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PRELIMINARY ANTIBACTERIAL TESTING OF BIOSYNTHESISED SILVER NANOPARTICLES AGAINST THE MARINE AQUATIC PATHOGENS *Vibrio alginolyticus* AND *Vibrio harveyi*

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Abstract

Aquaculture is an up and rising industry that that contributes to the economy of Malaysia. However, fish and shellfish-related diseases have become a leading cause of loss of product and, in turn, loss of money to fish farmers as well as the government. The pathogens that cause these diseases are resistant to commercially available preventive and curing techniques, thus giving rise to new techniques. Biologically synthesised silver nanoparticles are predominantly known for their antibacterial potential and therefore were chosen as the antibacterial agents to combat these pathogens. Forty samples of biosynthesised silver nanoparticles were tested against the marine pathogens *Vibrio alginolyticus* and *Vibrio harveyi* for inhibition zones. Samples of *Serratia* sp. strains AQ5-NT14 and AQ5-NT39 showed the biggest zones and were the most effective against both *Vibrio* strains.

INTRODUCTION

Aquaculture has expanded and developed rapidly in Malaysia in recent decades. The government has given out incentives and support systems to boost the aquaculture industry to increase the country's economy and source of food due to the increasing population [1]. The Department of Fisheries Malaysia, under the National Agro-Food Policy (NAFP) has set a target for the production of fish to be at 794,000 tonnes by 2020 [2]. Although the aquaculture industry has significantly benefited the country, there are yet problems that have arisen in relation to development and aquaculture practice primarily the occurrences and spread of many fish and shrimp-related diseases caused by microorganisms in farms and hatcheries throughout the country [3, 4]. If the issue is not dealt with, farms and hatcheries will suffer significant yield and economic losses.

The cause of fish and shrimp-related diseases are microorganism such as bacteria, fungus and yeast, of which bacteria have been proven to be the primary cause. Vibriosis is one of the most prevalent causes of fish and shrimp mortality in the aquaculture industry, which is contributed by the *Vibrio* spp. strain of bacterium on the global scale [5]. This happens due to the lack of proper handling, pollution, poor water quality and overcrowding in farms and hatcheries [6]. There are a number of Vibrio sp. that are highly pathogenic and able to cause severe damage to the host. These strains include *Vibrio harveyi*, *Vibrio parahaemolyticus, Vibrio alginolyticus, Vibrio anguillarum*, *Vibrio vulnificus* and *Vibrio splendidus*. The most common symptoms of vibriosis in shellfish and fish are the darkening of the outer body, anaemia, dermal haemorrhages, red spots on the body, intestinal hyperaemia and congestion and swelling of the spleen, liver and kidney in fish, visible large open ulcers, eye lesions as well as visible rot and paleness of gill of fish are observed for chronic infections [7]. *Vibrio* spp. attacks the host through penetration via oral routes, dermal routes due to the presence of wounds, contaminated food sources, and contact between infected and non-infected fishes or shrimp, thus causing severe damage to the host's internal organs

resulting in death [8].

Silver nanoparticles are promising antibacterial agents that can significantly change their physical, chemical and biological properties owing to their surface-to-volume ratio [9]. Among various synthesis methods, the biological means of synthesis is by far the simplest, non-toxic, dependable, rapid as well as able to produce nanoparticles of well-defined size and shape under optimum conditions [10]. Silver nanoparticles trigger cell death via a primary action of cytotoxicity. The action of silver nanoparticles is complex and centred around their size and shape. The toxicity of silver nanoparticles depends primarily on their size and shape; other factors such as surface charge, functionalisation and core structure are also accounted for the biological action of the nanoparticles. These factors play a crucial part in the mechanism of action towards bacterial cells in terms of cellular uptake, cellular activation and intracellular distribution [11-15].

Being one the strongest strains of the *Vibrio* sp., *Vibrio alginolyticus* and *Vibrio harveyi* are resistant to many antibiotics such as ampicillin and vancomycin and are only susceptible to antibiotics such as Oxytetracyline, Ofloxacine and Kanamycine [16]. However, over the years, researchers have encountered a problem in which these strains are developing resistance against the antibiotics as mentioned above, resulting in various means that emerged to overcome the issue. Silver nanoparticles play their role by being excellent antibacterial agents, which have been proven to have bactericidal effects against various pathogens. Van et al. [16] reported that the minimum inhibitory concentration of silver nanoparticles required to kill these strains of Vibrio completely is 25 ppm. In contrast, Khanh and Cu [17] reported that chemically synthesised silver nanoparticles could inhibit Vibrio alginolyticus and Vibrio harveyi at 12.5 ppm completely. Thus, it can be concluded that different means of synthesis produces nanoparticles of various size and shape have different effects at different concentrations.

This present study aims to synthesise silver nanoparticles via biological means and test its antibacterial activity against the pathogenic *Vibrio alginolyticus* and *Vibrio harveyi* to give a preliminary antibacterial result for further antibacterial research and to provide additional as well as new knowledge to existing data in the field.

MATERIALS AND METHODS

Materials

Analytical grade silver nitrate (AgNO3) was purchased from Systerm. Marine broth and Muller Hinton Agar were purchased from Himedia and Tryptic soy broth was purchased from Biokar diagnostics. The materials were prepared as per manufacturer's instruction.

Preparation of bacterial culture

Marine bacteria isolated from soil samples collected from Port Dickson, Negeri Sembilan and Bagan Lalang, Selangor, Malaysia were used as nano factories [18,19]. Serial dilution and culture transfer methods were used as reported by De Silva et al. [18] and Noor et al. [19] to obtain single strain bacteria, which were then subjected to screening for the ability to biosynthesise silver nanoparticles based on the biosynthesis method.

Biosynthesis of silver nanoparticles

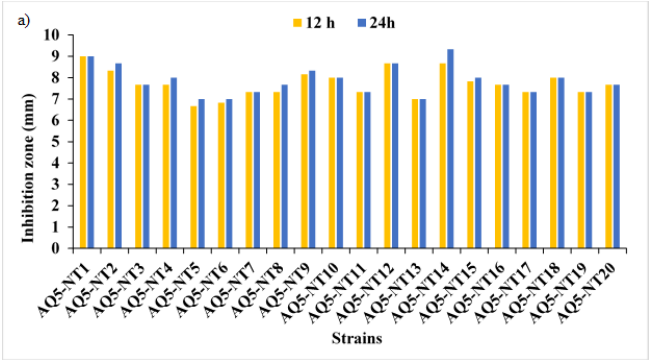
The biosynthesis of silver nanoparticles was done based on the report by De Silva et al. [18]. Extracellular means of synthesis were done using a bacterial supernatant mixed with equal parts of the 0.5 M silver nitrate solution prepared based on the manufacturer's instruction. The mixture of bacterial supernatant and silver nitrate solution was left in a dark condition at room temperature for three days with continuous stirring until a colour change from milky white to brown was observed. A control was run alongside the samples to observe colour change. The synthesised silver nanoparticles were removed by simple filtration (0.1 µm) and maintained in liquid form to ensure that the particles are monodispersed.

Antibacterial testing

Antibacterial testing was done based on the Kirby-Bauer method or agar disk diffusion method [20]. Bacterial cultures of *Vibrio alginolyticus* and *Vibrio harveyi* were prepared based on the 0.5 McFarland standard. Disks with the size of 6 mm were prepared using filter paper and a two-hole puncher machine and autoclaved to sterilise the disks, which were then used as a medium that held the nanoparticles. The sterilised disks were dipped into the silver nanoparticles solutions and allowed to air dry in the laminar flow for 15 min, while negative and positive controls using distilled water and 0.5 M silver nitrate solution were prepared. Muller hinton agar plates were prepared based on manufacturer's instruction, divided into six quadrants and labelled with negative control, positive control and silver nanoparticles sample codes. The cultured bacteria were streaked onto the plates using sterile cotton swabs and the disks were placed onto the respective quadrants. The plates were incubated at 30°C for 24 hours with observations of the halo zones formation and recorded at 12-hour intervals. Triplicates were done for each silver nanoparticle sample.

RESULTS AND DISCUSSION

Forty bacterial strains showed positive results for the synthesis of silver nanoparticles based on colour change. The samples were subjected to further characterisation and proceeded to antibacterial testing against the marine bacterial pathogens *Vibrio alginolyticus* and *Vibrio harveyi*. **Figures 1** and **2** show the data for inhibition zones of the 40 silver nanoparticle samples against *Vibrio alginolyticus* and *Vibrio harveyi*, respectively. Based on **Figure 1**, it can be concluded that samples AQ5-NT1, AQ5-NT2, AQ5-NT12, AQ5-NT14, AQ5-NT27 and AQ5-NT39 showed the best zones of inhibition for *Vibrio alginolyticus*. In contrast, samples AQ5-NT2, AQ5-NT2, AQ5-NT3, AQ5-NT7, AQ5-NT14, AQ5-NT35 and AQ5-NT39 showed the best zones of inhibition for *Vibrio harveyi* (**Figure 2**). However, samples AQ5-NT14 and AQ5-NT39 were the most effective silver nanoparticle samples and showed the biggest zones of inhibitions against both *Vibrio alginolyticus* and *Vibrio harveyi.*



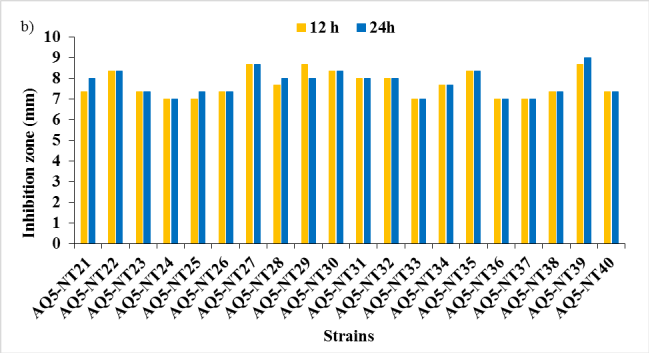


Figure 1. Diameter of inhibition zones of silver nanoparticles against *Vibrio alginolyticus* at a) AQ5-NT1-AQ5-NT20 and b) AQ5-NT21-AQ5-NT40 at 12 hours and 24 hours.

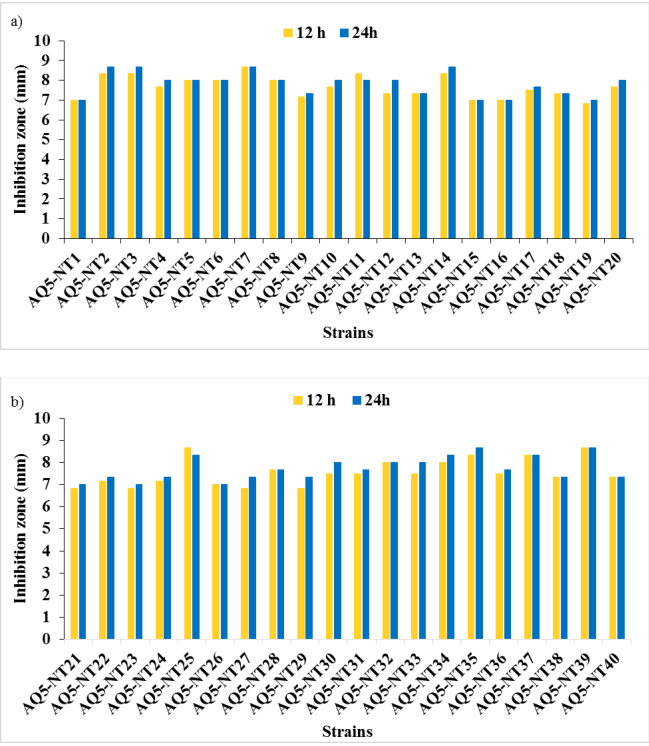


Figure 2. Diameter of inhibition zones of silver nanoparticles against *Vibrio harveyi* at a) AQ5-NT1-AQ5-NT20 and b) AQ5-NT21-AQ5-NT40 at 12 hours and 24 hours.

Figure 3 and **4** display the inhibition zones of samples AQ5-NT14 and AQ5-NT39 against the Vibrio strains. The bigger inhibition zones recorded for AQ5-NT14 and AQ5-NT39 for both *Vibrio alginolyticus* (**Figure 3**) and *Vibrio harveyi* (**Figure 4**) may be due to the reason that the samples contained silver nanoparticles with well-defined size presumably smaller sized nanoparticles. Studies have proven that small sized nanoparticles are mostly effective in killing pathogenic bacteria as compared to larger size nanoparticles due to their larger surface area to volume ratio that allows them to efficiently bind to bacterial cells, penetrate and cause cellular damage leading to cell death [21-23]. However, the bactericidal effects of silver nanoparticles depend on other factors such as shape, surface chemistry, crystallinity, capping agent, dose of AgNPs, bacterial strains and composition of culture media. Thus, silver nanoparticles' exact mechanism of action is yet to be fully understood [24].

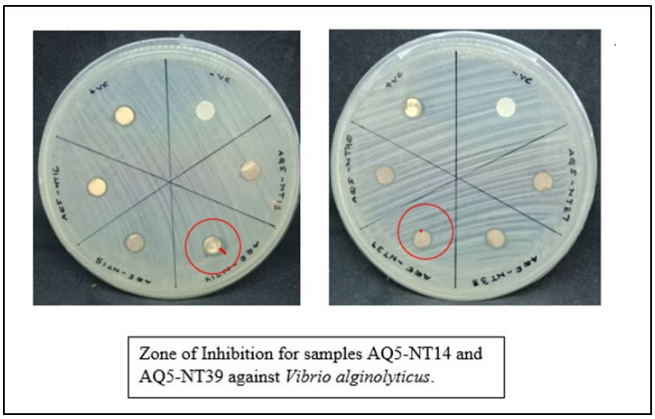


Figure 3. The inhibition zone after 24 hours of incubation with silver nanoparticles of sample AQ5-NT14 and AQ5-NT39 against *Vibrio alginolyticus*.

Vibrio alginolyticus and *Vibrio harveyi* are pathogenic strains of the *Vibrio* sp. that cause severe internal and external damages to various species of marine fish and shellfish [7]. Based on the antibacterial results obtained, *Vibrio harveyi* showed smaller inhibition zones for all silver nanoparticle samples than *Vibrio alginolyticus,* proving that the strain is stronger than the latter. This might be due to the presence of different thicknesses of biofilms surrounding the *Vibrio* sp. that causes a delay in the ability of the silver nanoparticles to penetrate the bacterial cells [25]. *Vibrio* sp. are motile bacteria with polar flagella that allow them to produce flagella protein, which then reduces the stability of the nanoparticles reducing antibacterial effects [26]. *Vibrio harveyi* may have produced a high amount of flagella protein, thus resulting in lower antibacterial activity.

CONCLUSION

Based on the results obtained, silver nanoparticles synthesised from bacterial samples AQ5-NT14 and AQ5-NT39 showed the biggest inhibition zones against *Vibrio alginolyticus* and *Vibrio harveyi*. This proved that silver nanoparticles are good and efficient antibacterial agents. Primarily, this ability of silver nanoparticles is contributed by the small size and well-defined shape of nanoparticles and various other factors. This paper adds to existing knowledge on the topic and provides additional information on the potential presence of biofilms and secretion of flagella protein by the pathogens as a means to develop resistance against silver nanoparticles, thus allowing future improvements.

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