Optimization of silver nanoparticles synthesis by the green method using *Streptomyces* sp. SSUT88A and their antimicrobial activity against *Pseudomonas aeruginosa*

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**Abstract.** The green method has become an environmentally safe and valuable alternative to synthesizing silver nanoparticles (AgNPs). The AgNPs has been applied as antimicrobial agents, which their toxicity depends on several variables that generate different ability to inhibit pathogenic bacteria. Therefore, the optimization of AgNPs synthesis plays an important role in providing good antimicrobial activity. In this study, the synthesis of AgNPs was carried out with three different parameters: time of incubation, pH, and temperature to inhibit *Pseudomonas aeruginosa* growth using cell-free supernatant of *Streptomyces* sp. SSUT88A. The UV-Vis spectroscopy and antimicrobial activity were measured to obtain the optimum condition for each condition. The 74.12 nm in the spherical shape of AgNPs were optimized at 37˚C, under pH 7 for five days. The synthesized AgNPs exhibited antimicrobial activity against *P. aeruginosa* 1287 and multidrug-resistance *P. aeruginosa* N90PS.

**Keywords:** AgNPs, antimicrobial activity, optimization, *Pseudomonas aeruginosa*, *Streptomyces* sp. SSUT88A

1. **Introduction**

*Pseudomonas aeruginosa* is aerobic gram-negative bacteria that belongs to the family of *Pseudomonadaceae*. This bacterium is known as an opportunistic pathogen microorganism. *Pseudomonas aeruginosa* is a prevalent source of hospital-acquired infections and is particularly dangerous in the patients of intensive care units (ICU). *Pseudomonas aeruginosa* infections have been linked to a high risk of morbidity and mortality in various populations, especially patients with healthcare-associated pneumonia, chronic lung illness, and cystic fibrosis. [1-3]. Because of its innate resistance to several antibiotics (β-lactam and penem groups of antibiotics) and its ability to acquire additional resistance mechanisms to multiple classes of antibiotics, including Beta-lactams, aminoglycosides, and fluoroquinolones, it is difficult to treat infections produced by this bacterium. On
the other hand, *P. aeruginosa* contains a wide range of intracellular and extracellular factors that offer its virulence pathogenesis, allowing the pathogen to be extremely adaptable [4, 5]. Antibiotics misuse has led to antibiotic-resistant microorganisms for whom there are limited therapeutic alternatives [1]. *Pseudomonas aeruginosa* was included in the critical category of priority pathogen list by World Health Organizations (WHO), in which new antimicrobial agents research and development are urgently needed [6].

Currently, nanotechnology-based therapies have opened up new opportunities in the combat against multidrug-resistance bacteria [7, 8]. It deals with materials with lengths ranging from 1 to 100 nanometres [9]. Silver nanoparticles (AgNPs), known as a powerful antimicrobial agent, have received much interest [10, 11]. Due to their antimicrobial capabilities, AgNPs have been applied in the biomedical field as drug and gene delivery, diagnostics, biosensors, medical devices, and implant materials [12, 13]. The development of methods for AgNPs synthesis is currently a significant problem due to its high reactivity and low stability [9]. The synthesis of AgNPs can be done in three ways: physically, chemically, or biologically. However, several disadvantages such as using toxic chemical agents and expensive were found in the chemical and physical method, respectively [11, 14-16]. The utilization of bacterial extracts in the green production of AgNPs has opened up new nanotechnology possibilities in recent years. Due to its simple, cost-effective, high-yielding, and environmentally friendly methods, this approach may be a possible option for synthesizing AgNPs [14]. Bacteria are useful microorganisms for studying how AgNPs are synthesized. They are abundant, fast-growing microorganisms that easily adapt and regulate the growth condition [17]. Essential physicochemical factors that govern the antimicrobial action of AgNPs are their size, charge of surface, shape, colloidal state, and concentration of AgNPs [11, 16]. Therefore, the condition during AgNPs synthesis needs to be optimized.

The synthesis of AgNPs in this study was conducted by utilizing the green technique using *Streptomyces* sp. SSUT88A. The conditions were optimized by modifying incubation time, pH, and temperature during the synthesis process. UV-Visible spectroscopy, dynamic light scattering (DLS), scanning electron microscope (SEM), and Energy dispersive spectroscopy (EDS) were used to characterize the synthesized AgNPs. The antimicrobial properties against non-resistant and multidrug-resistant *P. aeruginosa* were also carried out.

2. Material and methods

2.1. Chemicals

AgNO$_3$ was purchased from POCH SA™, Poland. Mueller Hinton Agar (MHA), yeast extract, malt extract, and D-glucose were obtained from HIMEDIA, India.

2.2. Microorganisms

*Streptomyces* sp. SSUT88A was isolated from a soil sample at Sakaerat Environmental Research Station, Thailand. The *P. aeruginosa* 1287 and multidrug-resistance *P. aeruginosa* N90PS were employed to estimate the antimicrobial activity of synthesized AgNPs. The multidrug-resistant *P. aeruginosa* N90PS was isolated from a clinical sample at Suranaree University of Technology, Thailand.

2.3. AgNPs biosynthesis

*Streptomyces* sp. SSUT88A was cultured on 100 mL of ISP2 liquid medium consist of (g/L): 4 g yeast extract, 10 g malt extract, and 4 g D-glucose. The isolate was then cultured at 37 °C under 200 rpm agitation for three days. The cell mass of the *Streptomyces* sp. SSUT88A was collected and washed with sterile water three times. The cell filtrate was incubated at sterile water at 37 °C for 24 h. The cell-free supernatant was collected by filtration through the Whatman filter paper No. 1 (Whatman, England). For the AgNPs synthesis procedure, a silver source of 1 mM silver nitrate (AgNO$_3$) in distilled water was used. Sets of the reaction mixtures (20 mL) containing cell-free supernatant and 1 mM AgNO$_3$ in
the ratio of 1:1 were prepared under different pH (6, 7, and 8) and incubated under the dark condition at 30 and 37 °C for 7 days.

2.4. UV-Visible spectroscopy
The obtained AgNPs were characterized using a UV-Visible spectrophotometer (Thermo Scientific Multiscan GO, Finland) in the range of 300 to 600 nm from day 0 to day 7. The surface plasmon resonance of AgNPs ranges from 380 to 450 nm.

2.5. Scanning electron microscopy (SEM) and Energy dispersive spectroscopy (EDS) analysis
The morphology of optimized AgNPs was studied using a scanning electron microscope (SEM, JEOL model JSM800F, Japan). EDS (OXFORD Instrument) was used to confirm elemental silver and other chemical compositions in the sample.

2.6. Dynamic light scattering (DLS) analysis
The Zeta sizer instrument measured the zeta potential and hydrodynamic particle size distribution of AgNPs (Malvern Instrument Corp, 28 Malvern, USA).

2.7. Antimicrobial activity assay
The antibacterial activity of synthesized AgNPs was evaluated against non-resistant P. aeruginosa 1287 and multidrug-resistant P. aeruginosa N90PS using an agar well diffusion assay. The 5 × 10^5 CFU/mL of mid-log phase test pathogens were seeded onto MHA plates. Then, a 6-8 mm hole was punched aseptically, and a volume of 100 µL of 0.2 mg/mL AgNPs samples was introduced into the well. The plates were incubated at 37°C. After 24 h, antibacterial activity was recorded by measuring the diameter of the zone of inhibition. The experiments were conducted in triplicate.

2.8. Statistical analysis
The statistical analysis of the data was executed using an independent sample t-test and one-way analysis of variance (ANOVA) using IBM SPSS Statistics Version 23.

3. Results

3.1. Synthesis of AgNPs
The synthesis of AgNPs was carried out by mixing 1 mM AgNO₃ with cell-free supernatant of Streptomyces sp. SSUT88A. Visual observation of cell-free supernatant incubated with AgNO₃ showed a color change from yellow to brown (figure 1a). The UV-Visible absorbance was employed to indicate the synthesis process in this investigation. The surface plasmon resonance of AgNPs was confirmed by the UV-Vis spectrum, which displayed a single broad peak at 418 nm (figure 1b).

Figure 1. Synthesized AgNPs. a). Visual color change of the mixture and b). UV-Visible spectrum of AgNPs.
3.2. The effect of temperature and incubation time

The effects of reaction temperature and incubation time on the speed of bio-reduction of Ag\(^+\) to Ag\(^0\) were substantial in optimization studies. The absorbance at 418 nm was observed during synthesis every 24 h for seven days. At a fixed AgNO\(_3\) concentration of 1 mM, variation in the reaction was observed to synthesize AgNPs by varying temperatures, 30 ºC and 37 ºC (figure 2). Since the change in the color of the mixture corresponded to the AgNPs formation, the UV-Vis spectra were recorded from day 0 to day 7 per 24 h intervals (figure 2). Optimum synthesis of AgNPs occurred at a temperature of reaction 37ºC. The UV-Vis spectra from both mixtures exhibited an increase in peak intensity as well as an increase in time. After 5 and 6 days at 37ºC and 30ºC, respectively, there was no change in peak intensity, indicating that the reaction was completed.

![Figure 2. UV-Vis spectra of AgNPs synthesis from different temperatures.](image)

The inhibition zone formation revealed the antimicrobial activity of AgNPs that measured in millimeters (mm). The inhibition zone was formed in both synthesized AgNPs from day 5 reaction against *P. aeruginosa*, as shown in table 1. The synthesized AgNPs from the mixture at 37ºC exhibited significant antimicrobial activity against the tested pathogen than the mixture at 30ºC. The inhibition zone was 22±1 mm and 19±3.6 mm on *P. aeruginosa* 1287 and multidrug-resistant *P. aeruginosa* N90PS.

| AgNPs Sample | *P. aeruginosa* 1287 | *P. aeruginosa* N90PS |
|--------------|----------------------|-----------------------|
| Temp 30 ºC   | 15±1\(^a\)           | 11±2\(^a\)            |
| Temp 37 ºC   | 22±1\(^b\)           | 19±3.6\(^b\)          |

Values are mean±SD (n=3). Superscript indicated a significant difference (p<0.05).

3.3. The effect of pH and incubation time

The effects of incubation time and pH on the bio-reduction of Ag\(^+\) to Ag\(^0\) also played a significant role in the optimization studies. The pH variation of 6, 7, and 8 was used for the AgNPs synthesis reaction. Compared to the UV-Vis spectrum obtained at 418 nm from synthesized AgNPs, the optimum synthesis of AgNPs occurred at the mixture with pH 7, followed by a mixture of pH 8 and 6 (figure 3). Based on the UV-Vis spectra at 418 nm, the mixture with pH 7 showed a higher absorbance than others (figure 3).
Figure 3. UV-Vis spectra of synthesized AgNPs from different pH.

The antimicrobial activity of synthesized AgNPs at day 5 was tested against *P. aeruginosa* 1287 and multidrug-resistant *P. aeruginosa* N90PS, which showed in table 2. With inhibition zones of 22 mm and 19 mm, respectively, the mixture at pH 7 demonstrated the best antibacterial effect against *P. aeruginosa* 1287 and multidrug-resistant *P. aeruginosa* N90PS. However, it did not show any significant difference in antibacterial activity with the mixture at pH 8.

**Table 2. Antimicrobial activity of AgNPs in different pH of synthesis.**

| AgNPs Sample | *P. aeruginosa* 1287 | *P. aeruginosa* N90PS |
|--------------|---------------------|-----------------------|
| pH 6         | 12±1                | 9±3                   |
| pH 7         | 22±2                | 19±3                  |
| pH 8         | 20±3                | 16±1                  |

Values are mean±SD (n=3). Superscript indicated a significant difference (p<0.05).

3.4. Characterization of optimized AgNPs

The AgNPs synthesis using *Streptomyces* sp. SSUT88A was optimized at 37ºC, under pH 7 for 5 days. The synthesized AgNPs are spherical form, as evidenced by SEM observation (figure 4a). Figure 4b showed the EDS spectrum of AgNPs at 3 keV indicated the presence of elemental AgNPs. Other signal spectra of C, O, and Na were also observed. It might be due to the signal of biological molecules that appear in the cell-free supernatant. The result of size determination using DLS showed the average hydrodynamic size of AgNPs was 74.12 nm (figure 4c) with the zeta potential value of -31 mV (figure 4d).

4. Discussion

Because of its unique features, the preparation of AgNPs is getting a lot of attention these days. Metal nanoparticles have been synthesized using various physical and chemical approaches; nonetheless, a simple and environmentally friendly method must be developed [15, 16, 18]. This study was carried out on the synthesis of AgNPs by *Streptomyces* sp. SSUT88A isolated from Sakaerat Environmental Research Station, Thailand. Without the use of toxic ingredients, biological synthesis was an environmentally friendly method. *Streptomyces* are nano factories for generating non-toxic and clean AgNPs manufacturing methods [15, 19, 20]. The appearance of the color change of the mixture from yellow to brown was a clear indication of the formation of AgNPs, due to the surface plasmon resonance of AgNPs during the synthesis [21, 22]. Mohamedin et al. [23] reported a similar finding in *Streptomyces*
viridodiastaticus SSHH-1 for AgNPs production. In the current study, UV-Visible absorbance was used to indicate the synthesis process. AgNPs have a peak between 410 and 440 nm, common for metal nanoparticles with sizes of 2 to 100 nm. [24, 25]. In the current study, the maximum absorbance was observed at 418 nm, which was confirmed their hydrodynamic size using DLS, and it found the average hydrodynamic diameter was 74.12 nm. A similar result was also reported on the synthesis of AgNPs using *Streptomyces rochei* MLM13 which showed maximum absorbance at 410 nm with an average size of 85 nm [22].

![Figure 4. Characterization of AgNPs. a) Morphology of AgNPs; b). Elemental composition of AgNPs; c) Size of AgNPs; and d). Zeta potential of AgNPs.](image)

During the synthesis, the temperature and pH play a significant role in the optimization process. Compared with the UV-Vis spectrum obtained from synthesized AgNPs at 418 nm, the reaction at 37°C showed a higher absorbance than the mixture from 30°C. Khalil *et al.*, Soman and Ray, and Kredy [26-28] found that the synthesis of AgNPs was made possible by increasing the reaction temperature, which resulted in a rapid decrease of Ag⁺ and subsequent homogeneous nucleation of silver nuclei. In the end, it is widely assumed that a higher temperature promotes nucleation, whereas a low temperature promotes growth [29].

On the other hand, the mixture’s pH plays a vital role in synthesizing controlled shaped and sized AgNPs. Because the nucleation process for AgNPs in an acidic environment is slow, only a small number of particles may form. While the accessibility of -OH ions at high pH led to a rapid nucleation process, resulting in many particles [30]. In the current study, the optimum pH for the synthesis of AgNPs using cell-free supernatant of *Streptomyces* sp. SSUT88A was 7. A similar report on the synthesis of AgNPs using *Givotia moluccana* leaf extract showed that at neutral pH, the formation of AgNPs occurs rapidly because the phenolic group present in the leaf extract is ionized [31].
Zeta potential analysis was used to decide the long-term stability of nanoparticles and the surface charge of the nanoparticles. A stable nanoparticle shows the zeta potential value of more than +30 mV or less than -30 mV [32-34]. In the present study, the zeta potential value was -31 mV. The zeta potential measurement indicated that AgNPs have a negative surface charge and are well dispersed in the water. The presence of the biological molecules in the cell-free supernatant was affecting the zeta potential value since its molecules are involved in the capping and stabilizing agents [16, 35]. Scandorizeiro et al. [36] observed a negative zeta potential value in the biosynthesis of AgNPs using Fusarium oxysporum, and Ninganagouda et al. [37] reported a negative zeta potential value in the biosynthesis of AgNPs using Aspergillus niger. Dispersions with a low zeta potential value will eventually agglomerate due to interparticle attractions. On the other hand, the zeta potential of nanoparticles is highly influenced by the pH and electrolyte concentration of the nanoparticle [38].

In the current study, synthesized AgNPs showed antimicrobial activity against multidrug-resistant P. aeruginosa N90PS. It has been known that treatment of drug-resistant P. aeruginosa necessitates significant investment in the development of highly effective and safe bactericides [39]. Carbapenems are the possible option for treating multidrug-resistant P. aeruginosa at the moment [40]. AgNPs are metal nanoparticles with antimicrobial action and were effective against both Gram-positive and Gram-negative bacteria [41-43]. The antibacterial properties of AgNPs are related to the particle size [11, 39, 44]. Several biologically synthesized AgNPs are effective against multidrug-resistant pathogens such as Staphylococcus aureus, Acinetobacter baumannii, and P. aeruginosa [45, 46]. The ability of AgNPs to lyse the bacterial cell wall, enabling the cytoplasm content to leak, blocking the respiratory chain, and damage the DNA, explains their bactericidal and bacteriostatic capabilities [39, 47-49].

5. Conclusion
A green technique was used to synthesis AgNPs using cell-free supernatant of Streptomyces sp. SSUT88A. The reduction rate of Ag⁺ to Ag⁰ takes place at an optimized temperature of 37 °C under pH 7 for 5 days. The AgNPs had a spherical form and -31 mV of zeta potential, with an average hydrodynamic size of 74.12 nm. The AgNPs showed antimicrobial activity against P. aeruginosa 1287 and multidrug-resistant P. aeruginosa N90PS.

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