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ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES AGAINST *Aeromonas* spp. AND *Vibrio* spp. ISOLATED FROM AQUACULTURE WATER ENVIRONMENT IN THUA THIEN HUE

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Abstract: A silver nanoparticle solution prepared at the Center for Incubation and Technology Transfers was used in the current study. The nanoparticles have an average size of 15.0 nm. The silver nanoparticle solution exhibits an antibacterial activity to *Aeromonas hydrophyla* and *Aeromonas caviae* isolated from fresh water fish ponds and *Vibrio harveyi* and *Vibrio alginoliticus* isolated from white shrimp ponds. The silver nanoparticle solution at a concentration of 25 ppm inhibits *A. caviae* and *A. hydrophila*, and the peak attenuation time was 24 hours after exposure to the bacteria. The solution at a concentration of 12.5 ppm also inhibits *Vibrio harveyi* and *Vibrio alginoliticus*, and the peak attenuation time was 48 hours after exposure to the bacteria.

Keywords: antibacterial activity, silver nanoparticles, *Aeromonas* spp. and *Vibrio* spp.

1 Introduction

In recent years, aquaculture has significantly developed all over the world. However, the disease situation is very complex, causing great economic losses. In particular, *Aeromonas* spp. and *Vibrio* spp. are two groups of bacteria that cause disease in freshwater and saltwater animals. They are present in aquaculture pond water with high density in polluted environments and readily penetrating and causing disease to aquatic animals, they are ready to invade and cause diseases for aquatic animals. So, many disinfectants and antibiotics are used to prevent the harmful effects of these two groups of bacteria.

However, the current situation of using chemicals and antibiotics is no longer the optimal solution to solve environmental problems and diseases in aquaculture due to the consequences regarding many issues such as environmental pollution, antibiotic residues in aquatic products, and antibiotic resistance. Therefore, looking for a new solution to treat environmental pollution and to prevent disease is an urgent issue for aquaculture [22].

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Silver nanoparticles prove to be highly biocidal and environmentally friendly [16]. Nanotechnology has attracted many aquaculturists [25]. In aquaculture, nanoparticles are used in alimentation, treatment of water culture medium and effluents, and control of infectious diseases [9]. This paper presents the silver nanoparticle preparation and investigates the antimicrobial properties of silver nanoparticles on *Aeromonas hydrophyla, Aeromonas caviae, Vibrio harveyi* and *Vibrio alginoliticus* isolated from aquaculture ponds.

2 Materials and methods

2.1 Materials

The chemicals used to prepare silver nanoparticles include $AgNO_3$ (Germany), glycerol (China), poly(vinylpyrrolidinone) (PVP) (India). All the chemicals are of analytical grade.

The bacterial cultured and isolated medium is nutrient agar (NA) and thiosulphate citrate bile salt agar (TCBS), and the growth medium is Trypticase soy agar. The media were sterilized at 121 °C for 15 minutes.

The media for biochemical reactions are saccharose, glucose, lactose, maltose, kia medium, MR environment, mannitol, citrate, and tryptone liquid.

Bacterial strains: *Aeromonas hydrophyla* and *Aeromonas caviae* were isolated from the water of freshwater fish ponds; *Vibrio harveyi* and *Vibrio alginoliticus* were isolated from the water of white shrimp ponds. Pathogenic bacteria were identified on the basis of the methods developed by Bergey [2].

2.2 Device

The microwave oven (Samsung), magnetic stirrer with heating (Italy), UV-VIS Spectrometer U-2910 (Japan), transmitted electron microscope (TEM) Philips CM 120, Ultrasonic processor (USA), microbiological safety cabinets (Japan), and autoclave Sterilizer (Japan) were used in the study.

2.3 Methods

Preparation of silver nanoparticles

10,8g of PVP of PVP was added to 300 mL of glycerol stirred with a magnetic stirrer until PVP was completely dissolved at 90–100 °C. Next, 1,08g of silver nitrate was added to 200 mL of glycerol and the mixture was stirred until silver nitrate was completely dissolved. The $AgNO_3$ solution was added dropwise to the PVP solution, and the mixture was stirred thoroughly and exposed to ultrasound for 5 minutes. This mixture was put into the microwave for the reaction.

Physical and chemical analysis of silver nanoparticles

The absorption spectrum of silver nanoparticles was measured using a UV-Vis spectrophotometer. Silver nanoparticles have a characteristic yellow color and absorption peaks in the range of 400–450 nm [6, 14, 21].

The Transmitted Electron Microscope (TEM) technique was used to determine the size and shape of the silver nanoparticles.

Collection, culture and isolation of bacteria

Sampling method: The water samples were collected from freshwater fish ponds (freshwater samples) and white shrimp ponds (saline samples). 10 water samples were collected from 5 locations, including 4 corners (1–2 m from shore) and 1 center of each aquaculture pond. At each site, two samples were collected: one 40 cm under the surface and the other 30 cm above the bottom.

The bacterial isolation and identification were performed according to Bergey (1957) [2].

Antimicrobial properties of silver nanoparticles

Silver nanoparticles at 6 concentrations, namely 0, 6.25, 12.5, 25, 50, and 75 ppm were put in different plastic wells. Bacterial agar was cut with a specialized tool into disks of 5.5 mm in diameter, then placed in wells and soaked for 24 hours; each treatment was repeated 3 times. Next, this bacterial agar was cultured on nutrient agar plates for *Aeromonas* bacteria and TCBS agar plates for *Vibrio* bacteria. The growth of bacteria on the agar plates was examined during 1 day, 2 days, and 7 days [22].

To determine the optimal antimicrobial time, 0.5 mL of bacteria (10^6 CFU/mL) was spread on Petri dishes containing NA medium for *Aeromonas* bacteria and TCBS medium for *Vibrio* bacteria. After a minute, 3 holes/agar plate with a diameter of 3 mm were drilled. Subsequently, the silver nanoparticles solutions with optimal inhibitory concentration were injected into the holes in the agar plates. The plates were incubated at 37 °C; each treatment was repeated 3 times. The diameter of the sterile circle was measured after 12, 24, 48, and 72 hours [22].

Data analysis

The data were analyzed using one-factor ANOVA, and the differences between the treatments were compared using Minitab version 16.2.0 and Microsoft Excel 2007.

3 Results and discussion

3.1 Physical and chemical properties of silver nanoparticles

According to published studies, silver nanoparticles can inhibit the growth of bacteria, attack and break down cell membranes of nearly 650 pathogenic bacteria [11]. Silver and silver compounds have inhibitory activity and destroy bacteria, viruses, algae and fungi. Unlike other heavy metals (lead, mercury, etc.), silver is not toxic to humans [8, 13]. The important factors that determine the antimicrobial properties of silver nanoparticles include the size, shape, electrical charge, concentration, and glue state [5]. The nanoparticle size is important with bactericidal effect. The smaller the nanoparticles are, the stronger the antibacterial activity is. Small-sized nanoparticles have a large exposure area for bactericidal action at low concentrations [15, 24].

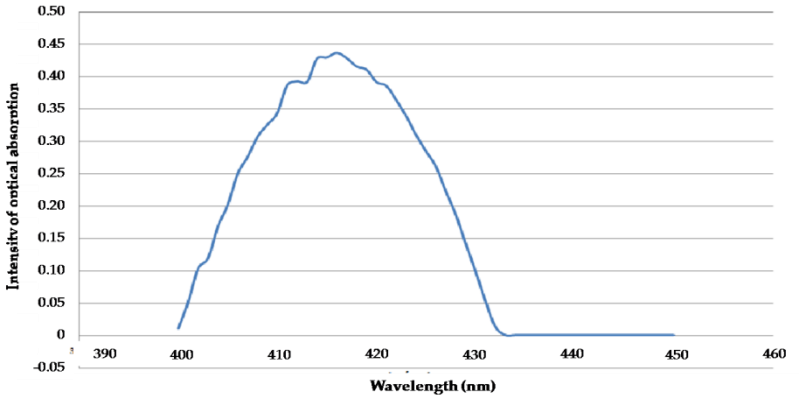

Figure 1. UV-Vis spectrum of silver nanoparticle solution

In this study, the silver nanoparticles were yellow with a maximum absorption wavelength λ_{max} = 415 nm (Figure 1).

The UV-Vis absorption spectrum of the silver nanoparticle solution depends on the shape and size of the silver nanoparticles. Silver nanoparticles with a spherical structure and a diameter of 10–40 nm have maximal peaks of absorption spectra between 410 and 427 nm; When the particle size is around 40–90 nm, the peak of the spectrum is absorbed at wavelength of 480 nm, and when the silver nanoparticles have bar and strings structures the peak of the absorption spectrum moves to the wavelength of 350–380 nm [10, 21].

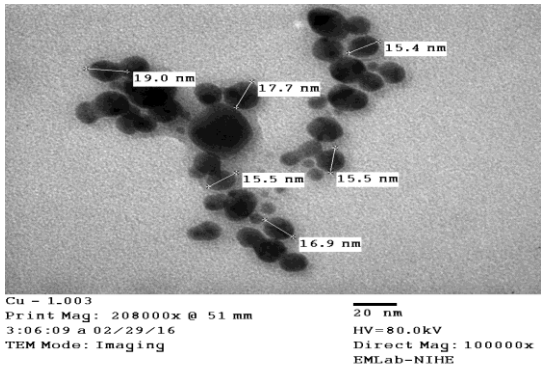

Figure 2. TEM image and distribution of silver nanoparticles

(*The numbers display the diameter of silver nanoparticles.*)

Our results show that the UV-Vis absorption spectra of the silver nanoparticle solution have an absorption peak at 415 nm, confirming that the synthesis is successful [3, 19]. The TEM image confirms that the silver nanoparticles are spherical with an average particle size of 15.0 nm (Figure 2).

3.2 Isolation of *Aeromonas* spp and *Vibrio* spp. from aquaculture ponds

The results of the culture, isolated from the water samples after 24 hours are shown in Table 1, Figure 3 and Figure 4.

Table 1. Characteristics of colonies isolated from water samples of aquaculture ponds after 24 hours

Characteristics of colony	NA medium		TCBS medium	
	1NN species	2NN species	1NM species	2NM species
Colony shape	Smooth, shiny, evenly	Smooth, shiny, evenly	Round, uneven	Smooth, shiny, evenly
Colony color	Milk-yellow	Milk-white	Dark blue	Yellow
Diameter of colony (mm) (TB ± SE)	1,88 ± 0,1	2,25 ± 0,2	3,10 ± 0,1	2,25 ± 0,2
The smell of colony	Awful smell	No smell	–	–
Changing media	–	–	No changing medium	From blue to yellow

Note: – indefinite

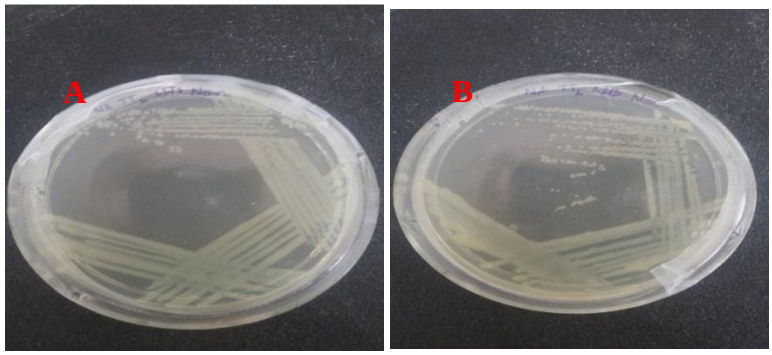

Figure 3. Bacterial colonies isolated on NA medium: A: 1NN species; B: 2NN species

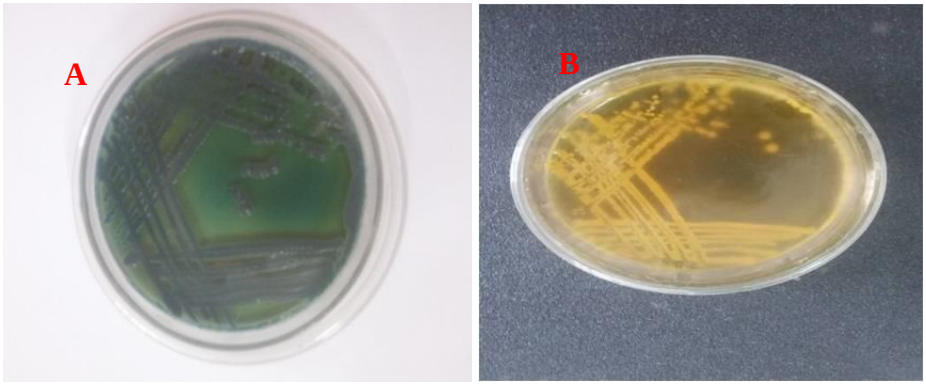

Figure 4. Bacterial colonies isolated on TCBS medium: A: 1NM species; B: 2NM species

From the four colonies isolated on both culture media, we dyed Gram according to the method of Christian Gram (1884) and observed under the 100× optical microscope [2]. All four species of bacteria are colored Safranin dye and pink, which indicates that they are Gram-negative bacteria.

There are differences in bacterial species. The 1NN species have negative reactions to VP, slightly produced from glucose and a positive reaction on H_2S production, while the 2NN species give the opposite reaction; the 1NM species have a positive reaction in lactose fermentation, methyl red test, and negative reaction in saccharose fermentation, while the 2NM species give the opposite reaction (Table 2).

On the basis of the biochemical and morphological characteristics of the bacteria isolated, the different characteristics of these species, and the classification of Bergey [2], we conclude that 1NN species is *A. caviae*; 2NN species is *A. hydrophyla*; 1NM species is *V. harveyi* and 2NM species is *V. alginolyticus*. These strains will be used in experiments to determine the antimicrobial concentration and optimal antimicrobial time of silver nanoparticles.

Table 2. Biochemical characteristics of bacteria isolated from water samples of aquaculture ponds

Index	1NN species	2NN species	1NM species	2NM species
Gram staining	–	–	–	–
Glucose fermentation	+	+	+	+
Lactose fermentation	–	–	+	–
Saccharose fermentation	+	+	–	+
Methyl red test	–	–	+	–
KIA test	+/+	+/+	+/–	–/+
Citrate test	–	+	–	–
Slightly produced from glucose	–	+	+	+
Indol	+	+	+	+
Voges Proskauer test	–	+	–	–
Mobility	+	+	+	+
Ability to produce H_2S	+	–	–	–
Conclusion	*A. caviae*	*A. hydrophyla*	*V. harveyi*	*V. alginolyticus*

Note: (+): Positive; (–): Negative

3.3 Antibacterial activity of silver nanoparticles to bacteria isolated from aquaculture ponds

Bacterial inhibitory concentration

When the nano level increases, the ability to inhibit the bacteria increases (Table 3).

Table 3. Bacterial inhibitory concentration of silver nanoparticles to *A. caviae*, *A. hydrophyla*, *V. alginolyticus* and *V. harveyi*

Concentration of silver nanoparticles (ppm)	Growth of bacteria after											
	1 day				2 days				7 days			
	AC	AH	VA	VH	AC	AH	VA	VH	AC	AH	VA	VH
75.0	–	–	–	–	–	–	–	–	–	–	–	–
50.0	–	–	–	–	–	–	–	–	–	–	–	–
25.0	–	–	–	–	–	–	–	–	–	–	–	–
12.5	–	–	–	–	+	–	–	–	+	+	–	–
6.25	+	+	–	–	+	+	+	–	+	+	+	+
0	+	+	+	+	+	+	+	+	+	+	+	+

Note: *Positive; (–): Negative ; AC: A. caviae; AH: A. hydrophyla; VA:* V. *alginolyticus; VH: V. harveyi*

Silver nanoparticles are able to inhibit *A. caviae* and *A. hydrophyla* at 12.5 ppm after 1 day of culture, but after 2 days, the *A. caviae* growth restores. After 7 days, the *A. hydrophyla* growth restores. Silver nanoparticles at 25 ppm inhibit both *A. caviae* and *A. hydrophyla* which do not grow again after 7 days of culture.

According to Shaalan et al. (2017), the minimum inhibitory concentration (MIC) of silver nanoparticles to the growth of *A. hydrophila* and *A. salmonicida* is 17 μg/mL (with silver nanoparticles having an average size of 21 nm) [17]. Mahanty et al. (2013) use silver nanoparticles synthesized with carica papaya leaf extracts (papaya). The silver nanoparticles are 25–40 nm in size, round, irregular, and are used to study the antibacterial activity of *A. hydrophila*. The results show that silver nanoparticles synthesized from papaya leaves have the highest antimicrobial activity at 153.6 μg/mL [12].

Silver nanoparticles can inhibit *V. harveyi* and *V. alginolyticus* at a concentration of 6.25 ppm after 1 day of culture. However, *V. alginolyticus* recovers after 2 days, and *V. harveyi* recovers after 7 days. At 12.5 ppm, silver nanoparticles inhibit both *V. harvey* and *V. alginolyticus* completely.

According to Van et al. , the minimum inhibitory concentration of silver nanoparticles for *Vibrio harveyi* and *Vibrio parahaemolyticus* is 25 ppm. At this concentration, silver nanoparticles are more effective against *Vibrio harveyi* and *Vibrio parahaemolyticus* than Oxytetracyline, Ofloxacine and Kanamycine [22].

In the study of Bahabadi et al., silver nanoparticles in two different sizes (16.62 and 22.22 nm) are used to evaluate the antibacterial activity against *V. harveyi*. They found that small silver nanoparticles are faster and stronger in antimicrobial activity than large silver nanoparticles [1].

Vaseeharan et al. use silver nanoparticles synthesized with tea leaf extracts to destroy the *Vibrio harveyi* pathogenic to Indian shrimp. They indicate that the number of *V. harveyi* colonies on agar plate LB agar is inversely proportional to the silver nanoparticles concentrations, and *V. harveyi* do not grow (increase OD value) at 35 μg/mL silver nanoparticles concentration [23].

The antimicrobial properties of silver nanoparticles are derived from the chemical nature of Ag^+ ion as follows: (1) Ag^+ ions strongly associate with peptidoglycan, which constitutes the bacterial cell walls, and inhibit the transport of oxygen into the cell to paralyze the bacteria; (2) after the Ag^+ ions act on the bacterial cell membrane, they penetrate inside the cell and react with the sulfhydryl groups of the oxygen metabolizing enzyme molecules to inactivate the enzyme, resulting in the inhibition of bacterial cell respiration; (3) Ag^+ ions bind to the base of the DNA molecule and disable the power of phosphate thus preventing the DNA replication process [10]. Silver nanoparticles with size 1–100 nm and high surface energy are capable of slowly releasing Ag^+ ions into the solution; therefore, silver nanoparticles have longer

antimicrobial efficacy than colloidal silver and silver in the form of blocks [4]. Steuber et al. suggest a mechanism of antimicrobial activity of Ag^+ ions against *Vibrio alginolyticus*, in which flavin adenine dinucleotide (FAD) was isolated from holo-enzyme Na^+ -NQR (Na^+-translocating NADH:ubiquinone oxidoreductase) that inactivates the enzyme. The antiseptic effect of silver nanoparticles is also related to the prevention of sugar metabolism [20].

In this study, the minimum inhibitory concentration of silver nanoparticles for *A. hydrophyla* and *A. caviae* is 25 ppm, and for *V. harveyi* and *V. alginoliticus* is 12.5 ppm. The difference between the studies is mainly due to the size difference of the silver nanoparticles. The particle size and concentration play an important role in the antimicrobial properties of nanoparticles. The smaller the nanoparticles are, the larger the surface area is, thus increasing their antimicrobial activity [18].

Optimal antimicrobial time of silver nanoparticles

For each minimum inhibitory concentration of silver nanoparticles on four bacterial species, we set up experiments and observed the antibacterial activity over different time periods: 12, 24, 48, and 72 hours to determine when the antibacterial activity is strongest.

The antimicrobial effect over time is similar between *A. caviae* and *A. hydrophyla*. The strongest antibacterial activity is found after 24 hours with the sterile ring diameter for *A. caviae* at 15.33 mm and for *A. hydrophyla* at 14.67 mm, and it is significantly different from that of other times ($p < 0.05$). After this time, the sterile ring diameter decreases (Table 4).

The sterile ring diameter for *V. harveyi* and *V. alginolyticus* increased from 12 to 48 hours and reaches the maximum at 48 hours for *V. harveyi* at 17.33 mm and *V. alginolyticus* at 18.00 mm, and it is significantly different from that of other times ($p < 0.05$). After 48 hours, the sterile ring diameter decreases (Table 4).

Table 4. Antibacterial activity of silver nanoparticles

Bacteria	The concentration of silver nanoparticles (ppm)	The diameter of the sterile ring (mm)			
		12 hours	24 hours	48 hours	72 hours
A. caviae	25.0	$14.00^b \pm 0.57$	$15.33^a \pm 0.33$	$11.33^c \pm 0.33$	$7.33^d \pm 0.33$
A. hydrophyla	25.0	$12.67^b \pm 0.33$	$14.67^a \pm 0.33$	$11.33^c \pm 0.33$	$7.33^d \pm 0.33$
V. haveyi	12.5	$12.33^c \pm 0.33$	$15.66^b \pm 0.33$	$17.33^a \pm 0.33$	$13.33^c \pm 0.33$
V. alginolyticus	12.5	$11.33^c \pm 0.33$	$14.66^b \pm 0.33$	$18.00^a \pm 1.00$	$12.00^c \pm 1.00$

Note: Values on the same row with different characters (a, b, c, d) are significantly different ($p < 0.05$).

When the culture time is longer, the number of silver ions decreases, which reduces the antibacterial activity of silver nanoparticles, and the bacteria can regenerate.

4 Conclusion

The silver nanoparticles are yellow with a maximum absorption wavelength λ_{max} = 415 nm. They are spherical with an average diameter of 15.0 nm and resistant to four species of bacteria isolated from the water of aquaculture ponds. In particular, their minimum inhibitory concentration for *A. caviae* and *A. hydrophyla* is 25 ppm, and for *V. alginoliticus* and *V. harveyi* is 12.5 ppm. Their antimicrobial activity is highest for *A. caviae* and *A. hydrophyla* at 24 hours, and for *V. alginolyticus* and *V. harveyi* at 48 hours after exposure to bacteria. Thus, the silver nanoparticles have great potential for treating aquaculture pond water environments to reduce the pathogenic bacteria in the culture environments. In order to be able to apply to production, it is necessary to study the scale of aquaculture ponds.

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