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Antibacterial Activity of Silver Nanoparticles Synthesized Using Tyrosine as Capping and Reducing Agent

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ABSTRACT

Synthesis and antibacterial activity of silver nanoparticles (AgNPs) using tyrosine as a capping and reducing agent has been performed. The purpose of this research was to understand the effect of silver nanoparticles on antibacterial activity. AgNPs synthesis was performed by combining silver nitrate solution (AgNO3) as a precursor to amino acid tyrosine and heating it in a boiling water bath until the appearance of a color shift from colorless to yellow. Antibacterial activity test of silver nanoparticles (AgNPs) was performed with the bacteria *Bacillus subtilis* and *Escherichia coli* using the well diffusion method in solution. The well diffusion method was carried out by making a hole in the agar base media with a diameter of 6 mm. TEM characterization showed the formation of AgNPs has been successful. It was also known that the AgNPs have a round shape with an average particle size of 13.628 nm. $AgNO_3$ solution had antibacterial activity greater than silver nanoparticles. Ag^+ ions were converted into silver nanoparticles to reduce the toxic properties of Ag^+ so that it was safe for human health. The antibacterial activity in solution has a stronger effect against *Bacillus subtilis* (gram-positive) than *Escherichia coli* (gram-negative).

Key words: antibacterial activity, capping agent, reducing agent, silver nanoparticles, tyrosine.

1. INTRODUCTION

Nanotechnology is the technology that manipulates and controls of materials on the nanoscale using scientific knowledge of various industrial and biomedical applications [1]. The nanoparticle is a part of nanotechnology which is very popular with benefits and effects with multifield benefits and effects including environmental sustainability, etc, biomedicine, health care, agriculture, food, textiles, industry, electronics, and energy [2]. One of the most widely used nanoparticles in the medical field is silver nanoparticles (AgNPs) because they show the variation of extensive antibacterial activity [3]. Silver nanoparticles (AgNPs) have good antibacterial activity because of their large surface that allows direct contact with microorganisms. Silver nanoparticles (AgNPs) have been used for medical treatment without causing toxic effects on human skin. The antimicrobial activity of silver nanoparticles is influenced by several parameters such as composition, size, shape, chemical function, and surface charge, which are strongly influenced by the method of formation silver nanoparticles (AgNPs) [4].

Generally, the methods used for the synthesis of silver nanoparticles (AgNPs) are conducted using physicochemical techniques such as gamma-ray radiation, autoclaves, chemical reduction, laser ablation, microwave irradiation, electrochemical techniques, use of microemulsions, and photochemical reduction [5]. Among these methods, the chemical reduction method was chosen as the most effective method for producing silver nanoparticles. This is because the steps work easy, fast, inexpensive, and use low temperatures. In the chemical reduction method, metal ions are reduced by reducing substances by adding protective substances to stabilize nanoparticles. The stability of nanoparticles has a very important role especially when nanoparticles are characterized and applied in a product. Some stabilizers that are commonly used are chemical polymer materials such as PEG (polyethylene glycol), SDS (sodium dodecyl sulfate) [6] dan PVA (polyvinyl alcohol). The most common reducing agents used in the formation of silver nanoparticles are sodium borohydride, sodium citrate, hydrazine [7]. This reduction method has been done by another researcher [8]. Guzman et al. [8] uses silver nitrate as a bulk, hydrasin hydrate as a reducing agent and two stabilizing agents namely sodium citrate and SDS. Silver nitrate and sodium citrate were used. The sodium citrate is a reducing agent and stabilizing agent as well. The synthesis of nanoparticles was done using silver nitrate and p-Aminobenzoic Acid as a reducing agent and stabilizing agent. The synthesis was carried out at optimum conditions, namely pH 11 with a reaction time of 30 minutes and producing nanoparticle 12±7 nm.

The synthesis of nanoparticles is carried out using different reducing agents and stabilizers, but the chemicals used in the process are often toxic and dangerous to the environment, so they were not suitable for many applications. This creates

various green chemistry methods for the synthesis of AgNPs that were developed using biological sources environment-friendly and biocompatible. The sources are plant extracts, enzymes, microorganisms, and various biopolymers such as carbohydrates and proteins. Therefore, in this study, the synthesis of silver nanoparticles (AgNPs) was carried out using an environment-friendly material, the amino acid tyrosine, in which the amino acid tyrosine can act as a reducing agent and a stabilizer as well. Tyrosine is one of the amino acids which is needed for is needed for body growth because it can produce biomolecules and vitamins [9]. This amino acid acts as a reducing and capping agent in the silver nanoparticles (AgNPs) synthesis. It was reported that the utilization of tyrosine can influence the shape and the size of silver nanoparticles (AgNPs) [10]. Tyrosine has a phenolic group that acts as a reducing agent under alkaline conditions [11]. During the reduction process, the tyrosine molecule can also act as a capping agent to stabilize silver nanoparticles (AgNPs) in aqueous solutions. The Ag^+ ion was reduced to Ag^0 and changed in color from colorless to yellow [12].

Silver nanoparticles (AgNPs) have a crystal structure, and antimicrobial properties are very good, so they have been used for the treatment of medical diseases for more than 100 years, do not cause toxic to human skin. It is well known that Ag ions and Ag-based compounds are very toxic to microorganisms. It showed that the effect of biocidal is strong in 12 species of bacteria including *Escherichia coli*. For a long period of time, silver has been used to control bacterial growth in a variety of applications including dental care and burns. Silver nanoparticles (AgNPs) also have inhibitory effects on microorganisms and bactericides. It can be expected that large specific areas and the high atomic surface fraction of silver nanoparticles (AgNPs) will cause AgNPs to have high antimicrobial activity compared to bulk silver metal [13].

Silver nanoparticles (AgNPs) can be modified for better efficiency to facilitate its application. Silver nanoparticles (AgNPs) can be used as effective growth inhibitors in various microorganisms. Based on the differences in absorbing gram dyes, bacteria are divided into two groups, namely gram-positive bacteria and gram-negative bacteria. *Bacillus subtilis* is a group of bacteria of the family *Bacillaceae* that live in the human digestive tract and is pathogenic. These bacteria are gram-positive bacteria, rod-shaped, forming chains and spores, aerobic and often found in soil, water, air, and plants [14]. The infections caused by an explosion to *Bacillus subtilis* are meningitis, endocarditis, eye infections, and others [15]. *Escherichia coli* bacteria is one of the rod-shaped gram-negative bacteria and one of the aerobic or anaerobic facultative bacteria [16]. *Escherichia coli* has the biggest number in the digestive bacteria found in the digestive tract of livestock, especially poultry with value at 10^4-10^5 CFU/ml [17]. *Escherichia coli* can cause diarrhea because it produces enterotoxins known as Entero Toxigenic *Escherichia coli* (ETEC). *Escherichia coli* can also cause urinary tract infections in young women. The use of bacteria *Bacillus subtilis* and *Escherichia coli* because these two bacteria are pathogenic bacteria and can cause infections of the digestive tract in humans and animals [18]. Gram-negative bacteria have a lipid-rich outer membrane (as well as a plasma membrane) and a thin peptidoglycan layer. Gram-positive bacteria do not have an outer membrane, but are surrounded by layers of peptidoglycan much thicker than those present in Gram-negatives. Long anionic polymers threading through these layers of peptidoglycan have been called teichoic acids.

In this research, the synthesis of silver nanoparticles (AgNPs) was carried out using the amino acid tyrosine as a reducing and capping agent. The tyrosine amino group will reduce Ag^+ ions to Ag^0 and the carboxylate group will stabilize the AgNPs formed. The ability of the antibacterial activity of silver nanoparticles (AgNPs) is influenced by the physical characteristics of the nanoparticles such as size, shape, and surface properties. Therefore, synthesized silver nanoparticles (AgNPs) were characterized using UV-Vis Spectrophotometer and TEM. Colloidal silver nanoparticles (AgNPs) will be used to test antibacterial activity against gram-positive bacteria, *Bacillus subtilis* and gram-negative bacteria, *Escherichia coli*.
There are some application of nano particles in other areas [19-21]

2. EXPERIMENTAL

Synthesis of silver nanoparticles (AgNPs) in this research was performed out by reacting $AgNO_3$ using tyrosine amino acids as reducing and capping agent, so it can determine the effect of silver nanoparticles on antibacterial activity. The antibacterial activity test was carried out using 2 bacteria i.e *Bacillus subtilis* is gram-positive bacteria and *Escherichia coli* is gram-negative bacteria.

2.1 Materials

The materials used in this research were commercially obtained from Merck i.e. silver nitrate as a precursor of silver nanoparticles (AgNPs), hydrochloric acid, and potassium hydroxide. Commercially obtained tyrosine from Himedia was used as a reducing and capping agent. All reagents were used as received without further purification. Material for antibacterial testing was obtained from the Faculty of Veterinary Medicine UGM including agar solution, Nutrient Broth (NB), Mueller Hilton Agar (MHA), gentamicin antibiotics, alcohol 70 %, aquades, *Bacillus subtilis* as gram-positive bacteria and *Escherichia coli* as gram-negative bacteria.

2.2 Instrumentation

The characterization of silver nanoparticles (AgNPs) was performed by using *UV-Vis Spectrophotometer* (Shimadzu

Pharma Spec UV-1700) and *Transmission Electron Microscope* (TEM) JIOL series JEM-1400.

2.3 Procedure

A. Synthesis of silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were prepared in solution by chemical reduction method, using silver nitrate ($AgNO_3$) and tyrosine amino acid. Synthesis of silver nanoparticles (AgNPs) was performed by mixing 5 mL of 0.5 mM $AgNO_3$ solution with 5 mL of 3 mM tyrosine amino acid at pH 11 (volume ratio 1:1) and heating it in a boiling water bath for 45 minutes. The boiling was maintaine until appearance by the color was changed into yellow. This Silver nanoparticles synthesized was at optimum condition.

B. The Characterization of silver nanoparticles (AgNPs)

Silver nanoparticles (AgNPs) were characterized by UV-Vis Spectrophotometer in the wavelength range from 300 to 700 nm. Analysis of *Transmission Electron Microscope* (TEM) to visualize the shape and determine the size of the silver nanoparticles (AgNPs) synthesized.

C. Antibacterial activity

Preparation of media and materials

The media used consisted of 3 types was agar dilution, Mueller Hinton Agar (MHA) and Nutrient Broth (NB). Agar dilution is 1.8% agar in aquades. Preparing the Mueller Hinton Agar (MHA) is done by dissolving 4.5 g of Mueller Hinton Agar (MHA) in 250 distilled water. Each petri dish is filled with 50 mL of Mueller Hinton Agar (MHA). The test media was prepared by dissolving 0.4 g Nutrient Broth (NB) and 0.75 g agar in 50 mL of aquadest. Then Nutrient Broth (NB) prepared for inoculation, consisting of 0.048 g Nutrient Broth (NB) in 6 mL of distilled water. Nutrient Broth (NB) is prepared in a test tube and a base medium and the test is prepared in Erlenmeyer. All media covered with cotton, aluminum foil, paper and then wrapped in plastic. Samples to be tested for antibacterials are $AgNO_3$ solution, tyrosine amino acid solutions, and AgNPs solutions with various concentrations of $AgNO_3$, which are purified first.

D. Sterilization of tools and materials

Tools and media such as agar dilution, Mueller Hinton Agar (MHA) and Nutrient Broth (NB) are sterilized by autoclave for ± 1 hour at 121 °C.

E. Antibacterial activity test

First, inoculation was carried out by adding 1 mL of each of *Bacillus subtilis* and Escherichia *coli* bacteria into the Nutrient Broth (NB). Bacteria were incubated for 24 hours at 37 °C. Second, Mueller Hinton Agar (MHA) is poured into each petri dish until it hardens. After that, the bacteria are spread evenly to the base media using a cotton bud. The media were made wells each ± 6 mm in diameter, then injected with 50 µL of sample solution using a micropipette. After adding the test compound, incubation was then carried out for 24 hours at 37 °C. Antibacterial activity was calculated based on the inhibitory zone in mm unit diameters using the bar.

3. RESULTS AND DISCUSSION

In this research, silver nanoparticles were synthesized using the amino acid tyrosine as a reducing agent and stabilizer. The synthesis of silver nanoparticles has been successfully carried out under optimum conditions were pH 11 for 45 minutes. At pH 11 or under alkaline conditions, AgNPs can be formed. The tyrosine phenolic group will be protonated and form a negative charge under alkaline conditions, then the negative group will interact with positive metal ions which cause polarizability. Optimal tyrosine concentration of 3 mM to 0.5 mM $AgNO_3$. The optimal mole ratio of 0.5 mM $AgNO_3$ to tyrosine 3 mM is 1: 6. The optimal concentration of tyrosine 3 mM shows the absorbance peak absorption at the UV-Visible spectrophotometer is at a wavelength of 380 - 430 nm. This shows that at optimal tyrosine concentrations, the amount of tyrosine is sufficient to reduce Ag^+ particles in solution [22].

Saragih et al., [22] investigated the stability of silver nanoparticles indicates that no absorption peaks were shifted and widened. The absorbance of colloidal solution silver nanoparticles (AgNPs) absorption began to decrease at the measurement time of 6 days. The decrease in absorption intensity is due to a decrease in the number of silver nanoparticles, but the particle size does not increase and aggregation does not occur.

3.1 The Characterization of Silver Nanoparticles (AgNPs)

The silver nanoparticles (AgNPs) synthesized at optimal conditions was carried out until the color change from colorless to yellow. The color change indicates the formation of silver nanoparticles (AgNPs) and Ag^+ ions reduction to Ag^0 [12]. Metal nanoparticles have free electrons that can produce surface plasmon resonance (SPR) absorption bands. This is due to the reciprocal vibration of electrons from metal nanoparticles that are resonance with lightwave. The result of the peak shows the characteristics of the surface resonance of AgNPs [23]. The characterization of the color change that occurs showed in Figure 1.

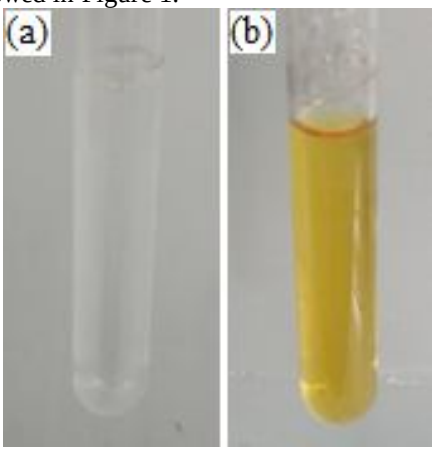

Figure 1: The color of AgNPs synthesis (a) before heating, and (b) after heating

The result of the UV-Vis absorbance spectrum obtained that a peak associated with surface plasmon resonance occurs at a wavelength maximum of 410 nm. According to Maliszewska & Sadowski [14], Silver nanoparticles (AgNPs) made by the reduction method show the presence of surface plasmon absorption bands with a maximum wavelength of around 400 nm. The peak with this wavelength range indicates the presence of silver nanoparticles (AgNPs) in solution. The absorbance band spectra of silver nanoparticles (AgNPs) produced at optimal conditions are shown in Figure 2.

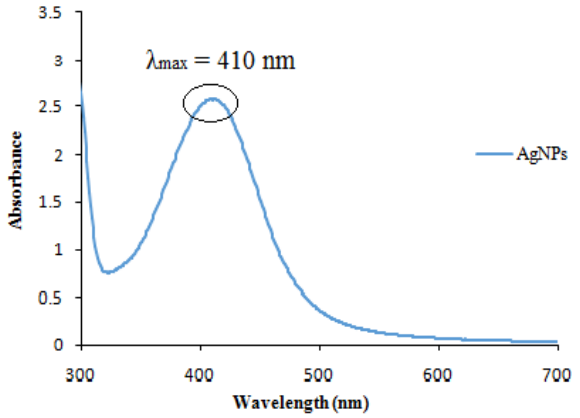

Figure 2: The absorption band spectra result from a reaction of synthesis AgNPs at optimal conditions

Analysis of *Transmission Electron Microscopy* (TEM) was used to determine the morphology and particle size of silver nanoparticles. The results of the TEM analysis were shown in Figure 3.

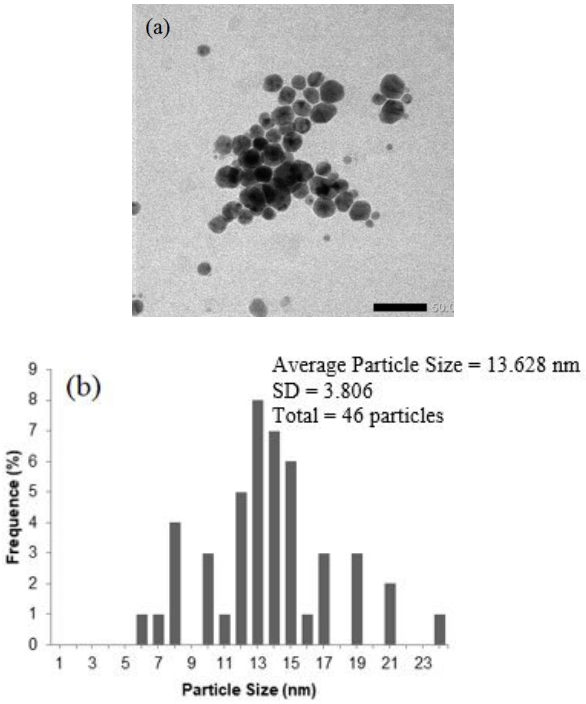

Figure 3: The TEM images (a) and particle size distribution (b) of synthesis AgNPs at optimal conditions

The results of TEM analysis showed that silver nanoparticles (AgNPs) have a round shape with almost the same size and no aggregate was formed. The appearance of absorption around 400 nm wavelength indicates that the particle has a spherical shape [6]. The particle size was analyzed using image-J software. The average size of silver nanoparticles (AgNPs) was 13,628 ± 3.8 nm. It showed that silver particles have size at the nanoscale (<100 nm). Moreover, aniline reducing agent to produce a round shape measuring 10-30 nm [24].

3.2 Antibacterial Activity

Antibacterial activity test of silver nanoparticles (AgNPs) was performed with the bacteria *Bacillus subtilis* and *Escherichia coli* using the well diffusion method in solution. The well diffusion method was performed by making a hole in the agar base media with a diameter of 6 mm. The solution used was injected into the hole. The antibacterial activity test was characterized by the appearance of a clear zone around the solution, the results of the antibacterial activity were shown in Figure 4 and Figure 5.

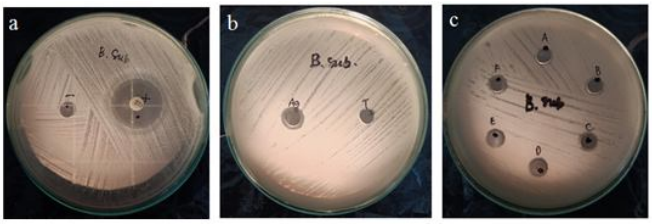

Figure 4: The results of antibacterial activity in *Bacillus subtilis* (a) control (+) and (-) (b) $AgNO_3$ and tyrosine solutions (c) variations in AgNPs concentration

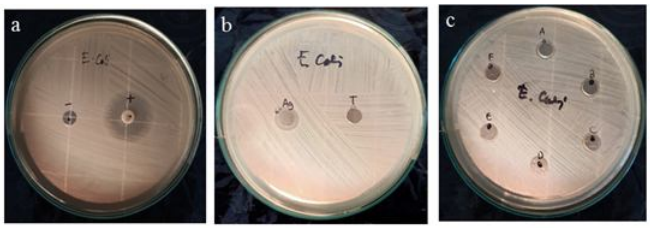

Figure 5: The results of antibacterial activity in *Escherichia coli* (a) control (+) and (-) (b) $AgNO_3$ and tyrosine solutions (c) variations in AgNPs concentration

The results of the antibacterial activity in Table 1 showed the presence of inhibitor zones, both in *Bacillus subtilis* and *Escherichia coli* bacteria. Gentamicin was used as a positive control on antibacterial and sterile aquades is used as a negative control on antibacterial. A positive control had inhibitory activity against bacteria, while negative control showed no inhibition zones around the sample.

Table 1: The results inhibition zone diameters of antibacterial activity

Sample	Inhibition Zones (mm) *Bacillus subtilis*	Category of antibacterial activity	Inhibition Zones (mm) *Eschericia coli*	Category of antibacterial activity
Controll (+)	23	Very strong	20	Very strong
Control (-)	0	No inhibition zones	0	No inhibition zones
$AgNO_3$ 0,5 mM	12	Strong	11	Strong
Tyrosine	0	No inhibition zones	0	No inhibition zones
AgNPs 0,5 mM	9	Medium	9	Medium
AgNPs 1 mM	9	Medium	9	Medium
AgNPs 1,5 mM	9	Medium	9	Medium
AgNPs 2 mM	9	Medium	9	Medium
AgNPs 2,5 mM	9	Medium	9	Medium
AgNPs 3 mM	9	Medium	9	Medium

According to Firyanto et al. [25], the strength of clear zone diameter on the inhibition of bacteria are categorized into four, i.e: inhibition zone 20 mm or more is categorized as very strong, inhibition zone 10-20 mm is categorized strong, inhibition zone 5-10 mm is categorized as medium, inhibition zone diameter 5 mm or less is categorized as weak. Based on these categories, the strength of antibacterial in samples with *Bacillus subtilis* bacteria can be grouped into very strong group, that is positive control, strong group is 0.5 mM $AgNO_3$ solution, medium group is silver nanoparticles (AgNPs) solution (0.5; 1; 1,5; 2; 2.5; and 3 mM), while the negative control and tyrosine have no inhibition zone. The strength of antibacterial in samples with *Escherichia coli* bacteria can be grouped into very strong group that is positive control, strong group is 0.5 mM $AgNO_3$ solution, medium group is silver nanoparticles (AgNPs) solution (0.5; 1; 1,5; 2; 2.5; and 3 mM), while the negative control and tyrosine have no inhibition zones of antibacterial.

$AgNO_3$ solution had antibacterial activity greater than silver nanoparticles. The antibacterial mechanism of AgNPs begins with the release of Ag^+ ions by silver nanoparticles and then the interaction between silver ions and sulfhydryl thiol groups on bacterial cell membrane proteins. The silver ion would replace the hydrogen cation (H+) from the sulfhydryl-thiol (-SH) group to produce a more stable S-Ag group on the surface of a bacterial cell, deactivate the enzyme and reduce membrane permeability. At the same time, silver ions would enter the cell and changed the structure of DNA and ultimately cause cell death [24]. It was known that Ag^+ had a role in the antibacterial activity as proven by inhibition zones that was larger than silver nanoparticles, but Ag^+ ions have disadvantages as they are heavy metals and toxic metals that have adverse effects and disease in human. Even colloidal silver or silver salt could cause skin diseases known as argyria, followed by direct contact with the skin and mucosa [26]. Ag^+ ions were converted into silver nanoparticles (AgNPs) to reduce the toxic properties of Ag^+ so that it was safe for human health that silver nanoparticles (AgNPs) were widely used in the medical field, silver nanoparticles were applied in wound dressings, surgical and coated bone instruments used with silver nanoparticles (AgNPs) [28]. Silver nanoparticles (AgNPs) also having safe properties for humans that could act as bacterial growth inhibitor bacterial growth. Silver nanoparticles (AgNPs) have been known as effective antibacterial agents with unique properties that increase biocompatibility because of their high ratio of surface area to volume, smaller particles (<100 nm), spherical shape, no charge, and non-toxicity to humans [29].

In this research, silver nanoparticles (AgNPs) in solution have a small equilibrium of Ag^+ ions. Furthermore, Ag^+ ions have a role in antibacterial activity tests. If Ag^+ ions from AgNPs are used as antibacterial, it could be ionized in the form of Ag^+ equilibrium and the equilibrium can continue to shift towards Ag^+ ions while function as antibacterial. Silver nanoparticles can also act as a reservoir of silver ions that can interact with the bacteria by invading their membranes, attacking the respiratory chain in their mitochondria, and causing cell death. Silver nanoparticles were able to store Ag^+ ions in a longer period of time so that testing with antibacterial, Ag^+ ions have released and produce inhibitor zones in the bacterial. In previous studies, smaller AgNPs would provide higher antibacterial activity because the smaller AgNPs size has a higher surface area which can increase the site of contact with the bacteria [30]. However, the results of this study are different. The higher antibacterial activity comes from $AgNO_3$ than AgNPs. It showed that Ag^+

ions used has a role in antibacterial activity because it affects AgNPs particle size and the presence of silver ions in AgNPs colloid solutions. The Ag^+ ions contained in silver nanoparticles (AgNPs) weren't as much as $AgNO_3$ so the detected antibacterial activity is smaller. Silver nanoparticles were able to store Ag^+ ions in a longer period of time so that testing with antibacterial, Ag^+ ions have released and produce inhibitor zones in the bacterial. The Ag^+ ions contained in silver nanoparticles (AgNPs) weren't as much as $AgNO_3$ so the detected antibacterial activity is smaller. Li et al. [31] proved that silver ions have similar modes of action to silver nanoparticles but stronger antibacterial activity than AgNPs. Tyrosine amino acids do not have inhibitor zones, It was due to amino acids in general didn't have antibacterial activity.

The results of variations in the concentration of silver nanoparticles (AgNPs) showed that the color of the solution becomes more concentrated along with the increasing concentration of $AgNO_3$ which could be seen in Figure 6. Its discoloration was caused by changes in the size or aggregation of silver nanoparticles. Variation in $AgNO_3$ concentration showed antibacterial activity, but there is no change in bacterial inhibition in both *Bacillus subtilis* and *Escherichia coli*. It was possible that Ag^+ ions from silver nanoparticles which have an effect on inhibition bacteria have almost the same amount so that with the increase in the concentration of silver nanoparticles (AgNPs) the results on antibacterial activity are still the same.

Figure 6: The color of variations in concentration of AgNPs solution at optimum conditions

The results showed that $AgNO_3$ solution in *Bacillus subtilis* bacteria experienced greater inhibition than *Escherichia coli* bacteria because of the difference in their structure groups of bacteria. *Bacillus subtilis* was a gram-positive and Escherichia coli was a gram-negative. Gram-*positive* and gram-negative bacteria have different structures in the cell wall. *Escherichia coli* as a gram-negative has a more complex cell wall structure than *Bacillus subtilis*. *Escherichia coli* was a gram-negative that was resistant to several antibacterials, it was because of the existence of three layers of cell walls in this bacterial so that some compounds were difficult to damage the tissue of the *Escherichia coli* bacterial cell wall. Gram-negative bacteria cell walls contain three layers, the outer layer was lipoprotein, the middle layer was lipopolysaccharide and the inner layer was peptidoglycan and the outer membrane in the form of a bilayer (have better resistance to compounds that enter or enter the cell) [14]. So Ag^+ ions from $AgNO_3$ were able to penetrate the cell wall of *Bacillus subtilis* bacteria more easily and produce an inhibition zone greater than *Escherichia coli* bacteria.

4. CONCLUSION

Antibacterial activity of silver nanoparticles (AgNPs) was successfully synthesized by using tyrosine as a reduction and capping agents. The color change of AgNPs from colorless to yellow. TEM characterizations showed that the particle was a round shape and had an average size of 13.628 nm. $AgNO_3$ solution had antibacterial activity greater than silver nanoparticles. Ag^+ ions were converted into silver nanoparticles to reduce the toxic properties of Ag^+ so that it was safe for human health. The antibacterial activity in solution has a stronger effect against *Bacillus subtilis* (gram-positive) than *Escherichia coli* (gram-negative).

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