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Biogenic synthesis of silver nanoparticles using ginger (*Zingiber officinale*) extract and their antibacterial properties against aquatic pathogens

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Abstract

With the development of aquaculture, there is an urgent demand for an alternative antibacterial agent to reduce the drug resistance and environmental pollution caused by the abuse of antibiotics. Recently, silver nanoparticles (AgNPs) have been viewed as a novel type of antimicrobial agents due to their unique advantages. In this study, AgNPs were biosynthesized with the ginger rhizomes extract. The biosynthesized AgNPs were characterised by UV–visible spectroscopy, transmission electron microscopy, X-ray diffraction and fourier transform infrared spectroscopy. Furthermore, the antimicrobial activities of the AgNPs were fully analyzed against six typical aquatic pathogens. The results indicated that the components in ginger extract could function as the chemical reductant to synthesize AgNPs. Moreover, compared with the AgNPs synthesized by chemical methods, the biosynthesized AgNPs were smaller, and had higher stability and antibacterial activity. Therefore, the biosynthesized AgNPs using ginger extract may have prospective applications in aquaculture.

Key words: ginger, silver nanoparticles, biosynthesis, antibacterial activity, aquatic pathogen

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1 Introduction

With the development of aquaculture, aquatic animal diseases caused by pathogens have become one of the biggest risk factors in the aquaculture industry (Francis et al., 2001). Among these pathogens, a substantial proportion accounted for the conditions of pathogenic bacteria. Fish farmers have been forced to use antibiotics to inhibit the infections. However, abuse of antibiotics could not only pollute environment, but could also lead to serious drug resistance of pathogens on a very large scale (Schmidt et al., 2000). Thus, there is an urgent demand for an alternative antibacterial agent to effectively control the aquatic pathogenic bacteria.

As effective antibacterial agents, silver nanoparticles (AgNPs) have attracted much attention in the past decade (Martinez-Gutierrez et al., 2010; Pavagadhi et al., 2014). AgNPs can be synthesized by a variety of methods, among which chemical reduction are the most commonly used (Darroudi et al., 2011). However, during the process, toxic chemicals are unavoidable as reducing agent and stabilising agent. Recently, the green synthesis of AgNPs has been developed via different biological systems, including bacteria, fungi, yeasts, algae or plants (Narayanan and Sakthivel, 2010; Hebbalalu et al., 2013; Miri et al., 2015). The extracts from living organisms can act as reducing and stabilising agents. There are many advantages in the synthesis of AgNPs via biological extract, such as eco-friendliness, low cost, mild reaction conditions and compatibility for biomedical applications. Moreover, the bioactive compounds in the extract are supposed to improve the characteristics of the nanoparticles (NPs). For example, the proteins in *Aspergillus oryzae* var. *viridis* extracts could cap the AgNPs and prevent their aggregation (Binupriya et al., 2010). Therefore, the biosynthesis of AgNPs would provide a new way for the development of novel antimicrobial agents.

Ginger (*Zingiber officinale*) is not only a well-known spice plant, but also a traditional oriental medicine used to treat many diseases such as cough, nausea and vomiting, colds, rheumatism, cardiac disorders, inflammation and tumours (Leach and Kumar, 2008; Dehghani et al., 2011; Ding et al., 2013). Ginger has been found to contain a variety of bioactive compounds, including alkaloids, flavonoids, zingiberene, gingerols and shogaols, most of which exhibit antioxidant activities (Shukla and Singh, 2007; Park et al., 2008; Butt and Sultan, 2011). Given that ginger is rich in antioxidants, the biomolecules in ginger extract are supposed to play a crucial role in the reduction of silver ions (Ag^+) to metallic AgNPs (Ag^0). Velmurugan et al. (2014) synthesized AgNPs using ginger root extract and proved their antimicrobial activity against food pathogens of *Staphylococcus* spp., *Listeria* spp. and *Bacillus* spp. However, to the best of our knowledge, there are no reports on antibacterial investigation of the ginger mediated AgNPs against aquatic pathogens.

In this study, the AgNPs were biosynthesized with the aid of

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ginger extracts at moderate temperatures within a short period of time. Moreover, antibacterial analysis showed that the biosynthesized AgNPs exhibited powerful antimicrobial activities against all tested aquatic pathogens, which revealed their new potential in aquaculture field.

2 Materials and methods

2.1 *Materials*

The dried rhizomes of experimental material ginger (*Z. officinale*) were purchased from a local market of Yantai, China. The ginger was washed thoroughly with tap water followed by distilled water, shade-dried for a week and stored at 4°C for further study. Silver nitrate ($AgNO_3$) and sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) were purchased from China National Pharmaceutical Group Corporation as analytical grade. Nutrient agar and nutrient broth were purchased from Sangon Biotech (Shanghai) Co., Ltd. Deionised water was used throughout the experimental procedure.

2.2 *Chemical synthesis of AgNPs*

The AgNPs were prepared by chemical reduction. In brief, 2 mL of 0.01 mol/L $AgNO_3$ was added to 16 mL of deionised water. After the solution was boiled, 2 mL of 0.002 g/mL sodium citrate was added drop by drop to the $AgNO_3$ solution with constant stirring. Boiling was continued for 1 h until the colourless mixed solution turned dark brown.

2.3 *Biosynthesis of AgNPs*

Ginger extract was prepared by cutting 5.0 g of rhizome into small pieces, which were refluxed with 100 mL of 70% ethanol at 70°C for 2 h. After cooling, the obtained extract was filtered through Whatman No. 1 filter paper and centrifuged. The supernatant was collected and stored at 4°C.

For the biosynthesis of AgNPs, 1 mL of ginger extract was added to 20 mL of the $AgNO_3$ solution (1 mmol/L) in a round-bottom flask. The mixture was heated at 85°C and color change of the solution was recorded within 20 min.

2.4 *Characterization*

The as-prepared AgNPs were characterised by UV–visible (vis) spectroscopy on a Shimadzu UV-2550 spectrophotometer in the range of 200–800 nm. The size and shape of AgNPs were analyzed by transmission electron microscopy (TEM, JEM 1011) at an acceleration voltage of 100 kV. The freeze-dried powder of the resulting AgNP solution was used for X-ray diffraction (XRD; Rigaku D/max-2500VPC) with Ni-filtered Cu Kα radiation at a scanning rate of 0.02°/s from 25° to 80°. The Fourier transform infrared spectroscopy (FTIR) spectra of the samples were recorded by FTIR (Nicolet Nexus 670).

2.5 *Antibacterial activity studies*

The antibacterial activity of the synthesized AgNPs was assayed by the agar well diffusion method (Wang et al., 2016). Six typical aquatic pathogenic strains of *Vibrio anguillarum*, *Vibrio alginolyticus*, *Aeromonas punctata*, *Vibrio parahaemolyticus*, *Vibrio splendidus* and *Vibrio harveyi* (stored in the Department of Marine Biological Technology, Ludong University) were used to determine the antibacterial activity of the AgNPs. The test bacteria were cultivated at 28°C overnight in 2216E liquid culture medium (tryptone 5.0 g/L, yeast extract 1.0 g/L, ferric phosphate 0.1 g/L, and seawater 1.0 L). About 100 μL of the bacterial culture suspensions with final concentrations of 10^6 cfu/mL (cfu is colony-forming units) was spread evenly on nutrient agar plates (tryptone 5.0 g/L, yeast extract 1.0 g/L, ferric phosphate 0.1 g/L, agar 15 g/L, and seawater 1.0 L). Oxford cups (8 mm in diameter) were gently placed on the inoculated agar plates, and 20 μL of the respective AgNPs samples was added to the cups. Sodium citrate and ginger extract served as controls. All plates were incubated at 28°C overnight. The zones of inhibition were measured and reported as an average. Experiments were performed in quadruplicate.

Minimum inhibition concentration (MIC) of synthesized AgNPs was determined by broth dilution assay. *V. anguillarum* was chosen as the model organism. The samples were diluted and added to 4 mL of 2216E liquid culture medium with tested bacterial concentrations of 10^6 cfu/mL (the final concentration of the silver sample was 0.8–50 μg/mL). The 2216E medium was used as negative control and 10^6 CFU/mL bacterial suspensions as positive control. Bacterial growth was observed after 24 h of incubation at 28°C. The lowest concentration of the prepared AgNPs at which no bacterial growth was observed in the culture suspensions was defined as the MIC. To ensure accuracy of results, the experiment was repeated three times.

For the bacterial inhibition kinetic test, *V. anguillarum* was also selected as the index bacterium and incubated overnight at 28°C. The bacterial culture suspension was adjusted to 10^6 cfu/mL, and then the two obtained AgNPs as well as ginger extract and sodium citrate was added at a final concentration of 10 μg/mL, respectively. The bacteria solution was incubated at 28°C while being shaken at 150 r/min. Bacterial growth rates were measured by monitoring the optical density at 600 nm (OD_{600}) at different time intervals using a UV–vis spectrophotometer (Shimadzu UV-2550). Normal saline was used as control. To ensure accuracy of results, the experiment was repeated three times.

2.6 *Stability experiment*

The stability of the AgNP hydrocolloid was determined by visual inspection after 90 days of storage in 4°C, followed by UV–vis spectroscopy and the agar well diffusion method.

3 Results and discussion

3.1 *Characteristics of AgNPs*

We synthesized two kinds of AgNPs: C-AgNPs by sodium citrate and B-AgNPs by ginger extracts. As shown in Fig. 1a, the colour of the reaction solution changed to dark brown with sodium citrate and golden yellow with ginger extracts, indicating the formation of AgNPs (Fig. 1a). The colour change is attributed to the excited surface plasmon resonance (SPR) of the AgNPs and the colour depth is dependent on the concentration and size of the NPs (Mulvaney, 1996). The formation of AgNPs was further examined by UV–vis spectroscopy. As shown in Fig. 1b, the absorption spectrum of C-AgNPs displayed a strong peak at 415 nm, whereas the B-AgNPs at 423 nm. Both of the characteristic peaks located in 400–500 nm, which was the λ_{max} range of typical AgNPs (Sadeghi and Gholamhoseinpoor, 2015). These results indicated that the components in ginger extract could function as the chemical reductant to synthesize AgNPs.

The morphology and size of the prepared AgNPs were evaluated by high-magnification TEM. The C-AgNPs were heterogeneously dispersed with the size range of 20–80 nm, the majority of which were polygonally shaped (Fig. 2a). By contrast, B-AgNPs were spherical and almost monodispersed with an average particle size of 10 nm (Fig. 2b). It is known that as for the AgNPs, the morphology and size are important criterions for any kind of

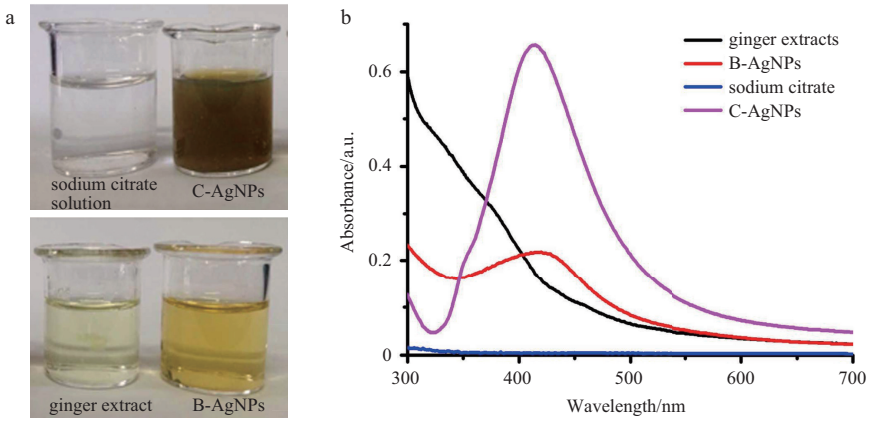

Fig. 1. Production of AgNPs using sodium citrate and ginger extract (a), and UV–vis absorption spectra of synthesized AgNPs (b).

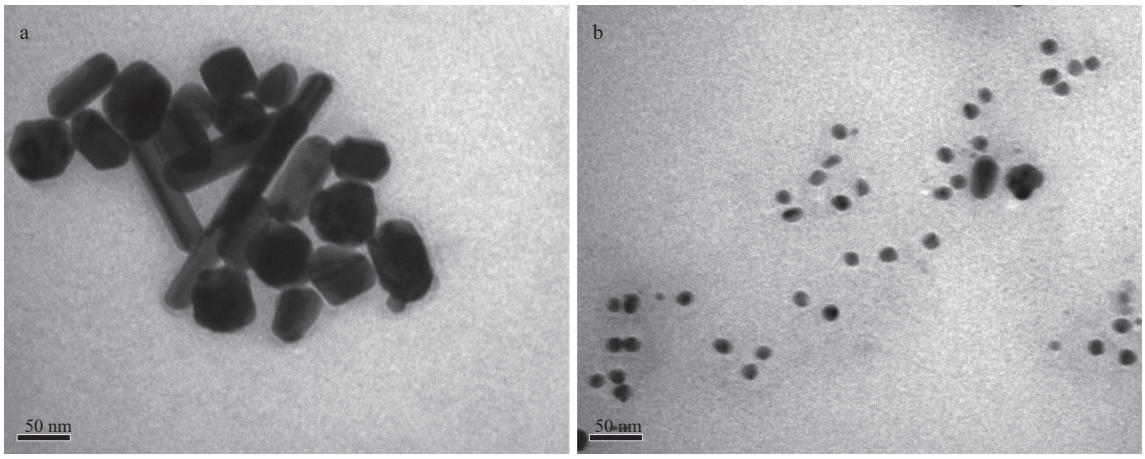

Fig. 2. TEM micrographs of C-AgNPs (a) and B-AgNPs (b).

application (Panáček et al., 2005). Previous studies also showed that spherical AgNPs with smaller size were anticipated to exhibit higher antibacterial properties (Pal et al., 2007; Singh et al., 2008). Hence, the results above indicated that the prepared B-AgNPs were at an ideal state as antimicrobial agents.

The crystalline structure of the synthesized AgNPs was investigated by XRD analysis (Fig. 3). In the case of C-AgNPs, four distinct diffraction peaks at 2θ=38.1°, 44.3°, 64.4° and 77.4° corresponded to the (111), (200), (220) and (311) planes of the face-centred cubic crystalline structure of Ag (JCPDS file No. 65–2 871). Thereby, the XRD pattern revealed that the sample was composed of crystalline face centered cubic (fcc) lattice structures of elemental silver. For B-AgNPs, two weak peaks centred at 2θ of 38.1° and 44.3° were observed because of the low concentration of AgNPs, which was consistent with the aforementioned results of UV–vis spectroscopy. Some of the unassigned diffraction peaks might be related to the crystallisation of bioorganic phases that attached to the surface of the NPs.

The size of the NPs was thus determined based on Scherer's equation:

$$D = k\lambda / \beta\cos\theta,$$

where D is the average crystal size, k is the Scherer coefficient (0.89), λ is the X-ray wavelength (λ=1.540 6×10^{-10} m), θ is Bragg's angle (2θ), β is the full width at half maximum (FWHM) in radians (Vidhu et al., 2011). The calculated grain sizes of the C-AgNPs and B-AgNPs were about 18.7 nm and 9.2 nm, respectively, which were in agreement with the results of TEM analysis.

The FTIR spectra of C-AgNPs, B-AgNPs and ginger extract in the range of 4 000–500 cm^{-1} were shown in Fig. 4. The ginger extract had peaks at 3 425.07, 2 919.28, 2 850.16, 2 361.78, 2 341.32, 1 735.22, 1 639.13, 1 382.13 and 1 083.70 cm^{-1}. Notably, the strong

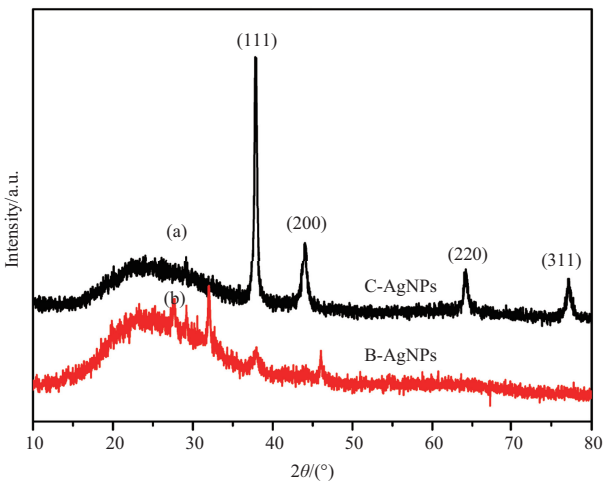

Fig. 3. XRD patterns of C-AgNPs (a) and B-AgNPs (b).

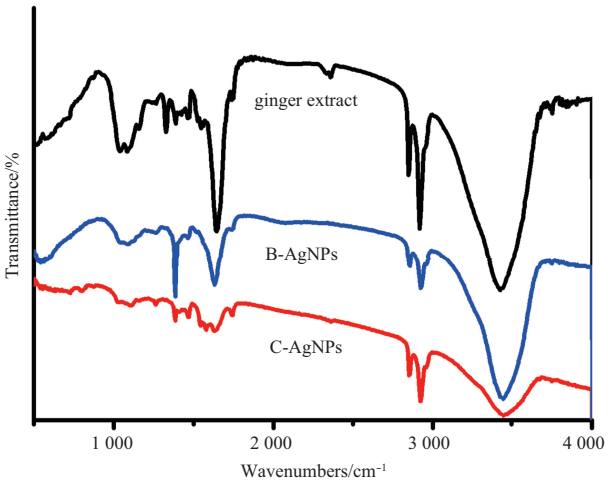

Fig. 4. FTIR spectra of C-AgNPs, B-AgNPs and ginger extract.

and wide band at 3 425.07 cm^{-1} referred to the strong stretching vibrations of the O-H functional group. The corresponding peaks at 2 919.28 and 2 850.16 cm^{-1} were caused by the C-H stretching vibrational mode, whereas the peaks at 1 632.28 and 1 735.22 cm^{-1} were attributed to the C=O stretching vibration of aldehydes and flavonoid. The peak at 1 382.13 cm^{-1} was attributed to the O-H deformation vibration of tertiary C-OH groups. Another weak peak near 1 083.70 cm^{-1} represented the C-O stretching vibration. The FTIR spectra of B-AgNPs shared very similar absorption peak positions with that of the ginger extract, which indicated the presence of ginger extract on the surface of the AgNPs. Besides, the peaks both at 3 400 cm^{-1} and 1 631.38 cm^{-1} of the B-AgNPs were obviously stronger than those of the C-AgNPs, which further confirmed that the compounds in ginger extract interacted with the surface of AgNPs.

3.2 *Antimicrobial activities of synthesized AgNPs*

The antibacterial activity of synthesized AgNPs against six typical aquatic pathogens was evaluated by the agar well diffusion method. As shown in Fig. 5 and Table 1, sodium citrate solution did not produce any inhibition zone against the tested bacteria; the ginger extract and C-AgNPs showed the inhibition zones which were slightly larger than the oxford cup, indicating

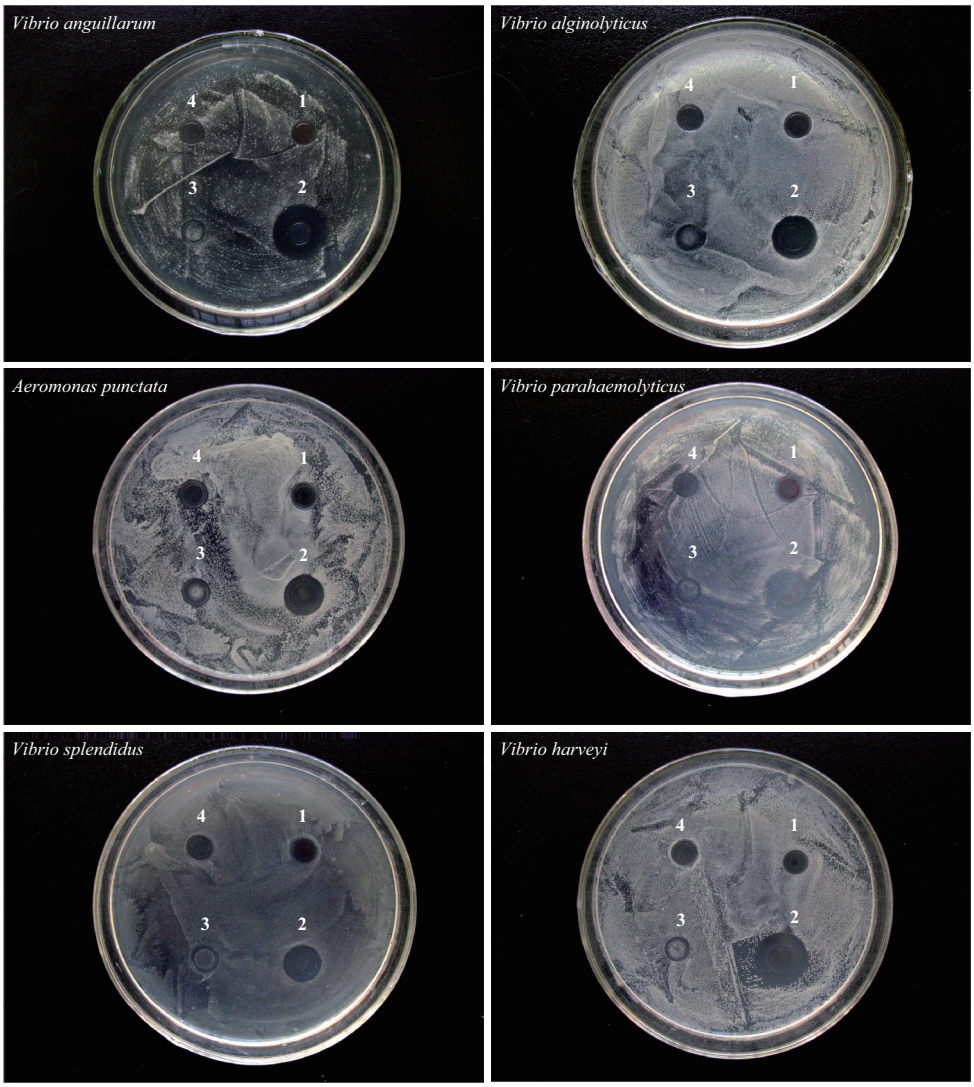

Fig. 5. Antibacterial effect of synthesized AgNPs against six different aquatic pathogens. 1. C-AgNPs, 2. B-AgNPs, 3. sodium citrate solution, and 4. ginger extract.

both of them had weak antibacterial activities. By contrast, B-AgNPs exhibited the significant inhibition zones against all of the tested bacteria, indicating that the B-AgNPs had a broad spectrum of antibacterial activities and was promising in the treatment of aquatic pathogenic bacteria.

For the MIC tests, gradiently diluted B-AgNPs and C- AgNPs were incubated with equal volumes of *V. anguillarum*, respectively. After 24 h of incubation, bacterial growth was studied by visual inspection. The MIC value of B-AgNPs was defined as 12.9 μg/mL and C-AgNPs was 50.0 μg/mL (Table 2). The result indicated that B-AgNPs showed relatively higher antibacterial activity than C-AgNPs.

Table 1. Diameter of inhibition zone (mm) of the synthesized AgNPs against various aquatic pathogenic bacteria

Pathogenic bacteria	Inhibition zone			
	Test		Control	
	C-AgNPs	B-AgNPs	Sodium citrate	Ginger extract
Vibrio anguillarum	8.4±0.03	15.8±0.05	NA	8.1±0.19
Vibrio alginolyticus	8.8±0.09	14.1±0.10	NA	8.0±0.00
Aeromonas punctata	8.5±0.04	13.5±0.07	NA	8.5±0.41
Vibrio parahaemolyticus	8.8±0.10	14.9±0.09	NA	8.2±0.21
Vibrio splendidus	8.4±0.08	11.1±0.02	NA	8.1±0.09
Vibrio harveyi	8.3±0.03	13.6±0.03	NA	8.2±0.12

Note: Values are expressed as the mean±standard error of the mean (n=3). NA means not appearing.

Table 2. Comparison of the MIC values of different samples (n=3)

Concentration of Ag/μg·mL^{-1}	B-AgNPs	C-AgNPs	Sodium citrate	Ginger extract
50.0	–	–	+	+
25.0	–	+	+	+
12.9	–	+	+	+
6.5	+	+	+	+
3.3	+	+	+	+
1.6	+	+	+	+
0.8	+	+	+	+
0.4	+	+	+	+

Furthermore, antibacterial activity kinetics for the silver nanoparticles samples as well as ginger extract and sodium citrate toward *V. anguillarum* were studied. The 10 μg/mL of each sample was incubated in bacterium suspensions, and bacterial growth rate was monitored by measuring the optical density of the culture solution at various time intervals (Fig. 6). For the negative control of normal saline, the optical density increased rapidly during the bacteria log phase, indicating the reproduction of *V. anguillarum*. The sodium citrate shared a similar growth curve of *V. anguillarum* with the negative control, while both C-AgNPs and ginger extract caused a growth delay of *V. anguillarum* and the growth curve increased gradually with a longer lag phase. Notably, *V. anguillarum* was completely inhibited as the optical density was almost the constant when treated with 10 μg/mL B-AgNPs. These results confirmed that the prepared B-AgNPs had powerful antibacterial activities against the aquatic pathogen.

AgNPs have been found to attach to the surface of the bacterial membrane, cause structural changes of the membrane, and finally lead to death of the microbe cells (Sondi and Salopek-Sondi, 2004; Kim et al., 2007). Generally, smaller AgNPs have a greater ability to penetrate cell membrane, and thus improve antibacterial activity (Singh et al., 2008; Dubey et al., 2010). In this study, B-AgNPs with smaller and more uniform size possessed significantly higher antibacterial activity than C-AgNPs, which is consistent with the previous reports. Besides, as the previous studies, the bioactive compounds in the ginger extract are supposed to improve the characteristics of the B-AgNPs, thereby partially contributing to the higher antibacterial activities of B-AgNPs (Niraimathi et al., 2013).

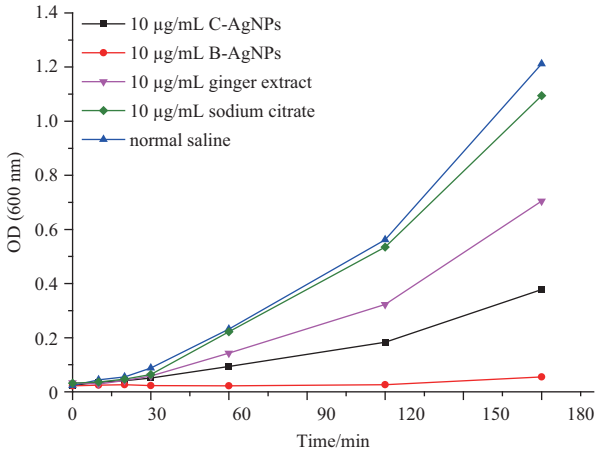

Fig. 6. Growth curve of *V. anguillarum* in 2216E media. C-AgNPs, B-AgNPs, ginger extract and sodium citrate (10 μg/mL) was added into the culture, respectively.

3.3 *Stability study*

The stability of AgNPs is a key factor that determines their antibacterial activity (Teeguarden et al., 2007). Thus, we further evaluated the stability of C-AgNPs and B-AgNPs after 90 days. The UV–vis absorption spectra showed that an obvious red-shift was observed in the surface plasmon peak of C-AgNPs, while the shift was almost negligible in that of B-AgNPs (Fig. 7). The results indicated that C-AgNPs were agglomerated and B-AgNPs were still well dispersed after 90 days. In addition, B-AgNPs still showed remarkable diffusion zones against the tested aquatic pathogens after 90 days, whereas C-AgNPs no longer produced inhibition zones (data not shown). Velmurugan et al. (2014) proved that the existence of phenolic compounds, terpenoids and proteins in ginger extract may prevent agglomeration and

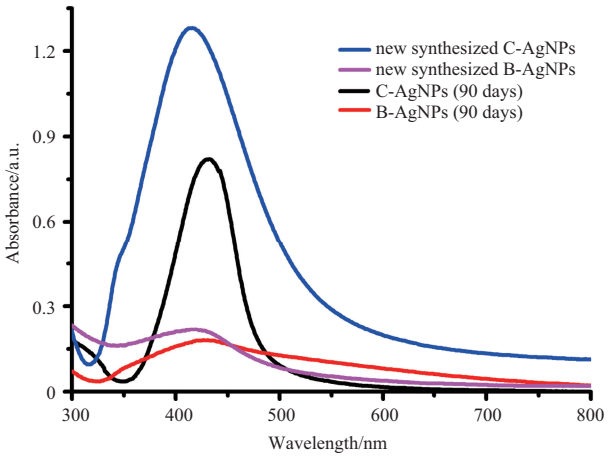

Fig. 7. Stability analysis of C-AgNPs and B-AgNPs stored for 90 days by UV–vis spectroscopy.

enhance stabilisation by covering the NPs. Therefore, the utilization of ginger extract during NP synthesis is beneficial to the stability of B-AgNPs.

4 Conclusions

In this study, B-AgNPs and C-AgNPs were synthesized by ginger extracts and sodium citrate, respectively. TEM studies revealed that B-AgNPs were superior to C-AgNPs in either size or morphology. XRD and FTIR analyses confirmed that the active components of ginger extract could reduce Ag^+ and stabilize the AgNPs. Moreover, the B-AgNPs showed higher antibacterial activities against aquatic pathogens than C-AgNPs. Besides, B-AgNPs maintained better dispersion and higher stability than C-AgNPs after 90 days. Our results indicated that the ginger extract could effectively synthesize the AgNPs at moderate temperature, and this eco-friendly method may have prospective applications in aquaculture.

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