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Screening of Endophytic Bacteria from Surian Leaves (*Toona sinensis* (Juss.) M.roem) as Silver Nanoparticles Reducing Agent

Gemmy Sarina, Dini Hanifa, Armenia, Akmal Djamaan*
(Faculty of Pharmacy, University of Andalas, Padang 25163, West Sumatra, Indonesia)

Abstract:
Background*: The silver nanoparticle (AgNPs) biosynthesis is an interesting issue due to thehigher needs in various fields. The development of environmentally friendly technologies has been made by using primary and secondary metabolite reducing agents from microorganisms and plants. The biosynthesis of AGNPs can be made using only plant extracts, microorganisms or enzymes. The endophytic bacteria is a bacteria that lives and symbiotics with host plants, also produce potential metabolite compounds that can be used as a reductor to produce AgNPs.*
Materials and Methods*: Endophytic bacteria were isolated from Surian leaves (Toona sinensis (Juss.) (M.roem)) obtained from the Medicinal Plant Garden of Andalas University. The bacteria were then purified by the streak plate method based on morphological characteristics. Their ability to reduce silver nitrate ($AgNO_3$) to AgNPs were then screened by mixing each of the5 mL bacterial supernatant with a 15 mL $AgNO_3$ 3 mM solution, and then was shaken at 150 rpm, 37^oC in a dark condition for 72 hours. The change in color of the solution from yellow to brown indicated the formation of AgNPs which was confirmed by UV-Vis spectrophotometry at a wavelength of 300-800 nm.The highest absorbance of nanoparticle suspension indicated the most AgNPs is producted.*
Results*: The results showed that 10 endophytic bacterial were isolated from Surian leaves (BES_01 – BES_10) based on the macroscopic characteristics. Around nine of ten of the bacterial were Gram positive bacteria and only one of them (BES-02) was gram negative bacteria. From the UV-Vis spectrophotometer data, showed that the nanoparticles produced using those bacterial absorb the visible light at the wave length of 405-434 nm with the absorbance (from BES-01 to BES-10) of 2.214, 1.654, 2.547, 2.831, 2.139, 2.653, 2.213, 2.432, 2.299 and 2,293.*
Conclusion*: It is indicated that the ten endophytic bacteria isolate from Surian leaves are potential to be used as bioreductor to produced AgNPs, with the BES-04 as a most potential one.*
Key Word*: Isolation; Endophytic bacteria*; *Toona sinensis (Juss.) (M.roem) Silver nanoparticle; Bioreductor*

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I. Introduction

The development of science and technology is currently growing rapidly, especially in the material field[1]. Nanotechnology is a concern of researchers because of its wide application in the fields of materials science, medical science, biology, physics and chemistry[2]. One of the nanoparticles that are currently being developed is AgNPs. Silver is a metal group that has antimicrobial activity[3]. AgNPs have more potential than silver ions[4]. The development of nanotechnology in silver formulations can modulate metals into nano sizes that drastically change the chemical, physical and optical properties of metals so that their antimicrobial properties are more potential[5].

Synthesis of AgNPs can be carried out by chemical, physical and biological reduction (Green synthesis). Biological synthesis using reducing agent from plant extracts, microorganisms and enzymes have gained a lot of interest because it is environmentally friendly, does not use toxic chemicals, does not require high pressure, temperature and energy and its high compatibility for biomedical use[6].

One of the potential microorganisms as reducing agent of AgNPs is endophytic bacteria. Endophytic bacteria are bacteria that grow in healthy plant tissue and able to protecting plants from phytopathogens also increasing plant growth under normal and challenging conditions[7]. These endophytic bacteria produce secondary metabolites with antimicrobial properties and are thought to have the ability to act as reducing agent of AgNPs. The content of different metabolite compounds from plant extracts or microorganisms results in different nanoparticle properties[8]. In this research, isolation of endophytic bacteria from Surian leaves and screening for AgNPs reducing agent will be carried out.

II. Materials And Methods

Materials

Materials used in this research were $AgNO_3$ (Merck®), aquadest (Bratachem®), antiseptic alcohol 70% (Bratachem®), Nutrient Broth (NB) (Merck®), Nutrient Agar (NA) (Merck®), sodium hypochlorite, violet crystal, lugol, safranin, ethanol 96% and H_2O_2.

Collection of *Toona sinensis* (Juss.) M.roem leaves samples

Toona sinensis (Juss.) M.roem leaves were collected from the Medicinal Plant Garden, Andalas University. The leaves were cut with a sterile knife, washed with sterile aquadest then put into sterile plastic.

Sterilization of *Toonasinensis* (Juss.) M.roem leaves surface

The collected leaves were cut about 1 cm^2. The leaves were disinfected with 70% ethanol for 5 minutes, sodium hypochlorite 2% for 6 minutes, 70% ethanol for 30 seconds to remove sodium hypochlorite and then rinsed three times using sterile aquadest. The aquadest from last rinse then poured into a petri dish containing NA media as control[9].

Isolation and purification of endophytic bacteria

The sterilized leaves were sliced off with a sterile knife and inoculated on NA media and then incubated at 27^oC for 3 x 24 hours. Growing bacterial colonies were purified using the streak plate method on NA medium[9].

Identification of bacterial isolates

Identification of bacterial isolates was done by observing macroscopic and microscopic characteristics of bacteria. Macroscopic observation was carried out by directly observing the colony characteristics of bacterial isolate including: color, shape, edge and elevation of the colony[10]. Microscopic observations were done by Gram staining method.

Production of bacterial isolates stock

All of pure bacterial cultures that had been isolated were inoculated on slant NA and stored at 4^oC as a collection of bacterial isolates.

Cultivation of endophytic bacterial from *Toona sinensis* (Juss,) M. Roem leaves

Each pure bacterial culture was subcultured onto NB media and shaked at 250 rpm for 72 hours at 37^oC.

Screening for silver nanoparticle (AgNPs) reducing agent

To determine the bacteria that able to reduce $AgNO_3$ to AgNPs, screening for $AgNO_3$ reducing agent was carried out. The bacterial culture on NB media was centrifuged at 8000 rpm for 15 minutes to collect the supernatant. 3 mM of $AgNO_3$ solution was prepared using aquadest. A total of 15 mL of 3 mM $AgNO_3$ was mixed with 5 mL supernatant per bacterial culture in a 50 mL Erlemeyer flask. All samples were shaked at 150 rpm, 37^oC and maintained in dark conditions for 72 hours. The change in color of the solution from yellow to brown indicated the formation of AgNPs which later confirmed by UV-Vis spectrophotometry at wavelengths of 300-800 nm. UV-Vis spectrophotometric analysis was carried out in a quartz cuvette using aquadest as a reference solvent. The absorption spectrum at the wavelength region ± 400 nm indicated the formation of AgNPs. The highest absorbance of nanoparticle suspension shows the most production of AgNps [11,12].

III. Results

Isolation of endophytic bacteria from Surian leaves (Figure 1) collected from the Medicinal Plant Garden, Andalas University resulted in 10 isolates based on morphological characteristics (Table 1). The leaves placed on the NA media were surface sterilized and the final rinse water from surface sterilization process was used as a control (Figure 2). Bacterial isolates were purified by the streak plate method (Figure 3).

Figure 1. *Toona sinensis* (Juss.)(M.roem) leaves samples

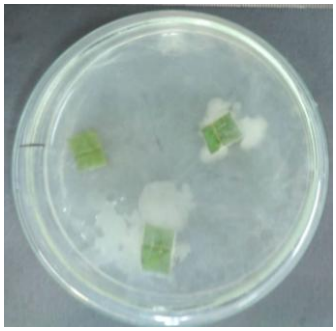
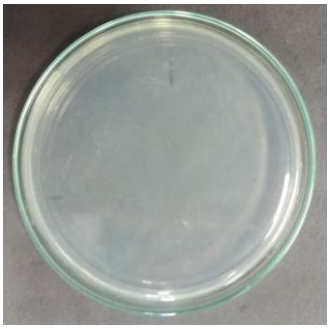
a.  b.  c. 

Figure 2. (a) Sterile Surian leaves on NA medium, (b) Surian leaves after 24 hours incubation, (c) Control

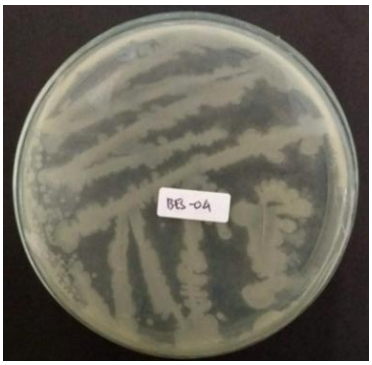


Figure 3: Examples of endophytic bacteria pure isolates from Surian leaves (BES-04)

Table 1. Macroscopic and Microscopic Observations of Bacterial Isolates

Isolate Code	Colony Morphology				Gram Staining
	Coloration	Shape	Edge	Elevation	
BES-01	White	Circular	Undulate	Raised	Positive
BES-02	Yellowish	Circular	Entire	Raised	Negative
BES-03	White	Circular	Undulate	Raised	Positive
BES-04	White	Irregular	Lobate	Raised	Positive
BES-05	White	Irregular	Irregular	Raised	Positive
BES-06	White	Circular	Undulate	Raised	Positive
BES-07	White	Irregular	Lobate	Raised	Positive
BES-08	White	Irregular	Irregular	Raised	Positive
BES-09	White	Irregular	Lobate	Raised	Positive
BES-10	White	Irregular	Irregular	Raised	Positive

Ten bacterial isolates were screened to determine potential bacteria as AgNPs reducing agent. From the results of visual observation screening, the color changes happened from yellow to brown and gave UV-Vis absorbtion at wavelengths between 405-434 nm for all bacterial isolates (Figure 4). From the results of UV-Vis spectrophotometry, the most optimum bacterial isolate as silver nanoparticles reducing bacteria was BES-04 which was a Gram-positive bacterium (Figure 6).

(a). 

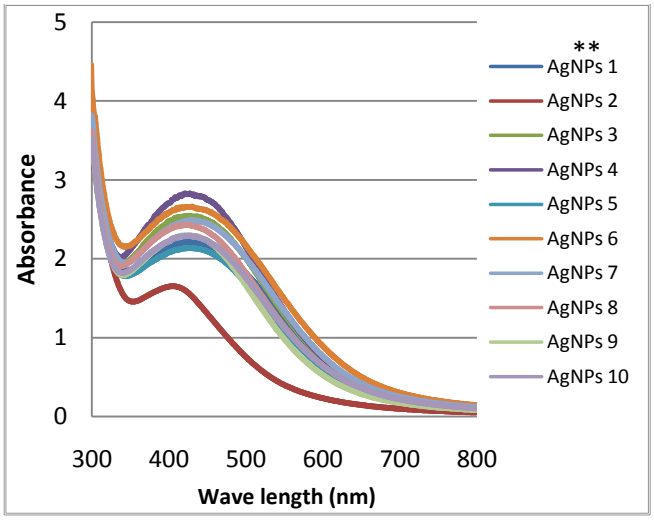
(b). 

Figure 4. (a) Visual color change from yellow to brown (b) The UV-Vis spectra profile of AgNPs

**AgNPs1 :AgNPs biosynthesis results from BES-01
AgNPs2 :AgNPs biosynthesis results from BES-02
AgNPs3 :AgNPs biosynthesis results from BES-03
AgNPs4 :AgNPs biosynthesis results from BES-04
AgNPs5 :AgNPs biosynthesis results from BES-05
AgNPs 6 : AgNPs biosynthesis results from BES-06
AgNPs 7 : AgNPs biosynthesis results from BES-07
AgNPs 8 : AgNPs biosynthesis results from BES-08
AgNPs 9 : AgNPs biosynthesis results from BES-09
AgNPs 10 : AgNPs biosynthesis results from BES-10

Table 2. Absorbance and wavelength of AgNPs

Reducing agent	Wavelength (nm)	Absorbance (a.u)
BES-01	427	2.214
BES-02	405	1.654
BES-03	426	2.547
BES-04	427	2.831
BES-05	426	2.139
BES-06	434	2.653
BES-07	426	2.213
BES-08	420	2.432
BES-09	429	2.299
BES-10	422	2.293

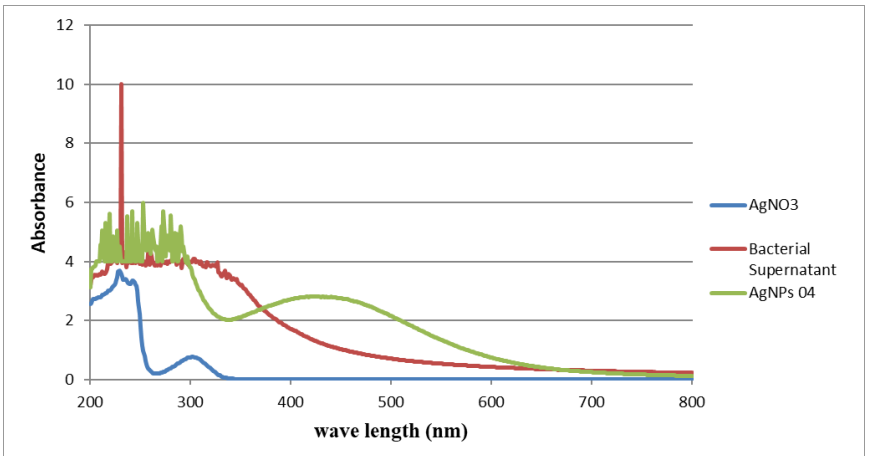


Figure 5. The UV-Vis spectra profile of $AgNO_3$, formed bacterial supernatant and AgNPs 04

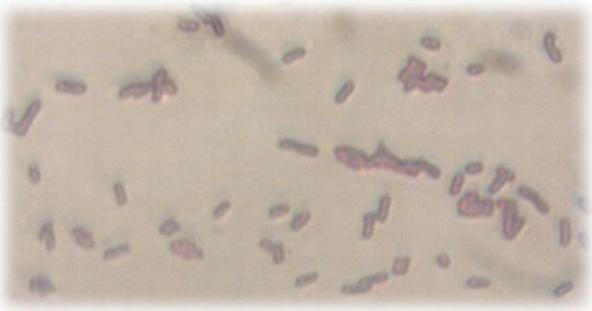


Figure 6. The microscopic profile of BES-04 isolated bacteria after Gram staining

IV. Discussion

Around ten isolates of endophytic bacteria have been successfully isolated from Surian leaves (*Toona sinensis* (Juss.) (M.roem)) taken from the Medicinal Plant Garden, Andalas University, Padang, West Sumatra of Indonesia. Surian leaves were surface sterilized so that other bacteria than endophytic bacteria are sterile from the leaf surface. To be sure, the last rinse water was used as a control (Figure 2). From the results obtained, there was no bacterial growth in the NA media with the addition of last rinse water. About nine out of ten bacterial isolates were Gram positive bacteria and one bacterial isolate was Gram negative bacteria (Table 1).

The isolation of endophytic bacteria from surian leaves aimed to obtain potential bacteria as AgNPs bioreductors. In some literature, various bacterial strains such as *Bacillus amyloliquefaciens*, *Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Escherichia coli* and *Bacillus licheniformis* were used effectively for the synthesis of AgNPs[13].

The bacteria synthesize AgNPs by two methods, which are extracellular and intracellular processes[14]. In the process of biosynthesis, reducing agents and stabilizers are replaced by metabolites produced by endophytic bacteria such as the nitrate reductase enzyme. The nitrate reductase enzyme plays a role in bioreduction of Ag^{+} to Ag^{o}, reducing nitrate to nitrite and nitrite to nitrogen gas (Figure 7)[14,15]. In general, AgNPs biosynthesis has 3 stages, namely reduction, growth and stabilization[16,17].

In this study, researchers conducted AgNPs biosynthesis using an extracellular method at 37^{o}C, using a supernatant of endophytic bacteria as a reducing agent. The resulting AgNPs have UV-Vis absorbtion at wavelength between 405-434 nm (Table 2). Das *et al*. has carried out extracellular biosynthesis of AgNPs at room temperature using *Bacillus cereus* isolated from heavy metal contaminated soil. From this research, the formed AgNPs have UV-Vis absorption at wavelength of 450 nm. Whereas Dani *et al*., carried out AgNPs extracellular biosynthesis using *Bacillus cereus* isolated from Tembagapura Papua soil at 37^{o}C, having UV-Vis absorption at wavelength of 414 nm. The maximum wavelength of absorption is influenced by the shape and size of the nanoparticles. Therefore it can be concluded that the type of bacteria and the conditions of synthesis process can affect the size or shape of the formed nanoparticles.

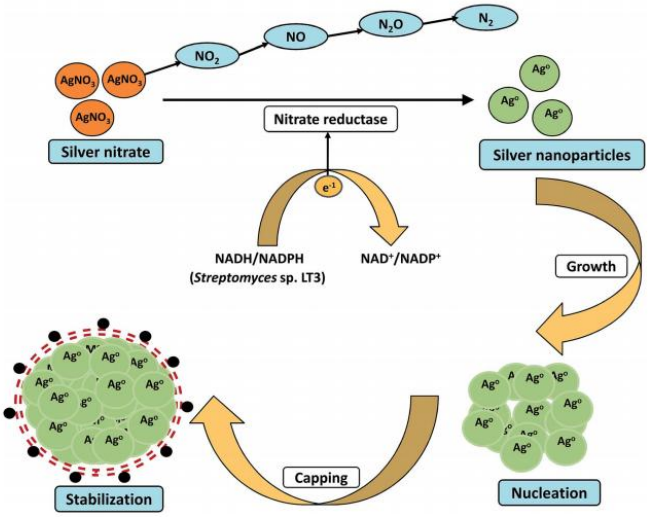


Figure 7. Schematic mechanism of bioreduction and stabilization of silver nanoparticles by the nitrate reductase enzyme[14]

The indicator of AgNPs formation can be seen visually, which is from the change of color from light yellow to brown. This condition then confirmed using a UV-Vis spectrophotometer[20]. The color change occurs due to the reduction of Ag^+ to Ag^0 by compounds present in the bacterial supernatant. The screening result showed that all isolate bacteria (BES-01 to BES-10) were able to reduce $AgNO_3$ to AgNPs. All colloids were changed color from light yellow to brown. The confirmation using UV-Vis spectrophotometer for all colloids showed an absorption in the wavelength area between 405-434 nm which indicated the formed AgNPs.

Each bacterium has a different reduction ability. This can be seen from the absorbance and wavelength values of UV-Vis spectroscopy. From the results obtained, BES-04 was the bacterium that produced the most amount of silver nanoparticles with absorbance value of 2,831 and wavelength of 427 nm. While the bacteria that produce the least amount of silver nanoparticles in the same time period was BES-02 with absorbance value of 1.654 and wavelength of 405 nm. BES-04 was a Gram-positive bacterium with bacilli shape (figure 6). The content of different metabolite compounds from bacterial supernatants resulted in different nanoparticle properties. Biological reducing agents significantly affects physical and chemical properties such as shape, size, zeta potential and stability of formed AgNPs[8].

Figure 5 showed the spectrum of $AgNO_3$, bacterial supernatants and AgNPs. $AgNO_3$ and bacterial supernatants do not have absorption at wavelengths ± 400. Whereas AgNPs showed the characteristic of absorption peaks at wavelength of 427 nm. The phenomenon absorption formation at 427 nm wavelength occurred due to surface resonance of Plasmon (SPR). The SPR peak is very sensitive to the shape and size of the nanoparticles[21].

Future research is expected to optimize the synthesis of AgNPs for BES-04 bacteria, produce a suitable AgNPs formula and conduct pharmacological tests such as application of wound healing medicine.

V. Conclusion

Around 10 endophytic bacteria were isolated from Surian leaves (BES_01 – BES_10) based on the macroscopic characteristics. As much as nine of ten of the bacteria were classified as Gram positive and one of them (BES-02) was Gram negative. From the UV-Vis spectrophotometer data, indicated that the nanoparticles produced using those bacterial absorb the visible light at the wavelength of 405-434 nm. It also observed that the ten endophytic bacteria were isolated from Surian leaves are potential to be used as bioreductor to produced AgNPs, with the BES-04 isolate is the most potential one.

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