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Isolation of Endophyte Bacteria from Water Hyacinth Plants (Pontederia crassipes) and Testing Their Activity as Silver Nitrate ($AgNO_3$) Bioreductors in The Formation of Silver Nanoparticles (AgNPs)

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Abstract
Nanotechnology plays an important role in various sectors, with the ability to optimize material properties at the nanometer scale. One significant innovation is silver nanoparticles (AgNPs), which have wide applications in textiles, cosmetics, and biomedicine due to their antimicrobial and antioxidant properties. In this study, endophytic bacteria found in water hyacinth plants (Pontederia crassipes) were used as bioreductors in the formation of silver nanoparticles, endophytic bacteria were isolated from several parts of the water hyacinth plant which were from the leaves, stems and cobs or hyacinth parts of the plant, endophytic bacteria found or obtained were then tested in a 3 mM $AgNo_3$ solution by means of, pure endophytic bacteria were fermented into NB (Nutrient Broth) media for three days or 78 hours, then the fermented bacteria were centrifuged at a speed of 1000 rpm for 15 minutes, and the supernatant formed was tested with an $AgNO_3$ solution with a concentration of 3 mM for 3 days or 78 hours, To evaluate the formation of silver nanoparticles, the color change from pale yellow to brownish yellow and brown was observed, then the absorbance of the sample was measured with a UV-Vis spectrophotometer at a wavelength of 300-800 nm at the specified time. Isolation of endophytic bacteria carried out on water hyacinth plants contained 12 endophytic bacteria with the codes BEPC 1, BEPC 2, BEPC 3, BEPC 4, BEPC 5, BEPC 6, BEPC 7, BEPC 8, BEPC 9, BEPC 10, BEPC 11, and BEPC 12. After testing as a bioreductor in AgNO3 solution, UV-Vis spectro analysis showed that there were 3 pure isolates that were able to reduce $AgNO_3$, namely in isolate codes BEPC 2, BEPC 3, and BEPC 12, where each isolate had a wavelength and absorbance of BEPC 2 (Wavelength 435.00 nn and absorbance 3.141), BEPC 3 (Wavelength 425.50 nm and absorbance 3.042) and BEPC 12 (Wavelength 427.50 nm and absorbance 2.227). Conclusion: Endophytic bacteria from water hyacinth plants have the potential as bioreductors of silver ions $AgNO_3$ to form silver nanoparticles, namely with bacterial isolate codes BEPC 2, BEPC 3, and BEPC 12.

Keywords: *Nanotechnology; silver nanoparticles; Pontederia crassipes; environmentally friendly.*

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I. Introduction

Nanotechnology plays a vital role in the modernization of many sectors of technology, environmental science, food technology, biotechnology, information science, energy, transportation and so on. Nanotechnology has significantly influenced the growth of material science research by tailoring the properties of materials at very small scales. Most nanoparticles are synthesized by top-down approaches such as sputtering, lithography, ball milling and bottom-up approaches such as hydrothermal, pyrolysis, spray, chemical vapor deposition, solvent gel process. Especially in the case of chemical methods, the size and shape of nanoparticles are controlled by changing the concentration of the chemical and reaction conditions. However, these methods are toxic, expensive and potentially hazardous which can cause unwanted by-product diseases to overcome these problems, green synthesis approaches are attracting much attention due to their low cost, biocompatibility and environmental friendliness [1].

Nanotechnology plays a vital role in the modernization of many sectors of technology, environmental science, food technology, biotechnology, information science, energy, transportation due to its unique properties combined with relatively low production costs. These special properties have previously determined AgNP [2] for applications in various fields such as functional textiles, medicine, cosmetics, ecology and many others. In the field of cosmetics, AgNP has recently been used as an additive due to its antiseptic and preservative functions, for example in acne care products [3].

The antibacterial activity of AgNPs is based on several interactions of the particles with the bacterial body. Very small AgNPs can penetrate the bacterial membrane and enter the cytoplasm where they induce

strong oxidative stress [4]. Larger nanoparticles accumulate on the bacterial membrane and form aggregates. This process damages the integrity of the membrane leading to cell death [5].

Microorganisms that have the potential to form AgNPs include endophytic bacteria. Endophytic bacteria are bacteria that grow in healthy plant tissue and can protect plants from phytopathogens, increasing plant growth in normal and challenging conditions [6]. These endophytic bacteria have secondary metabolite compounds that are antimicrobial and are considered to have the ability to reduce silver ions [7].

Biological synthesis approach of nanoparticles is environmentally friendly, non-toxic nanoparticles, various types of organisms found in plants, fungi, yeast, actinomycetes and bacteria for nanoparticle synthesis [8]. Environmentally friendly synthesis methods are more accessible because of their non-toxic nature compared to physical and chemical methods that show toxicity to the environment, physical and chemical methods are said to be dangerous because chemicals are flammable and the chemicals used cannot be disposed of in the free environment [9].

The advantages of biosynthesis compared to chemical and physical synthesis methods are that it is environmentally friendly, more stable in the long term, cost-effective, easy to scale up for large-scale nanoparticle synthesis and do not require high temperatures, pressure, energy and/or toxic chemicals [10]. Bacteria-based synthesis methods are more economical, simple, reproducible and require less energy when compared to chemical synthesis routes [11].

Pontederia crassipes, also known as water lily, water hyacinth, camalote, water flower, water drum, lechuguilla, nymph or huachinango is a flowering aquatic species native to the Amazon basin (mostly in Brazil) [12]. Researchers estimate that 10 Pontederia crassipes plants can reproduce into 655,360 plants, covering about half a hectare in 8 months. Pontederia crassipes has been reported to contain many biochemical compounds and 13–17% tannins in its leaves, roots and flowers. Some of the tannic acids present in Pontederia crassipes are gallic acid, catechin, chlorogenic acid, vanillic acid, p-coumaric acid, ferulic acid, rutin, quercetin. Due to it is abundant in nature, it can be used as a source of vegetable tannin extraction [13]. Therefore, researchers are interested in isolating endophytic bacteria found in water hyacinth plants and screening tests as bioreductors against $AgNO_3$.

II. Methods

Materials

Water hyacinth plants had taken from Talago Kotobaru, Tanah Datar District, West Sumatra, Indonesia and identified at the Herbarium Laboratory of Andalas University (ANDA). $AgNO_3$ (Merck®), distilled water (Bratachem®), Nutrient Broth (NB) (Merck®), Nutrient Agar (NA) (Merck®), sodium hypochlorite, crystal violet, lugol, safranin, 96% ethanol, H_2O_2, $FeCl_3$, Mayer's reagent, concentrated HCl, Liebermann-Burchard and magnesium powder, methyl paraben, glycerin, polyethylene glycol, carbopol 940 and triethanolamine.

Surface sterilization of Pontederia crassipes leaves. The collected leaves were cut into approximately 1 cm2. The leaves were disinfected with 70% ethanol for 5 minutes, 2% sodium hypochlorite for 6 minutes, 70% ethanol for 30 seconds to remove sodium hypochlorite and then rinsed three times using sterile distilled water. The last rinse water was put into a petri dish containing NA media as a negative control and incubated for 3x24 hours. If there is no bacterial growth on the media, it indicates that the surface of the surian leaves has been sterile from bacteria.

Isolation and purification of endophytic bacteria

Sterilized leaves were cut at the veins with a sterile knife and inoculated on NA media. Then the leaves were incubated at 37oC for 3 x 24 hours. The bacterial colonies that grew were purified using the streak plate method on NA medium. Pure isolates of bacteria were identified based on morphological characteristics referring to Bergey's Manual of Determination of Microorganism.

Identification of bacterial isolates

Identification of isolates was carried out by macroscopic and microscopic morphological observation. Macroscopic observation is done by directly observing the characteristics of the isolate bacterial colony including: color, shape, margin and elevation of the colony. Microscopic observation is done by the Gram staining method. Gram staining is done by cleaning the object glass, air-dried over a spirit lamp. Then 1 loop of bacterial suspension is dropped on the object glass aseptically and fixed, then 1-2 drops of crystal violet are dropped for 2-3 minutes and washed with running distilled water then air-dried. 2-3 drops of lugol are added, left for 2 minutes and washed again with running distilled water then air-dried. Furthermore, the preparation is dripped with 96% alcohol until it is clear and washed again with distilled water then air-dried. The preparation is dripped with 1-2 drops of safranin, left for 2-3 minutes then washed again with running distilled water and air-dried. The preparation is observed under a microscope with a magnification of 10x100. Gram-positive bacteria will appear purple under the microscope, while Gram-negative bacteria will appear red.

Preparation of bacterial isolate stock

All pure bacterial cultures that have been isolated, inoculated on NA slant and stored at 4^{o}C as a collection of isolated bacteria

Preparation of $AgNO_3$ stock solution

0.1M $AgNO_3$ stock solution was made by dissolving 1.6987 grams of $AgNO_3$ solids with distilled water to a volume of 100 ml.

Screening of $AgNO_3$ reducing bacteria

To determine bacteria that are able to reduce $AgNO_3$ to form AgNPs, screening of $AgNO_3$ reducing bacteria was carried out. $AgNO_3$ 3mM solution was prepared by diluting $AgNO_3$ 0.1 M stock solutions. Each pure bacterial culture was taken 1 Ose then inoculated into NB media and shaken at a speed of 250 rpm, temperature 37^{o}C for 72 hours. A total of 15 ml of $AgNO_3$ 3mM was mixed with 5 ml of supernatant of each bacterial culture in a 50 ml Erlenmeyer flask. All samples were shaken at 150 rpm, 37^{o}C and stored in the dark for 72 hours. The color change of $AgNO_3$ solution from light yellow to brownish yellow indicated the formation of AgNPs and the formation of AgNPs was confirmed by UV-Vis spectrophotometry at a wavelength of 300-800 nm. UV-Vis spectrophotometry analysis was carried out in a quartz cuvette using distilled water as a reference solvent. The presence of an absorption spectrum in the wavelength region of 400-450 nm indicated the formation of AgNPs. Bacteria that produced the highest concentration of AgNPs were used for further AgNPs biosynthesis.

III. Results

Water hyacinth plants were isolated from various parts of the plant starting from the leaves, stems and cobs or hyacinths of the plant, the results of the isolation can be seen in Figure 1.

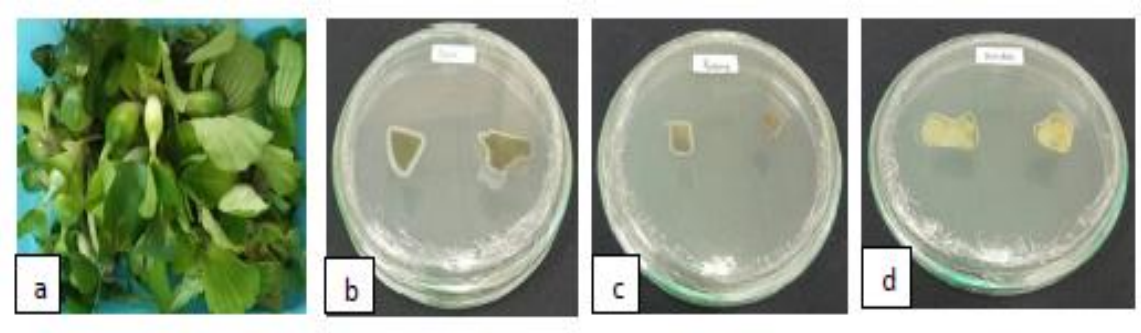

Figure 1 Image of water hyacinth plant from endophytic bacteria that were isolated in this study (a). Water hyacinth plant, (b). Leaves part, (c). Stem part, (d). Hyacinth part

From the results of endophytic bacterial isolation in water hyacinth plants shown in Figure 1. 12 types of pure isolates were obtained, but after being tested for $AgNO_3$ reducer screening, there were 3 isolates that had the potential to be $AgNO_3$ reducers, namely with the codes BEPC 2, BEPC 3 and BEPC 12, as can be seen in Figure 2.b. there was a color change where previously 15 ml of $AgNO_3$ solution with a concentration of 3 mM was added with 5 ml of bacterial supernatant.

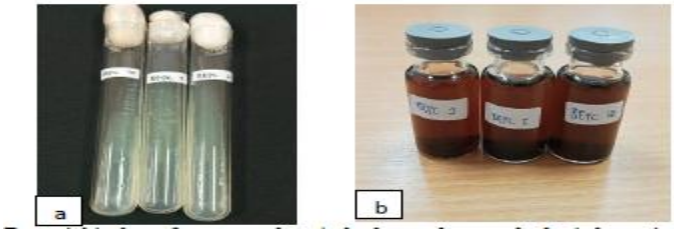

Figure 2 Bacterial Isolates From Water Hyacinth Plants Whose Endophytic Bacteria Were Isolated In This Study. (A). Pure Isolates of Bacteria With Codes BEPC 2, BEPC 3 And BEPC 12, (B). $AgNO_3$ Reducer

Silver nanoparticle biosynthesis was carried out by reducing silver ions using endophytic bacteria of water hyacinth plants as bioreductors. Silver nanoparticles were formed in yellow to brown colloids. Figure 3 shows the absorption spectrum of $AgNO_3$ with endophytic bacterial supernatant. The silver nanoparticles formed had maximum absorption in the wavelength range of 400-440 nm.

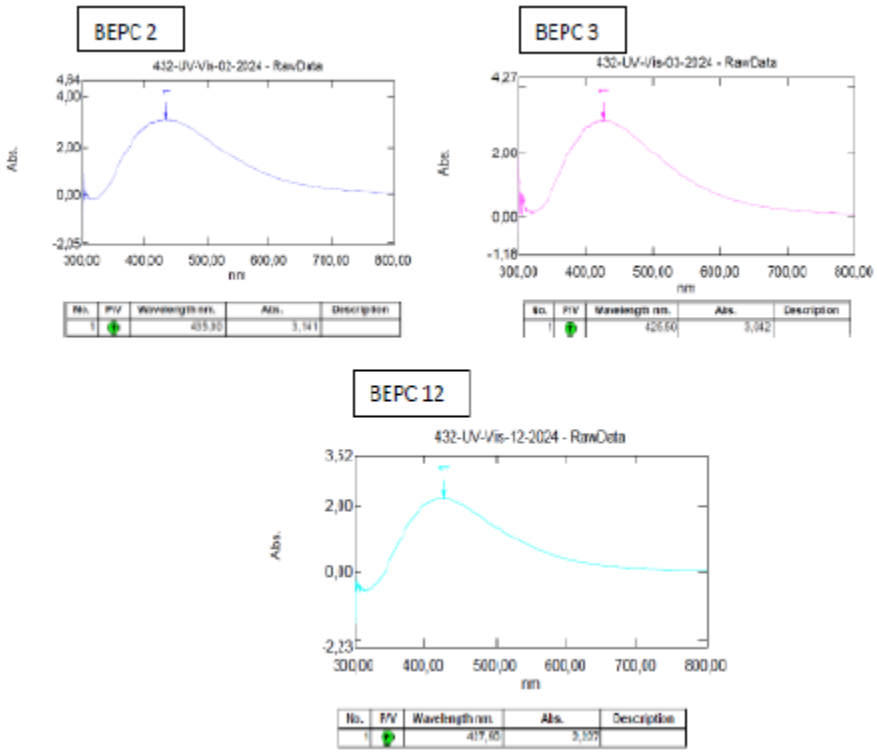

Figure 3 UV-Vis absorption spectrum showing the formation of AgNPS with maximum absorption at a wavelength of 400-500nm, with endophytic bacterial bioreductants isolated from water hyacinth plants

To identify the type of bacteria, Gram staining can be done as seen in Figure 4. The three types of bacterial isolates are included in gram positive which has a purple color in the Gram staining.

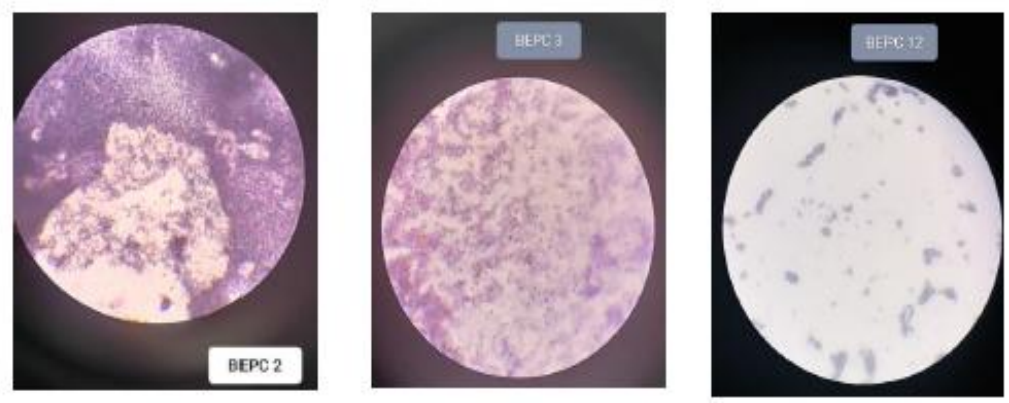

Figure 4 Appearance of The Gram Staining Results of 3 Endophytic Bacterial Isolates With Codes BEPC 2, BEPC 3, and BEPC 12 Isolated from The Water Hyacinth Plant

IV. Discussion

Endophytic bacteria in water hyacinth plants used in this study were obtained as many as 12 isolates of endophytic bacteria. Endophytes are microorganisms that are considered non-pathogenic symbionts found in plants because they do not cause disease symptoms in host plants. Soil and geographic conditions contribute to the type of endophytes isolated from plants [14].

Endophytic bacteria can live in the internal tissues of host plants without damaging host cells, usually living in intercellular spaces containing high amounts of carbohydrates, amino acids, and inorganic nutrients. Endophytic bacteria include a large number of Gram-positive and negative bacteria from the genera Alpha, Beta- and Gamma-proteobacteria, Actinobacteria and Firmicutes. Endophytic bacteria increase plant growth, have the capacity to dissolve phosphate and contribute to the process of nitrogen assimilation in plants [15].

Endophytic bacteria play an important role in the production of bioactive compounds. Bioactive compounds produced by endophytic bacteria such as alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, and azadirachtin natural insecticides. Bioactive compounds synthesized by endophytic bacteria help host plants to develop systemic resistance to pathogens, also used in the pharmaceutical industry as antibiotics, anti-cancer, anti-virus, anti-diabetic and other bioactive compounds [16].

Of the 12 bacterial isolates obtained after screening $AgNO_3$ reducers, there were 3 endophytic bacterial isolates that could reduce $AgNO_3$ into silver nanoparticles (AgNPs) as can be seen in Figure 2. The codes of the isolates that can reduce are isolates with codes BEPC 2, BEPC 3, and BEPC 12. Observations of the results of UV-Vis spectrophotometry can be seen in Figure 3, where in BEPC 2 (wavelength 435.00 nm, absorbance 3.141), BEPC 3 (wavelength 425.50 nm, absorbance 3.042) and BEPC 12 (wavelength 427.50 nm, absorbance 2.227), the process of the mechanism of silver nanoparticle synthesis in bacteria can be seen in Figure 5. This proves that endophytic bacteria found in water hyacinth plants can reduce $AgNO_3$.

One of the steps that can be taken to identify bacteria is by carrying out gram staining, as can be seen in Figure 4, that the 3 isolates that can reduce $AgNO_3$ are included in gram positive, which in Figure 4 shows that the gram staining that was carried out has a purple color.

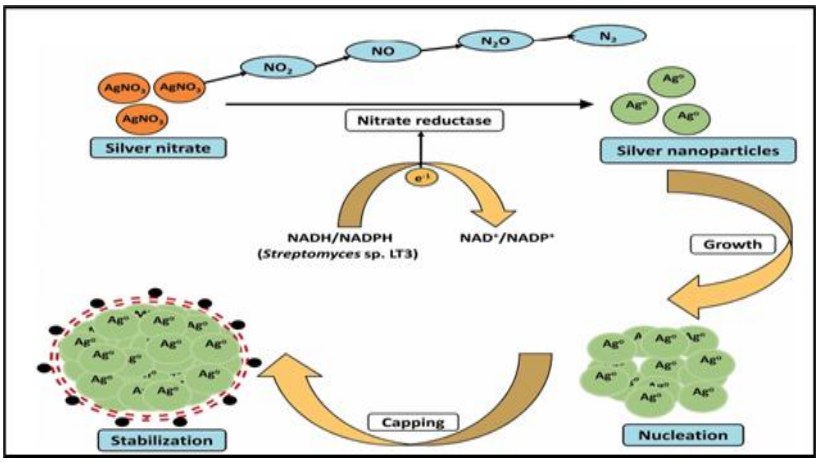

Figure 5 Theoretical mechanism of silver nanoparticle synthesis in bacteria [17]

This research has only reached the screening of silver nanoparticle reducers and still needs to be continued to the production stage in a bioreactor, isolation and characterization of the resulting AgNPs products and their applications in various fields.

V. Conclusion

From the results and discussion above, it can be concluded that there are 12 isolates of endophytic bacteria that have been isolated from water hyacinth plants, namely isolates with codes BEPC 1, BEPC 2, BEPC3, BEPC, 4, BEPC 5, BEPC 6, BEPC 7, BEPC 8, BEPC 9, BEPC 10, BEPC 11 and BEPC 12. From the 12 isolates obtained, screening was carried out as a reducer of AgNO3 to AgNPs, and after the screening was carried out, several isolates of endophytic bacteria were obtained that were able to reduce AgNO3 to AgNPs, as can be seen in the UV-VIS spectrophotometry results above, namely there are three isolates with codes BEPC 2, BEPC 3, and BEPC 12, where the three isolate codes have a higher spectral peak than several other isolate codes, so for further testing.

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