Indian J. Fish., 72 (4): 94-102, 2025 doi:10.21077/ijf.2025.72.4.170069-12

Available online at: epubs.icar.org.in/index.php/IJF

Antibacterial activity of green-synthesised silver nanoparticles (AgNPs) on major bacterial fish pathogens with emphasis on their extracellular enzymatic and haemolytic activities

Basanta Kumar Das[1*], Tamasi Senapati[2], Jyotirmayee Pradhan[3], Kausalya K. Nayak[4], Barsha Baisakhi[1,3], Debasmita Mohanty[1], Mala Kumari[1] and Swagatika Sahu[5]

[1]ICAR-Central Inland Fisheries Research Institute, Barrackpore, Kolkota - 700 120, West Bengal, India
[2]ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar - 751 002, Odisha, India
[3]Kuntala Kumari Sabat Women's College, Balasore - 756 003, Odisha, India
[4]Kshitrabasi Dayanand Anglo Vedic College, Nirakarpur, Khurdha - 752 019, Odisha, India
[5]Krishi Vigyan Kendra, Baliapal, Balasore - 756 027, Odisha, India

Abstract

Silver nanoparticles (AgNPs) were synthesised using a green synthesis apprach using silver nitrate, starch and glucose under optimised conditions. Morphometric characterisation of the green synthesised AgNPs was carried out by Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), dynamic light scattering (DLS), and X-ray diffraction (XRD). The antibacterial, extracellular enzymatic and haemolytic activities of AgNPs were evaluated against nine fish pathogenic bacteria *viz*., *Aeromonas hydrophila* (AH1), *Edwardsiella tarda* (ATCC15947), *Pseudomonas putida* (ATCC49128), *Pseudomonas aeruginosa* (ATCC35072), *Pseudomonas fluorescens* (PF1), *Vibrio alginolyticus* (ATCC17749), *Vibrio parahaemolyticus* (ATCC17802), *Escherichia coli* (EC1) and *Staphylococcus aureus* (ATCC6538). Bactericidal activity of of AgNPs was assessed using both minimum inhibitory concentration (MIC) and disc diffusion assays. In *A. hydrophila*, starch hydrolysis was completely inhibited from 72 h onwards in cultures exposed to both 50 µl and 100 µl of AgNPs. Similarly the haemolytic activity of *V. alginolyticus* showed a marked reduction from 24 h onwards at both AgNP exposure levels (50 µl and 100 µl). Bacterial sensitivity to AgNPs was found to vary among species, indicating pathogen-specific responses to AgNP treatment.

*Correspondence e-mail:
basantakumard@gmail.com

Keywords:
Bacterial sensitivity, Minimum inhibitory concentration, Nanomaterials in aquaculture, Virulence factors

Received : 09.08.2025
Accepted : 30.12.2025

Introduction

Miniaturisation is a fundamental objective of technological advancement aimed at developing smaller, faster, lighter and cost-effective devices with greater functionality while using fewer raw materials and consuming less energy. In this context, nanotechnology has brought a revolution in various fields, by focusing on structures in the size range of 1-100 nm in applied science and technology (Saxena *et al.,* 2010). Nanotechnology has seen an expansion in the bio-fabrication process of metal nanoparticles (MNPs) (Balantrapu and Goia, 2009). Among MNPs, silver nanoparticles (AgNPs) have drawn considerable attention for their high-efficiency, low-cost, distinct electronic and optical properties, enabling diverse industrial uses such as antimicrobial agents (Kheybari *et al.,* 2010), catalysts (Vasileva *et al.,* 2011), conductive coating and sensors (Singh *et al.,* 2009). However, many conventional physical and chemical methods implemented for the synthesis of AgNPs arecomplex, inefficient, costly and often non-biocompatible, involving the use of high energy, pressure, and temperature, as well as the use of toxic organic chemicals (Singh *et al.,* 2009). Though physical and chemical methods are widely employed for nanoparticle synthesis, the use of toxic reagents limits their practical applications. Thus, there is a growing demand to develop eco-friendly methods for the synthesis of nanoparticles that minimise environmental risks, while ensuring economic viability by limiting production costs.

Three fundamental requirements should be considered in the green synthesis of nanoparticles: (i) the choice of an environmentally safe and inexpensive solvent; (ii) the selection of a cost-effective and eco-friendly reducing agent; and (iii) the choice of renewable stabilising agents to protect the nanoparticle surface (Raveendran *et al.,* 2003). Accordingly, an eco-friendly approach for nanoparticle synthesis has several advantages, such as simplicity, cost-effectiveness, and compatibility for biomedical and pharmaceutical applications, as well as suitability for large-scale commercial production. In recent years, increasing attention has been directed towards green synthesis methods employing non-toxic materials, such as plant extracts for the synthesis of silver nanoparticles (AgNPs) (Ansari and Alzohairy, 2018; Pirtarighat *et al.,* 2019).

In the present study, starch was selected as the stabilising (capping) agent because of its renewable nature and ability to form stable dispersions in water. Previous studies have reported the use of starch as a capping agent for the production of uniform and monodisperse nanoparticles under the green synthesis strategy (Oluwafemi, 2009; Yakout and Mostafa, 2015). Owing to its natural abundance, biodegradability, and environmental compatibility, starch is widely recognised as an eco-friendly polymer for nanoparticle synthesis. Silver ions and silver-based compounds are well known for their toxicity to microorganisms. The antimicrobial activity of AgNPs is attributed to their accumulation in the bacterial cytoplasmic membrane, leading to increased membrane permeability and eventual cell death. It has been suggested that the free radicals generated on the surface of AgNPs play a major role in membrane damage in the antimicrobial mechanism (Kim *et al.,* 2007). Derivatives of silver and several silver salts are commercially applied as antimicrobial agents. However, the stability of these nanoparticles in the growth medium and aquaculture environments such as fishponds imparts greater retention time for bacterium-nanoparticle interactions, which determines their antibacterial property (Shrivastava *et al.,* 2007). The present study was carried out to synthesise AgNPs using a green approach with starch and glucose. The inhibitory potential of AgNPs was evaluated against major bacterial fish pathogens to assess their applicability as alternative antimicrobial agents in aquaculture.

Materials and methods

Test organisms

Fish pathogenic bacterial strains, *viz., Aeromonas hydrophila* (AH1), *Edwardsiella tarda* (ATCC 15947), *Pseudomonas putida* (ATCC 49128), *P. aeruginosa* (ATCC 35072), *P. fluorescens* (PF 1), *Vibrio alginolyticus* (ATCC 17749), *V. parahaemolyticus* (ATCC 17802), *Escherichia coli* (EC1), and *Staphylococcus aureus* (ATCC 6538) were used to evaluate the antibacterial sensitivity, extracellular enzymatic activity and haemolytic activity.

Green synthesis of silver nanoparticles and characterisation

AgNPs were synthesised by adopting the protocol of Raveendran *et al.* (2003). Briefly, an aliquot (100 µl) of a 0.10 M solution of $AgNO_3$ was added to 6.0 ml of a 0.17% (w/w) aqueous solution of soluble starch (Sigma). Prior to mixing, each solution was passed through inert nitrogen gas to remove dissolved oxygen. Then, a 150 µl aliquot of a 0.10 M aqueous solution of β-glucose (Sigma) was added as a reducing agent and stirred properly. The mixture was heated to 40°C and maintained at this temperature for 20 h to facilitate nanoparticle formation. The morphology and size distribution of the synthesised silver nanoparticles were analysed using transmission electron microscopy (TEM) (JEOL JEM-2100). Functional groups and chemical nature were characterised using an attenuated total reflectance–Fourier transform infrared (ATR-FTIR) spectroscope (Bruker VERTEX70). Particle size distribution, zeta potential and polydispersity index (PDI) of AgNPs were measured, using dynamic light scattering (DLS) was also ascertained. Additionally, the crystalline structure of the nanoparticles was analysed using X-ray diffraction (XRD) (SmartLabXE).

Antibacterial activity of AGNPs

The antibacterial activity of silver nanoparticles was evaluated by the disc diffusion technique (Chabbert, 1963). The bacterial fish pathogens maintained by the Fish Health Management Division of the ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), Bhubaneswar, were used. Sterile filter paper discs were impregnated with AgNP solutions at different concentrations (20, 30, 40 and 50 µl) and were placed on solidified agar plates previously inoculated with selected bacterial pathogens. Antibacterial activity was assessed by measuring the diameter of the clear inhibition zones around the discs after 24 h incubation at 37°C.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of AGNPs was evaluated by the tube dilution assay (Alderman and Smith, 2001). Briefly, 2 ml of bacterial suspension (1×10^7 CFU ml^{-1}) of the test organism was added to test tubes containing different concentrations of AgNPs (20, 40, 50, and 100 µl). The mixtures were incubated overnight, and the bacterial growth was assessed by visual observation of turbidity in each tube, which was visualised to determine the MIC. The lowest concentration of AgNPs showing no visible turbidity was recorded as the MIC.

Treatment of bacterial cultures with AGNPs

For each organism, 3 treatment sets were prepared in sterile test tubes. Set 1 (control) comprised only bacterial inoculum, set 2 consisted of bacterial inoculum along with 50 µl of AgNPs, and set 3 consisted of bacterial inoculum along with 100 µl of AgNPs. All these sets were incubated at 37°C for 24 h prior to testing for the effect of AGNPs on the extracellular enzyme activity, as well as the haemolytic activity of the bacterial strains.

Extracellular enzyme activity

Starch hydrolysis test: Starch hydrolysis test was performed to determine the ability of the bacterial strains to hydrolyse starch into simpler compounds like dextrin, glucose and maltose by the production of amylase enzyme. Bacterial cultures from the 3 sets

prepared as described above were spot inoculated onto starch agar plates containing nutrient agar with 1% soluble starch and then incubated at 37°C for 24 h. The plates were then flooded with iodine solution and kept for 5 min; thereafter, the excess iodine solution was decanted. The appearance of clear zones around the colonies indicated complete starch hydrolysis, whereas unhydrolysed starch produced a blue coloured background. Partial hydrolysis of starch due to α-amylase would lead to the formation of reddish-brown areas around the colonies. The observations were taken up to 96 h to monitor enzyme activity over time.

Gelatin hydrolysis test : The proteolytic activity of bacteria, indicated by the production of gelatinase enzyme, was detected by this test. Bacterial cultures from all the 3 sets were spot inoculated onto gelatin agar plates containing nutrient agar with 2% gelatin and were incubated at 37°C for 24-48 h. Then, acidic mercury chloride was flooded over the plates, kept for 5 min, and then the excess solution was decanted. The appearance of a clear zone around the colonies implied the hydrolysis of gelatin by the enzyme gelatinase. Unhydrolysed gelatin formed a white precipitate, leading to opacity. The observations were taken up to 96 h to monitor enzyme activity.

Casein hydrolysis test : This test detects the proteolytic activity of bacteria by measuring casein hydrolysis through the production of the caseinase enzyme. The 3 sets of bacterial cultures prepared as described above were spot inoculated onto casein agar plates with 1% casein and incubated at 37°C for 24 - 48 h. After the incubation, the plates were flooded with acidic mercury chloride and allowed to stand for 5 min, and excess solution was decanted. Formation of clear zones around the colonies indicated casein hydrolysis, whereas unhydrolysed casein forms a white precipitate, leading to opacity. The observations were taken up to 96 h to monitor enzyme activity.

Haemolytic activity

Haemolytic activity of the bacterial strains was assessed using blood agar medium, following exposure to silver AgNPs (as described earlier). Two types of haemolysis were observed: α-haemolysis, characterised by greenish discolouration with partial clearing around the colonies and β-haemolysis, characterised by complete lysis of erythrocytes resulting in a transparent clear zone around the colonies. The type of hemolysis was recorded, and the diameter of the zone was measured. Observations were made at 24 h intervals for up to 96 h.

Statistical analyses

Data from all experiments were expressed as Mean±Standard Error (SE) based on three independent replicates (n=3). Differences among treatments and exposure durations were analysed using two-way analysis of variance (ANOVA) using SPSS version 22 and significance was considered at $p < 0.05$.

Results

Synthesis and characterisation of silver nanoparticles

FTIR spectroscopy was employed to identify the functional groups present in biomolecules responsible for the bioreduction of Ag^+ ions and the capping/stabilisation of silver nanoparticles. The detected absorption bands were compared with standard reference values to assign the corresponding functional groups. The FTIR spectrum exhibited prominent absorption bands at 3304, 2944, 2889, 1646, 1418, 1334, 1148, 1078, 993, 926, 859, 760 cm^{1} are evident, indicating the presence of multiple functional moieties involved in nanoparticle formation and stabilisation.

In the spectra, the absorption band at 3304 cm^{1} corresponds to -OH stretching vibration, indicating the presence of alcohol and phenol. Bands at 2994 and 2889 cm^{1} indicated the C-H stretching of aromatic compounds. The peak at 1646 cm^{1} corresponds to C-N and C-C stretching (Prakash *et al.,* 2013), benzene ring containing aromatic compounds. Bands at 1418 cm^{1} exemplify N-H stretch vibration present in the amide linkages, and 1334 cm^{1} is characteristic of hydroxyl groups and phenolic hydroxyl groups, respectively. The C–O stretching and alkene groups (C–H stretching) are present at 1078 and 926 $cm^{1,}$ respectively. The peak near 833 and 760 cm^{1} is assigned to C=C-H and C–H bending. These functional groups have a role in the stability/capping of AgNPs, as reported in many studies (Fig. 1).

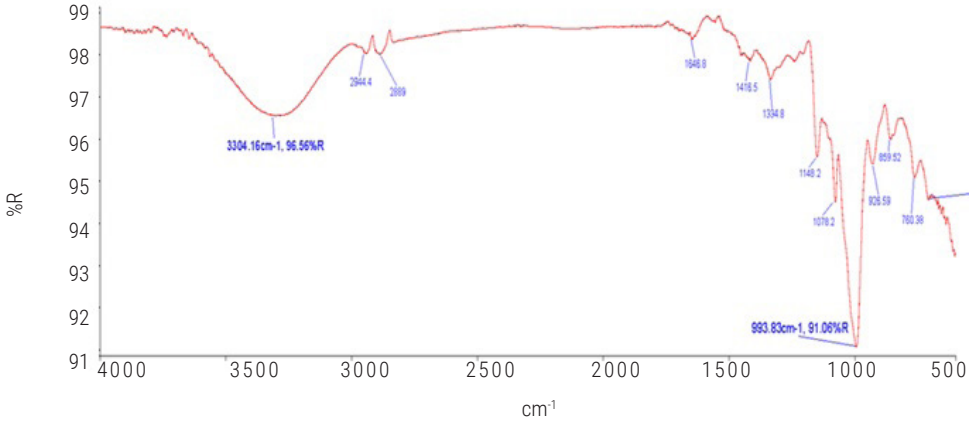


Fig. 1. FTIR spectra of green-synthesised AgNPs

DLS analysis confirmed that the green-synthesised silver nanoparticles were well dispersed in solution with an average particle size of 53.3±2.15 nm. The zeta potential measured was -22.9±0.79 mV, indicating good colloidal stability (Fig. 2a,b). TEM images (Fig. 3a, b) revealed that the nanoparticles had a spherical, homogenous structure and were uniformly dispersed. The XRD diffractogram of the synthesised AgNPs (Fig. 4) presented two prominent diffraction peaks at 33.2° and 61.7°, along with several weaker peaks, confirming the crystalline nature of the nanoparticles.

Antibacterial activity of AGNPs

The minimum inhibitory effects of silver nanoparticles against nine selected bacterial pathogens are shown in Fig. 5 . The maximum zone of inhibition (18.83±0.05 mm) was shown by the highest concentration of AgNPs (50 µl) against *A. hydrophila*. Maximum zone size was also exhibited by the highest concentration of AgNPs against *E. tarda*, *E. coli, V. alginolyticus* and *V. parahaemolyticus.* Similarly, the *Pseudomonas* spp. were also sensitive towards all four concentrations of AgNPs, and the highest concentration exhibited the maximum zone size. AgNPs at the lowest concentration showed a moderate zone of inhibition.

Minimum inhibitory concentration (MIC)

Detailed screening revealed that the MIC values of silver nanoparticles ranged from 20 µl to 100 µl among the tested bacterial fish pathogens (Table 1). The lowest MIC (20 µl) was observed against *V. alginolyticus*. In contrast, higher MIC values ranging from 40 to 100 µl were noticed against the rest of the pathogens.

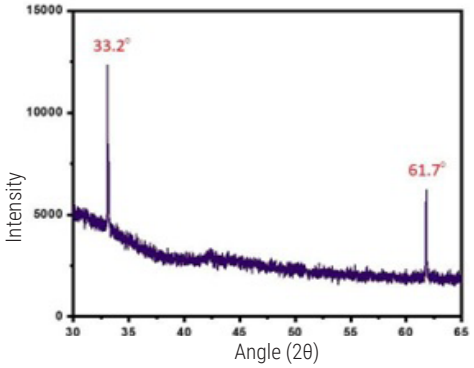
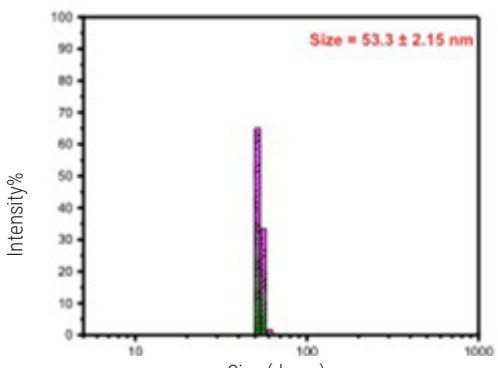
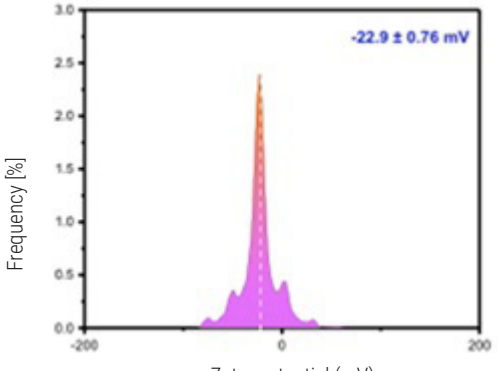


Fig. 4. XRD diffraction pattern of AgNPs

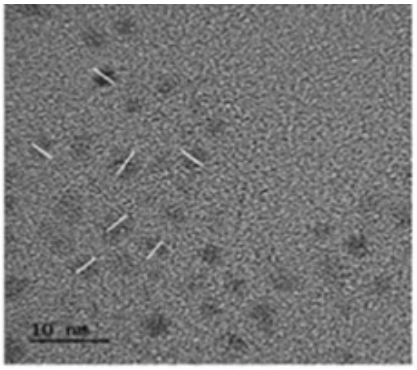


(a)

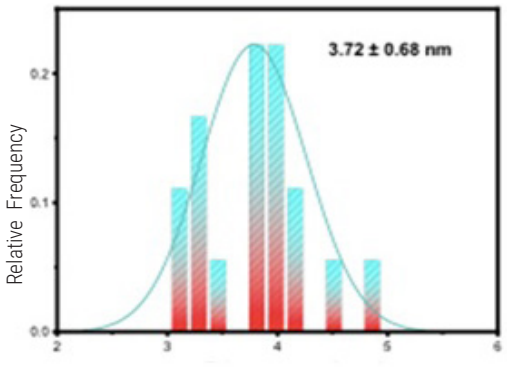


(b)

Fig. 2. Characterisation of AgNPs for particle size distribution and zeta potential analysis by DLS



(a)



(b)

Fig. 3. TEM micrograph and relative frequency of AgNPs

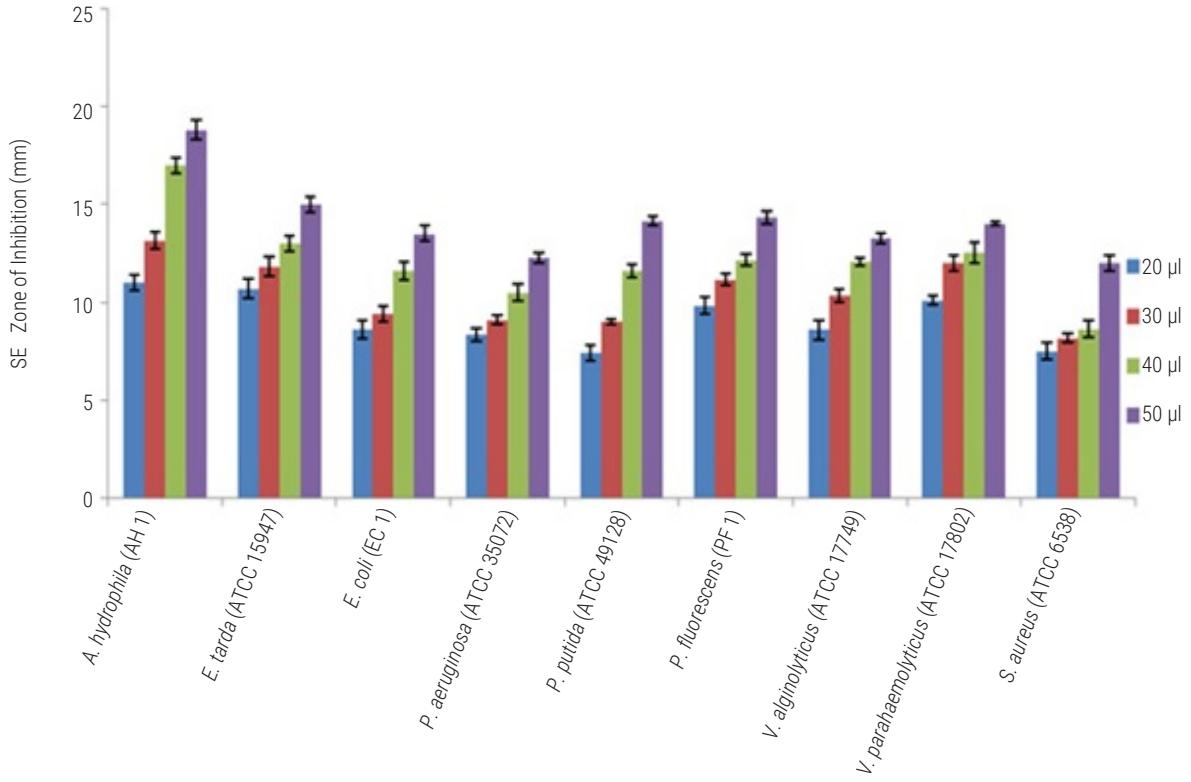


Fig. 5. Antibacterial activity of silver nanoparticles against selected bacterial fish pathogens assessed by the disc diffusion method after 24 h incubation. Data represent Mean±SE

Table 1. Minimum inhibitory concentration (MIC) of AgNPs against selected bacterial fish pathogens

Bacterial pathogens	MIC (µl ml^{-1})
A. hydrophila (AH 1)	40
P. aeruginosa (ATCC 35072)	40
P. fluorescens (PF 1)	50-100
P. putida (ATCC 49128)	50-100
E. coli (EC 1)	50-100
E. tarda (ATCC 15947)	50-100
V. alginolyticus (ATCC 17749)	20-40
V. parahaemolyticus (ATCC 17802)	50-100
S. aureus (ATCC 6538)	50-100

Effect of AGNPs on the extracellular enzymatic activity of bacterial fish pathogens

Amylase activity

The amylase activities of different bacterial pathogens exposed to AgNPs over a 96 h period are summarised in Fig. 6. The amylase activity at 24 h was higher in the control (1.8) compared to cultures exposed to 50 µl and 100 µl AgNPs. At 48 h, the enzyme activity was approximately similar in the control and the 50 µl AgNP-exposed cultures, whereas no starch hydrolysis was detected in the 100 µl AgNP-treated group from 48 h onwards. At 72 h, the control (1.41) exhibited higher enzymatic activity than the 50 µl AgNP-treated cultures. At 96 h, neither the control nor *A. hydrophila* exposed to AgNPs showed amylase activity. Overall, starch hydrolysis was highest at 24 h. Statistical analysis indicated that AgNP exposure significantly reduced amylase activity in *A. hydrophila*, *E. tarda*, and *P. aeruginosa*, compared with the control ($p<0.05$), with the highest inhibition observed at the higher AgNP concentration (100 µl) and at longer exposure durations.

Gelatinase activity

The gelatinase activities of different bacterial pathogens exposed to AgNPs over a 96 h period are summarised in Fig. 7. The gelatinase activity at 24 h was higher in *A. hydrophila* exposed to 100 µl (3.25) AgNPs than that of the control and 50 µl AgNP treatments. However, at 72 h and 96 h, no gelatinase activity was detected in *A. hydrophila* exposed to 100 µl AgNPs. For *E. tarda* and *P. aeruginosa*, gelatinase activity at 24 h was higher in the control (1.81) compared to the AgNP-exposed groups (50 and 100 µl). Overall, 50 µl AgNP exposure (2.65) showed the maximum enzymatic activity at 96 h relative to 24, 48 and 72 h incubation periods. Statistical analysis revealed that AgNP treatment caused a significant reduction in gelatinase activity compared with the untreated control ($p<0.05$), particularly at higher concentrations and longer exposure durations.

Caseinase activity

Fig. 7. presents the caseinase activity of different bacterial pathogens exposed to AgNPs over a 96 h period. At 72 h, *A. hydrophila* and *E. tarda* exposed to 50 µl AgNPs (1.88) showed higher enzymatic activity compared with the control and 100 µl AgNP-treated groups. However,

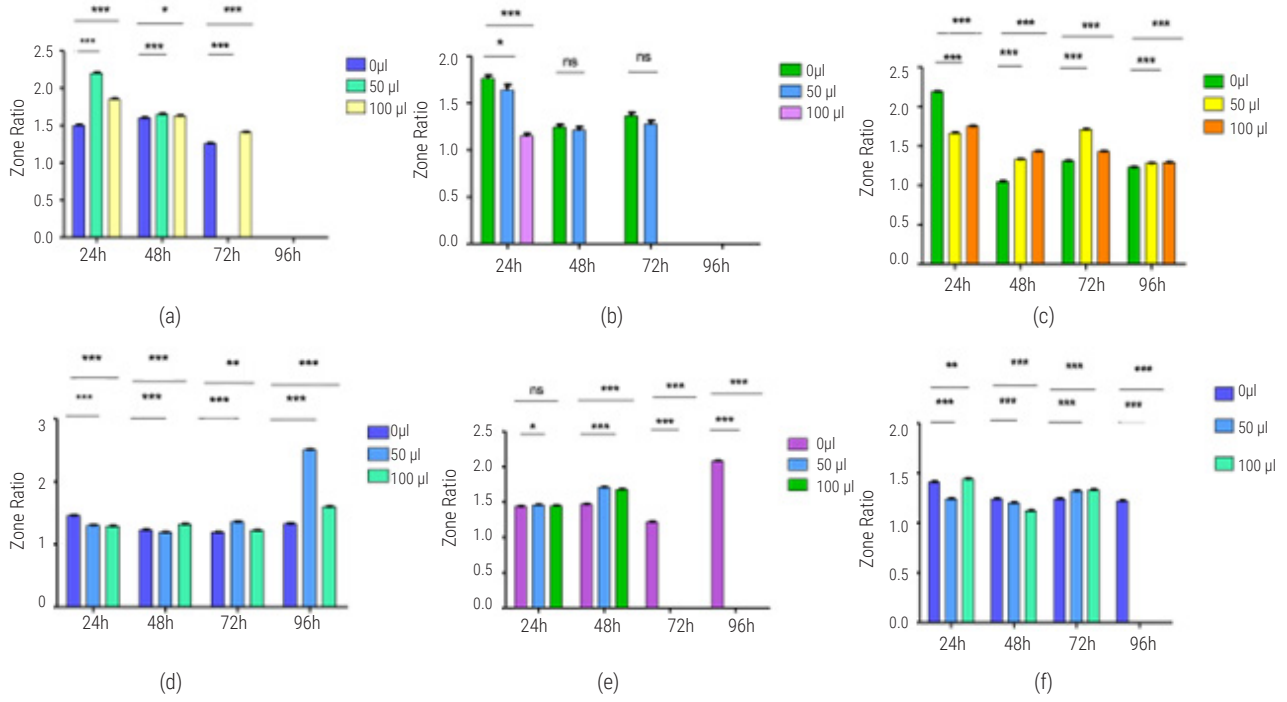


Fig. 6. Amylase activity (zone ratio) of bacterial fish pathogens exposed to AgNPs for 96 h. (a) *E. tarda* (ATCC 15947); (b) *A. hydraphila* (AH I); (c) *S. aureus* (ATCC 6538); (d) *P. putida* (ATCC 49128); (e) *P. aeruginosa* (ATCC 35072); (f) *P. fluorescens* (PF I). Data represent Mean±SE.

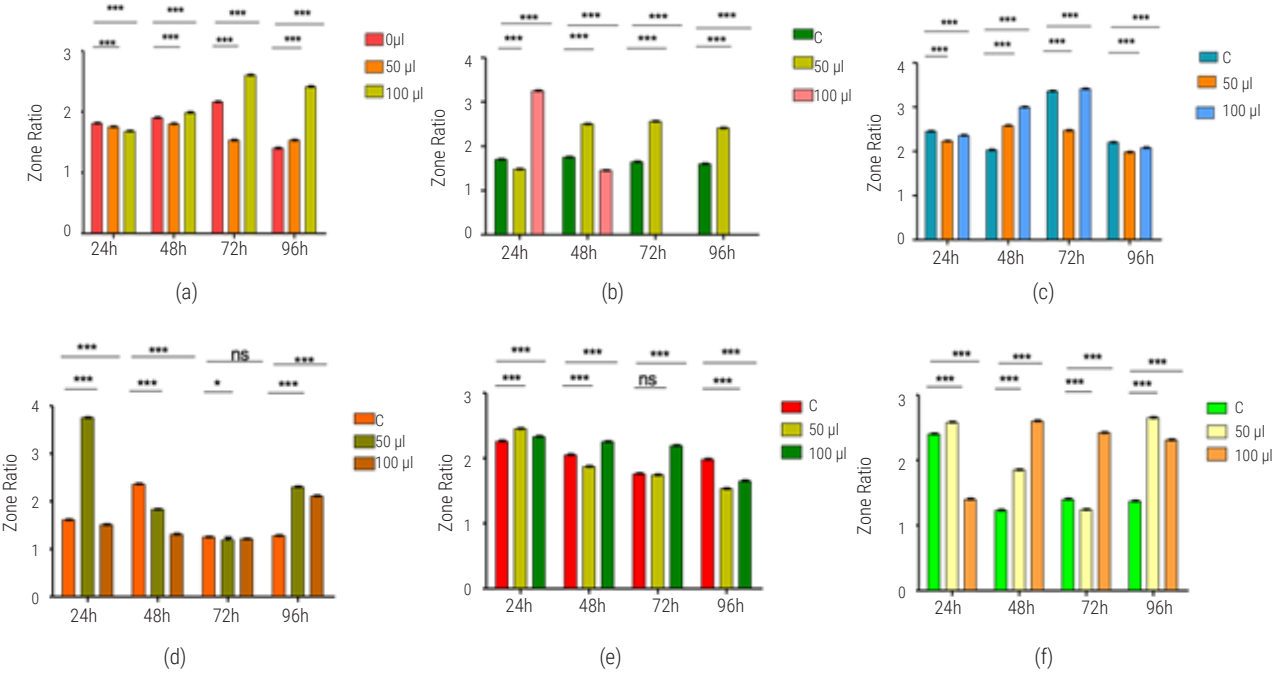


Fig. 7. Gelatinase activity (zone ratio) of bacterial pathogens exposed to silver nanoparticles for 96 h. (a) *E. tarda* (ATCC 15947); (b) *A. hydraphila* (AH I); (c) *S. aureus* (ATCC 6538); (d) *P. putida* (ATCC 49128); (e) *P. aeruginosa* (ATCC 35072); (f) *P.fluorescens* (PF I). Data represent Mean±SE

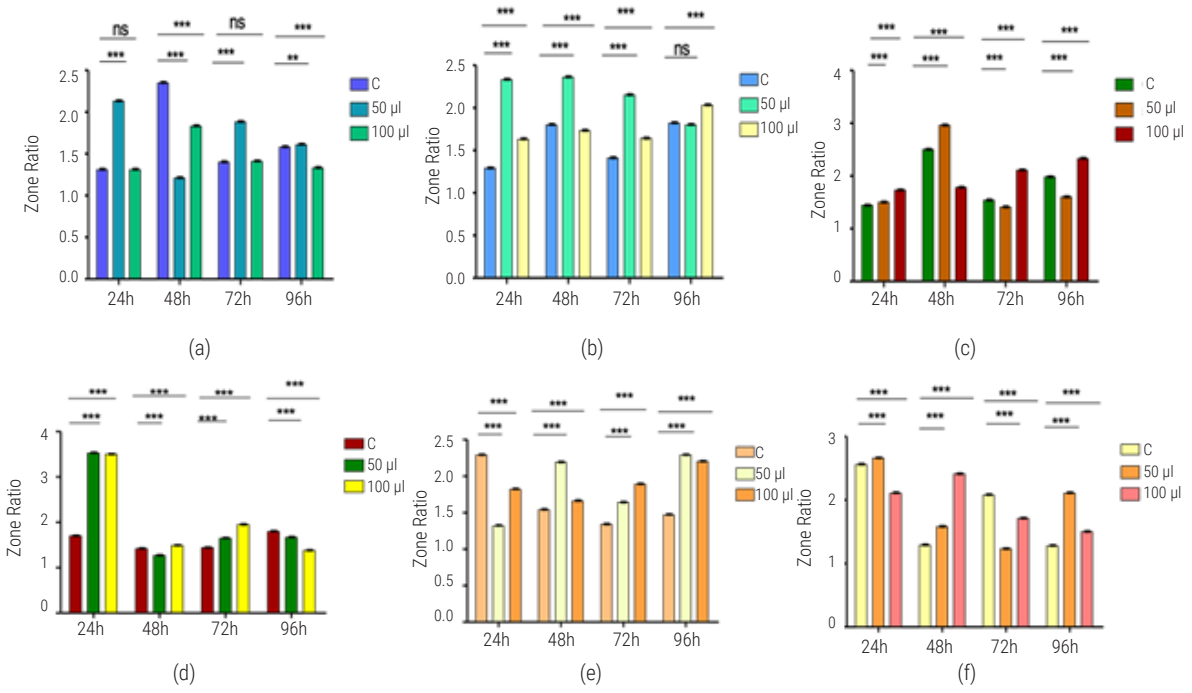


Fig. 8. Caseinase activity (zone ratio) of bacterial pathogens exposed to silver nanoparticles for 96 h. (a) *A. hydrophila* (AH I); (b) *E. tarda* (ATCC 15947); (c) *P. aeruginosa* (ATCC 35072); (d) *P. putida* (ATCC 49128); (e) *P. fluoresecens* (PF I); (f) *S. aureus* (ATCC 6538). Data represent mean±SE

across all treatments and time points, the maximum casein hydrolysis was observed at 24 h in cultures exposed to 50 µl AgNPs (2.35), compared with 24, 48 and 72 h incubation periods. Statistical analysis revealed that AgNP exposure significantly altered caseinase activity in *A. hydrophila*, *E. tarda*, and *P. aeruginosa* compared with the untreated control ($p<0.05$), with marked inhibition at 100 µl AgNPs and longer exposure durations.

Effect of AGNPs on haemolytic activity of bacterial fish pathogens

The haemolytic activity of bacterial pathogens exposed to AgNPs is summarised in Table 2. The haemolytic activity was measured at 24 h and 96 h for cultures treated with 50 µl and 100 µl AgNPs. The highest hemolytic activity of *A. hydrophila*, *V. alginolyticus*, *V. parahaemolyticus* and *E. tarda* was noticed in cultures treated with 50 µl AGNPs at both 24 h and 96 h. In the case of *E. coli*, haemolytic activity at both time points was higher in the 50 µl AgNP-treated culture, compared with the control and 100 µl AgNP treatment.

Discussion

The present study focused on the green synthesis of silver nanoparticles (AGNPs), which is based on three basic principles: the use of an appropriate solvent medium; environmentally benign reducing agents and non-toxic, eco-friendly stabilising substances to ensure nanoparticle stability (Raveendran *et al.,* 2003). We have used water as an environmentally benign solvent. Starch was used as a capping agent and glucose as a reducing agent under

Table 2. Haemolytic activity of bacterial pathogens exposed to AgNPs for 96 h. Data represent Mean±SE (n=3)

Bacterial pathogens	24 h			96 h		
	Control	50 µl	100 µl	Control	50 µl	100 µl
A. hydrophila	1.25±0.04	2.22±0.12	1.22±0.05	1.65±0.05	0	0
E. tarda	1.31±0.06	1.22±0.03	1.34±0.08	1.49±0.05	1.57±0.06	1.5±0.14
E. coli	1.24±0.04	1.42±0.06	1.21±0.06	1.45±0.05	1.47±0.04	1.34±0.05
P. aeruginosa	1.26±0.03	1.43±0.06	1.52±0.04	1.74±0.03	1.50±0.03	1.37±0.04
P. putida	1.14±0.04	1.31±0.04	1.71±0.04	1.17±0.04	1.22±0.02	1.18±0.03
P. fluorescens	1.24±0.04	1.51±0.05	1.56±0.06	1.5±0.05	2.01±0.11	1.64±0.06
V. alginolyticus	1.26±0.02	1.27±0.05	1.25±0.02	1.30±0.08	1.25±0.12	1.19±0.02
V. parahaemolyticus	1.1±0.01	1.42±0.05	1.12±0.04	1.17±0.03	0	0
S. aureus	1.25±0.05	1.27±0.03	1.45±0.07	1.44±0.03	1.38±0.04	1.58±0.04

mild heating conditions. The presence of starch in the mixture eliminates the need for relatively toxic solvents and contributes to the stabilisation of the synthesised nanoparticles. Physicochemical characterisation confirmed the effectiveness of the eco-friendly synthesis approach. DLS indicated that the AgNPs were uniformly distributed and colloidally stable, likely due to efficient surface capping by starch. TEM showed that the nanoparticles were spherical, homogeneous, and well dispersed, while XRD confirmed their crystalline nature corresponding to metallic silver, supporting their antimicrobial potential (Morones *et al.,* 2005).

The antibacterial efficacy of silver nanoparticles increased with concentration, as reflected by the enlargement of inhibition zones across all tested bacterial pathogens. The diameter of inhibition zones generally increased from 10 µl to 40 µl; beyond which only a marginal increase was observed. Enhanced antibacterial activity has been observed in silver nanoparticles modified by polymers such as PVP 360, and surfactants such as SDS and Tween 80 (Kvitek *et al.,* 2008). Other workers reported the effect of silver nanoparticles on the bacterial growth of *P. aeruginosa*, *E. coli* and *V. cholerae* using scanning electron microscope (SEM) techniques (Sondi *et al.,* 2003; Morones *et al.,* 2005). According to them, the cell damage observed may be due to the interaction of silver nanoparticles with phosphorus and sulphur-containing compounds such as DNA. Silver has a high affinity for such compounds and, to inactivate the bacteria, silver ions strongly interact with the available thiol groups of biomolecules (Matsumura *et al.,* 2002). It was noticed that the antibacterial activity of silver ions under anaerobic conditions was less potent than in an oxygen-rich environment. The prevention of DNA replication is due to interaction in the cell membrane (Matsumura *et al.,* 2002), which causes bacterial cell death (Feng *et al.,* 2000). The antimicrobial activities of silver nanoparticles against *E. coli* have been reported to be dependent on the chemisorbed silver ions and particle size (Sondi and Salopek-Sondi, 2004). In our study, the antimicrobial activities as reflected by the zone of inhibition in the disc diffusion method may be corroborated with the findings of Lok *et al.* (2007). Lee *et al.* (2003) reported that AgNPs inhibited *S. aureus,* which has potential applications in the textile industry. In our study, we have also found that AgNPs were effective against *S. aureus,* producing a zone of inhibition of 12 mm, which was lower than that of other Gram-negative bacteria. Among the tested pathogens, the lowest MIC was recorded in *V. alginolyticus,* followed by *A. hydrophila* and *P. aeruginosa*. These findings highlight the species-specific antibacterial response of fish pathogens to green-synthesised AgNPs.

Bacteria secrete a range of extracellular compounds, including those that degrade proteins like casein, gelatin and also hydrolyse starch, which contribute to their ecological success and adaptability. The enzymes secreted by both Gram-negative and Gram-positive bacteria are closely associated with virulence and pathogenesis. Previous studies have reported on the toxicity of AgNP to a variety of microorganisms, including changes in cell morphology and DNA damage, which may directly affect enzyme production and secretion (Sharma *et al.,* 2009). In the present study, amylase, gelatinase and caseinase activities were evaluated in *A. hydrophila, E. tarda, P. aeruginosa, P. putida, P. fluorescens* and *S. aureus* and found that amylase activity was completely inhibited in *A. hydrophila, E. tarda, P. aeruginosa, P. putida,* and *P. fluorescens* at 96 h when exposed to 50 and 100 µl concentrations of AgNPs. This inhibition is attributable to the bactericidal effect of AgNPs, as microbial growth was markedly suppressed and clear hydrolysis zones were absent. In some instances, faint microbial smears appeared; however, these did not produce discernible halo zones. However, in *P. putida,* the amylase activity of the treated AgNPs was much higher than that of the control, which may be indicative of a stress-induced adaptive or hormetic response. Low to moderate nanoparticle stress can trigger compensatory metabolic adjustments in bacteria, including upregulation of extracellular enzymes, as part of a survival strategy to maintain nutrient acquisition under adverse conditions. Such hormesis-like responses have been reported in microorganisms exposed to sub-lethal stressors, where enhanced enzyme production helps offset metabolic imbalance. So, at this point, we are unable to give a valid argument for higher amylase activity. However, further study, particularly focusing on cellular and morphological alterations are needed to elucidate this response. To the best of our knowledge, there are no prior reports addressing the effect of AgNPs on amylase, gelatinase and caseinase activities in major fish bacterial pathogens. Therefore, the findings of this study represent the first comprehensive assessment of AgNP-mediated modulation of these extracellular enzymes of fish bacterial pathogens and making direct comparison with earlier studies is difficult.

Gelatinase activity in *A. hydrophila* was completely inhibited after 72 h of exposure to 100 µl of AgNPs. But in all other bacterial species, gelatinase activity persisted up to 96 h. In control groups of *A.hydrophila, E. tarda, P. putida* and *S. aureus,* gelatinase activity showed a gradual decline over the 96h observation period. We also noticed that for these bacterial species, gelatinase activity was higher in AgNP-treated cultures compared to the controls. Whereas in the case of *P. aeruginosa* and *P. fluorescens*, lower gelatinase activity was recorded compared to the control group. As comparable reports on this aspect are not available, a definitive explanation for these contrasting responses cannot be provided at this stage. Further detailed investigations are needed to arrive at a conclusion.

Similar to gelatinase activity, caseinase activity was observed in all the bacteria over the 96h study period, although the responses showed variation without a definite trend. Most of the bacteria, including *E. tarda, P. aeruginosa, P. fluorescens* and *S. aureus,* exhibited higher caseinase activity at 100 µl AgNP concentration compared to 50 µl treatment and the control. To our knowledge, this study reports for the first time the changes in caseinase activity in fish bacterial pathogens in response to AgNPs interactions.

In vitro virulence assays were conducted to assess the effect of AGNPs on haemolytic activity, which is a key indicator of bacterial virulence, particularly ß-hemolysis. Haemolytic activity was completely inhibited in *A. hydrophila* and *V. parahaemolyticus* at both 50 and 100 µl AgNPs-treated cultures over a 96 h exposure period. In general, the haemolytic activity in AgNPs-treated cultures was higher than in the untreated controls at 24 h. However, prolonged exposure (96 h) at the higher AgNP concentration (100 µl) resulted in reduced haemolytic activity in *E. coli*, *P. aeruginosa*, *P. putida* and *V. alginolyticus,* compared with the control. Whereas *S. aureus*, *E. tarda*, and *P. fluorescens* exhibited higher haemolytic activity than the control even after 96 h of exposure at 100 µl concentration,

indicating persistence of virulence. These findings suggest that AgNPs can differentially modulate haemolytic activity and virulence among bacterial species, with certain pathogens exhibiting greater resilience to nanoparticle exposure.

Our study forms an initial step towards understanding the effects of green-synthesised silver nanoparticles on bacterial virulence and extracellular enzyme activities. Further comprehensive and long-term investigations are needed to elucidate the underlying mechanisms and to draw definitive conclusions.

Acknowledgements

The authors gratefully acknowledge the support and guidance provided by the Director, ICAR-CIFRI, Barrackpore and ICAR-CIFA, Bhubaneswar, for facilitating the necessary infrastructure and resources to carry out this research work.

References

Alderman, D. J. and Smith, P. 2001. Development of draft protocols of standard reference methods for antimicrobial agent susceptibility testing of bacteria associated with fish diseases. *Aquaculture*, 196 (3-4): 211-243. https://doi.org/10.1016/S00448486(01)00535-X

Ansari, M. A. and Alzohairy, M. A. 2018. One-pot facile green synthesis of silver nanoparticles using seed extract of *Phoenix dactylifera* and their bactericidal potential against MRSA. *eCAM.*, 2018 (1): 1860280. https://doi.org/10.1155/2018/1860280

Balantrapu, K. and Goia, D. V. 2009. Silver nanoparticles for printable electronics and biological applications. *J. Mater. Res*., 24 (9): 2828-2836. https://doi.org/10.1557/jmr.2009.0336.

Chabbert, Y. A. 1963. The antibiogram: sensitivity and resistance of bacteria to antibiotics. *Editions de la Tourelle*, 257.

Feng, Q. L., Wu, J., Chen, G. Q., Cui, F. Z., Kim, T. N. and Kim, J. O. 2000. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater.,* 52 (4): 662–8. https://doi.org/10.1002/1097-4636(20001215)52.

Kheybari, S., Samadi, N., Hosseini, S. V., Fazeli, A. and Fazeli, M. R. 2010. Synthesis and antimicrobial effects of silver nanoparticles produced by chemical reduction method. *Daru.*, 18 (3): 168-172.

Kim, J. S., Kuk, E., Yu, K. N., Kim, J. H., Park, S. J., Lee, H. J., Kim, S. H., Park, Y. K., Park, Y. H., Hwang, C. Y. and Kim, Y. K. 2007. Antimicrobial effects of silver nanoparticles. *NBM*, 3 (1): 95-101. https://doi.org/10.1016/j.nano.2006.12.001

Kvítek, L., Panáček, A., Soukupova, J., Kolář, M., Večeřová, R., Prucek, R., Holecová, M. and Zbořil, R. 2008. Effect of surfactants and polymers on stability and antibacterial activity of silver nanoparticles (NPs). *J. Phys. Chem. C.,* 112 (15): 5825–34. https://doi.org/10.1021/jp711616v.

Lee, H. J., Yeo, S. Y. and Jeong, S. H. 2003. Antibacterial effect of nanosized silver colloidal solution on textile fabrics. *J. Mater. Sci.,* 38 (10): 2199–2204. https://doi.org/10.1023/A:1023736416361.

Lok, C. N., Ho, C. M., Chen, R., He, Q. Y., Yu, W. Y., Sun, H., Tam, P. K. H., Chiu, J. F. and Che, C. M. 2007. Silver nanoparticles: partial oxidation and antibacterial activities. *J. Biol. Inorg. Chem*., 12 (4): 527-534. https://doi.org/10.1007/s00775-007-0208-z

Matsumura, Y., Yoshikata, K., Kunisaki, S. I. and Tsuchido, T. 2003. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl. Environ. Microbiol*., 69 (7): 4278-4281. https://doi.org/10.1128/AEM.69.7.4278-4281.2003 .

Morones, J. R., Elechiguerra, J. L., Camacho, A., Holt, K., Kouri, J. B., Ramírez, J. T. and Yacaman, M. J. 2005. The bactericidal effect of silver nanoparticles. *Nanotechnology*, 16 (10): 2346. 10.10880957-4484/16/10/059.

Oluwafemi, O. S. 2009. A novel "green" synthesis of starch-capped CdSe nanostructures. *Colloids Surf. B. Biol*., 73 (2): 382-386. https://doi.org/10.1016/j.colsurfb.2009.06.011.

Pirtarighat, S., Ghannadnia, M. and Baghshahi, S. 2019. Green synthesis of silver nanoparticles using the plant extract of *Salvia spinosa* grown *in vitro* and their antibacterial activity assessment. *J. Nanostruct., Chem*., 9 (1): 1-9. https://doi.org/10.1007/s40097-018-0291-4.

Prakash, P., Gnanaprakasam, P., Emmanuel, R., Arokiyaraj, S. and Saravanan, M. 2013. Green synthesis of silver nanoparticles from leaf extract of *Mimusops elengi*, Linn. for enhanced antibacterial activity against multi drug resistant clinical isolates. *Colloids Surf. B. Biointerfaces,* 108: 255-259. https://doi.org/10.1016/j.colsurfb.2013.03.017.

Raveendran, P., Fu, J. and Wallen, S. L. 2003. Completely "green" synthesis and stabilization of metal nanoparticles. *J. Amer. Chem. Soc*., 125 (46): 13940–13941. https://doi.org/10.1021/ja029267j.

Saxena, A., Tripathi, R. M. and Singh, R. P. 2010. Biological synthesis of silver nanoparticles by using onion (*Allium cepa*) extract and their antibacterial activity. *Dig. J. Nanomater. Biostruct*., 5(2): 427–432.

Sharma, V. K., Yngard, R. A. and Lin, Y. 2009. Silver nanoparticles: Green synthesis and their antimicrobial activities. *Adv. Colloid. Interface. Sci*., 145 (1-2): 83–96. https://doi.org/10.1016/j.cis.2008.09.002.

Shrivastava, S., Bera, T., Roy, A., Singh, G., Ramachandrarao, P. and Dash, D. 2007. Retracted: characterization of enhanced antibacterial effects of novel silver nanoparticles. *Nanotechnology*, 18 (22): 225103. 10.1088/0957-4484/18/22/225103.

Singh, M., Sinha, I. and Mandal, R. K. 2009. Role of pH in the green synthesis of silver nanoparticles. *Mater. Lett*., 63 (3-4): 425–427. https://doi.org/10.1016/j.matlet.2008.10.067.

Sondi, I.. Salopek-Sondi, B. 2004. Silver nanoparticles as antimicrobial agent: A case study on *E. coli* as a model for Gram-negative bacteria. *J. Colloid. Interface. Sci.,* 275 (1): 177–182. https://doi.org/10.1016/j.jcis.2004.02.012.

Sondi, I., Goia, D. V. and Matijević, E. 2003. Preparation of highly concentrated stable dispersions of uniform silver nanoparticles. *J.Colloid. Interface. Sci*., 260 (1): 75-81. https://doi.org/10.1016/S0021-9797(02)00205-9.

Vasileva, P., Donkova, B., Karadjova, I. and Dushkin, C. 2011. Synthesis of starch-stabilized silver nanoparticles and their application as a surface plasmon resonance-based sensor of hydrogen peroxide. *Col. Sur. A: Physicochem. Eng. Asp*., 382 (1-3): 203-210. https://doi.org/10.1016/j.colsurfa.2010.11.060.

Yakout, S. M. Mostafa, A. A. 2015. A novel green synthesis of silver nanoparticles using soluble starch and its antibacterial activity. *Inter. J. Cli. Exp. Med*., 8(3): 3538-3544.