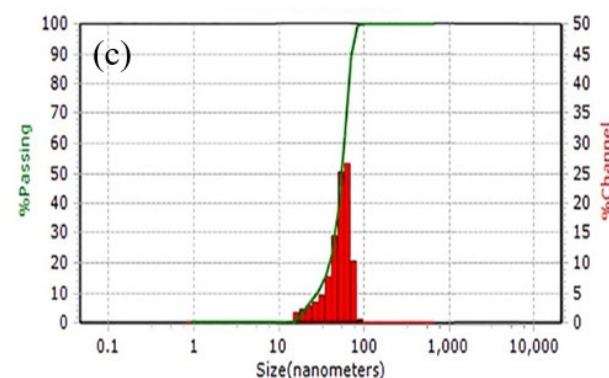
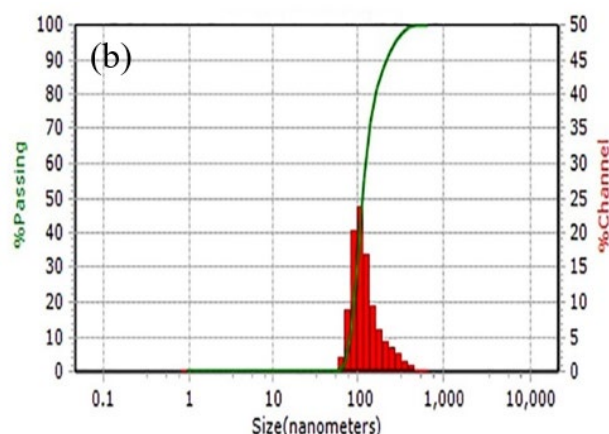
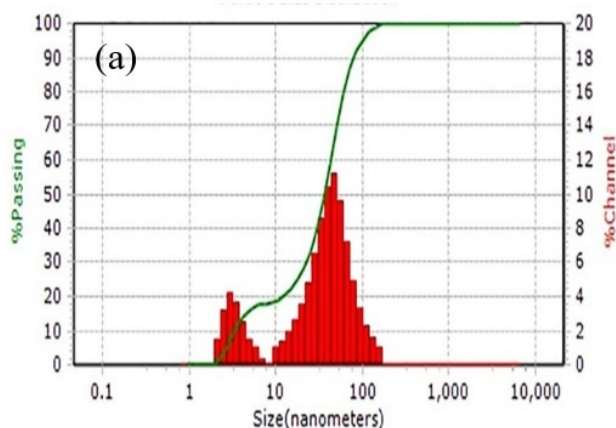


Fig 5: The Distribution of Nanosilver Size Resulting from the Synthesis Using; (a) NaBH_4 , (b) 3.0 g/L of Microalgae, (c) 5.0 g/L of Microalgae, (d) 6.0 g/L of Microalgae

4. Conclusion

This study has successfully synthesized nanosilver using *C.vulgaris* as a reductor and chitosan as a capping agent. The results indicated the presence of nanosilver particles through the brownish color of the solution and, confirmed by UV-visual spectroscopy, showed high absorbance at a wavelength of 400 nm. The results were also proved by EDS analysis confirming the nanosilver's presence in the solution. ANOVA analysis shows that the concentration of microalgae in nanosilver synthesis has a significant difference in nanosilver size at a significant level of 0.05. Current synthesis results in nanosilver with

particle size in an average of 41.4 ± 4.05 nm, 111.1 ± 12.95 nm, 53.4 ± 8.68 nm, and 51.6 ± 7.45 nm, after the reduction process using NaBH_4 , *C. vulgaris* with a concentration of 3.0, 5.0, and 6.0 g/L, respectively. The higher concentration of *C. vulgaris* with a chitosan capping agent, the higher the zeta potential value resulting in higher nanosilver stability. We can optimize the biosynthesis affecting variables and their interactions in the future to build an eco-friendly, advanced, inexpensive, and fast-grown field. So, resulted in nanosilver may be applied as an antibacterial agent.

Acknowledgments

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