Indian J. Fish., 72 (2): 103-116, 2025 doi:10.21077/ijf.2025.72.2.159295-12

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

Livestock waste-mediated biosynthesis of stable bactericidal silver nanoparticles and their chronic toxicity evaluation in fish using biomarkers

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Abstract

Silver nanoparticles (Ag-NPs) are contaminants of emerging concern. However, limited work has been done on chronic toxicity of Ag-NPs to fish using biomarkers. The present study elucidates the extracellular synthesis of non-agglomerated Ag-NPs using processing waste of sheep and swine by dispensing with additional capping agents and their characterisation, and toxicity evaluation on the physiological stress response of *Pangasianodon hypophthalmus* using biomarkers. Spectrophotometry, FTIR, DLS and HR-TM were employed for characterisation of Ag-NPs. Antioxidant, metabolic and acetylcholinesterase (AChE) enzyme activities were assayed using standard methods for chronic toxicity analysis of Ag-NPs to fish. Characterisation results showed a spherical shape of AgNPs with predominant size frequency between 5-20, 21-30 followed by 31-50 and 51-100 nm, capping by biomolecules, absorption at 400-410 nm with zeta potential of -27 and -32 mV. Sheep waste-derived Ag-NPs showed high bactericidal properties against fish pathogens. An enhancement in antioxidant and metabolic enzyme activities and inhibition of AChE activity were observed with increased sub-lethal ammonia concentration and temperature. Biosynthesis of Ag-NPs can be undertaken using animal wastes for their potential application in environment and health management of aquaculture based on characterisation, capping, bactericidal activity, biomarkers and physiological responses in fish.

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Keywords:
Animal waste, Aquaculture, Bactericidal activity, Capped silver nanoparticles, Chronic toxicity, Physiological responses

Received : 31.10.2024
Accepted : 26.06.2025

Introduction

Excessive and non-judicial use of antibiotic agents has significantly increased the emergence drug-resistant pathogens. Novel therapeutic approaches that replace inefficient antibiotics are in high demand to overcome increasing multidrug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR) microbes. A unique phenomenon of nanostructured materials enables novel applications, including various physicochemical properties (optical, electrical, magnetic.), such as large surface area, high surface energy and quantum confinement (Kumar and Seth, 2021).

Nanoparticles have unique properties due to their high specific surface area and the fraction of surface atoms, roughly 40-50%, which endows them with extraordinary potential for reactivity (Pournori *et al.,* 2017). Therefore, nanomaterials have attracted much attention for their distinct properties that are unavailable in conventional macroscopic materials (Annamalai and Nallamuthu, 2016). Due to their unique properties, nanomaterials have diverse applications in areas such as electronics, medical devices, cosmetics, food packaging, water treatment, fuel cells, biosensors and environmental remediation (Chakraborty and Krishnani, 2022). This has led to the large scale production of nanomaterials. Nanotechnology has transformative potential in aquaculture. with applications including rapid disease detection, development of DNA vaccines, targeted nutrient delivery,

water filtration and purification and water quality monitoring. Nanomedicine and nano-sensors can be employed to monitor aquaculture practices and improve fish health.

Silver nanoparticles (Ag-NPs) are one of the most exploited nanomaterials owing to their germicidal and anti-inflammatory properties. They are used in burn treatment, clothing materials, detergents, soaps, water and air filters, bedding and other medical as well as industrial textiles (Bar-Ilan *et al.,* 2009). About 30% of nanoproducts are known to contain Ag-NPs (Khan *et al.,* 2015). The stabilisation of Ag-NPs becomes a challenging research area because of their highly active surface atoms, the aggregation and deactivation by secondary nucleation as well as recrystallisation and hence restricts their wide industrial applications. Majority of nanoparticle synthesis methods depend on the use of chemical capping agents like surfactants, polymers, and thiols (Niu and Li, 2014), which have a strong interaction with the surface of Ag-NPs and hence act as good stabilising agents. However, these chemical capping agents are nonbiodegradable, toxic, and difficult to remove from the surface of the nanoparticles. Furthermore, concerns about the materials produced by these expensive chemical and physical methods usually carry traces of toxic chemicals, which could present catastrophic effects on the environment, leading to biological hazards (Saeb *et al.,* 2014; Sidhu *et al.,* 2022). There is always a pressing need to explore green capping agents to secure the biological system and the environment (Sharma *et al.,* 2019). Biogenic synthesis of nanoparticles is superior in comparison to the physical and chemical synthesis as it is cost-effective, eco-friendly, and involvement of biomolecule-based capping agents like proteins, amino acids, lipids, and carbohydrates, provide specific functional groups on the surface of nanoparticles (Chakraborty *et al.,* 2023; Das *et al.,* 2023). Various plant-derived products have been employed to synthesise nanomaterials (Sidhu *et al.,* 2022). However, animal-based materials are rarely used for the biosynthesis of nanoparticles, although biomolecules of animal wastes can serve as reducing and capping agents (Krishnani *et al.,* 2022). There is a need to improve nanoparticle characteristics such as reducing particle size, enhancing long-term stability and biocompatibility and increasing antimicrobial activity. This can be achieved by preventing agglomeration and effectively controlling surface energy, dispersion as well as electrostatic and steric interaction.

In the present scenario of climate change, rising water temperatures exacerbate the toxicity of metallic and ammonical pollutants (Kumar *et al.,* 2017; Abisha *et al.,* 2022). In aquaculture systems, ammonia nitrogen produced is the end product of protein catabolism and metabolism, is the most common environmental stressors (Arunkumar *et al.,* 2023). Similarly, the toxicity of heavy metals such as cadmium is known to increase with increase in temperature (Guinot *et al.,* 2012). These heightened toxicities adversely affect key physiological functions in fish, including thermal tolerance, growth, metabolism, food intake, reproductive success and the ability to maintain internal homeostasis. Meat production from farm to table generates wastes at various stages. The processing of animals such as cows, sheep, goats, pigs, chicken and turkeys produces byproducts such as bones, hides and blood. The meat/edible portion of these animals varies, with only 50-54% of a cow, 52% of sheep/goat, 60-62% of pig, 68-72% of chicken and 78% of turkey being utilised as meat and the remaining portion is turned into waste (Jha and Prasad, 2016). Wastes generated in processing house or abattoir is often discarded into the environment. If not properly managed, this can lead to unappealing and unhygienic surroundings. Therefore, effective utilisation of this waste to produce nanomaterials *via* biological methods is essential, along with a thorough understanding of the resulting products prior to their application. Several studies have explored the use of certain insects, fish and animal byproducts for nanoparticle synthesis including cockroaches (Jha and Prasad, 2013), fish scales of *Labeo rohita* (Sinha *et al*., 2014), cobwebs (Lateef *et al.,* 2016) and goat fur (Akintayo *et al*., 2020).

When applied within the permissible tissue retention limits, silver nanoparticles (AgNPs) offer protective benefits without posing any threat to environment (Chakraborty *et al.,* 2013). At a concentration 0.5 mg kg^{-1} feed, silver nanoparticles have been shown to provide protection against *Aeromonas veronii* biovar *sobria,* elevated temperatures and lead (Pb) toxicity (Kumar *et al.*, 2018a). Several ecological studies have been conducted on various species including *Labeo rohita, Lates calcarifer, Channa striata, Pangasianodon hypophthalmus* and zebra fish, to assess the environmental and biological impacts of nanoparticles (Sarkar *et al.,* 2014).

In the present study, ecotoxicity analysis was conducted on *P. hypophthalmus* fingerlings, as it is one of the most cultured species globally, owing to its high growth rate, strong disease resistance, good taste, high resistance to poor water quality and ability to thrive at high stocking densities. Silver nanoparticles were synthesized using waste materials. specifically sheep and swine intestines discarded during processing. These nanoparticles were then characterised, and their bactericidal activity was evaluated. The study also evaluated their toxicity by analysing the physiological stress responses of *P. hypophthalmus* under stressed environments, using biomarkers.

Materials and methods

Green synthesis of silver nanoparticles (Ag-NPs)

Pig rearing is an integral part of the livelihood and culture of the tribal population in the North-eastern states of India, where pork is a significant dietary component (Roy *et al.,* 2017). In this study, sheep and swine animal waste, primarily the intestine was collected directly from a slaughter house Lewduh Market, Shillong, Meghalaya, shortly after the animals were dissected. To eliminate undesired elements such as blood, undigested food, and potential metallic contaminants, the intestines were thoroughly washed multiple times with water.

Preparation of extract from sheep and swine intestines

To prepare the extract, a known quantity (2 g) of sheep and swine processing waste was gently ground using a porcelain mortar and pestle in the presence of 25 ml of distilled water, phosphate buffer (PB) or phosphate buffered saline (PBS), depending on the requirement. The resulting suspension was first filtered through an ordinary filter paper and subsequently using a syringe filter (0.45 μm) to obtain a clear extract. The pH of the extract was adjusted to 7.2 employing NaOH solution, as this pH is optimal for the activity of reducing agents and stabilisers involved in the

synthesis. Maintaining pH of the extract is critical as it significantly influences the size and shape of the silver nanoparticles formed during the synthesis process.

Biosynthesis of silver nanoparticles

Sheep and pig intestine extracts were mixed with a 3 mM silver nitrate ($AgNO_3$) solution at 1:1, 1:2, 1:3 and 1:4 ratios; then kept in a rotating shaker for 3 h at room temperature and subsequently incubated overnight for complete reduction of the $AgNO_3$. Once the suspension turned yellowish-brown, the mixture was centrifuged at 9000 rpm for 15 min and rinsed three times with distilled water to eliminate any unconverted silver ions. The silver nanoparticles were gathered in a pellet and preserved for later investigation.

Characterisation of silver nanoparticles

The biosynthesised Ag-NPs were assessed using a High-resolution Transmission Electron Microscope (HR-TEM-JEOL-JEM 2100 F 120/200 kV) operating at an acceleration voltage of 200 kV, a Dynamic Light Scattering (DLS) particles size analyser (Horiba Scientific Nanoparticles Analyser nano Partica SZ-100 series, Kyoto, Japan) and UV–Visible spectrophotometer equipped with a 1 cm quartz cell. The plausible interactions between Ag-NPs and the functional groups present in the sheep and swine intestinal extracts were investigated using Fourier Transform Infrared (FTIR) spectrometer (Thermo Fisher) using the potassium bromide (KBr) pellet technique. The sample was scanned from 4,000 to 400 cm^{-1} wave number.

Bactericidal activity

An agar well diffusion method was used to test the bactericidal activity of Ag-NPs synthesised using extracts of sheep and swine intestinal wastes. The Ag-NPs were tested against *Edwardsiella tarda* (ATCC 15947, Ref 0845P, LOT 845-38-30, HiMedia, India) and *Aeromonas hydrophilla* (ATCC 49140, Lot No. 637-55-6, Ref No. 0637P, HiMedia, India) both Gram-negative bacteria and *Micrococcus luteus* (ATCC 10240, lot 689-80-3, Ref 0689P, HiMedia, India), a Gram-positive bacterium. Bacteria from overnight cultures were poured onto a sterile agar plate and left for solidification. Four wells were prepared using a sterile micropipette tip, of which two of the wells were filled with 50 µl (250 µg) and 100 µl (500 µg) each of 5 mg of Ag-NPs per ml of sterile distilled water. As a control, 100 µl of streptomycin and 100 µl of sterile distilled water were added to the other two wells. After 24 h of incubation at 37°C, the antibacterial capabilities of the Ag-NPs were assessed using the zone of inhibition around the well.

Experimental animal, design and conditions for toxicity analysis of Ag-NPs

P. hypophthalmus fingerlings were procured from West Bengal. The fish were treated with a prophylactic dip in salt solution (2%) and then acclimatised for a period of two weeks prior to the start of the experiment. The experiment was carried out in a rectangular glass aquarium measuring 60"× 30" × 30". The acclimatised *P. hypophthalmus* fingerlings with an average weight of 8 g were divided into five groups in triplicate, following a completely randomised design, with each group containing 10 fingerlings. Throughout the course of the experiment, the fish were fed a practical diet (35 % protein) and non-stop aeration was provided to all the tanks employing a compressed air pump. Five experimental groups were designed *viz*., Control (no exposure to Ag-NPs and elevated temperature), T1 (exposure to Ag-NPs), T2 (concurrent exposure to Ag-NPs and ammonia), T3 (concurrent exposure to Ag-NPs and elevated temperature), and T4 (concurrent exposure to Ag-NPs, ammonia and elevated temperature). The duration of the exposure was 21 days during which 7 doses were administered. Each dose contained $1/10^{th}$ of the LC_{50} of Ag-NPs, with total ammonia-N maintained at 1.8 ppm using $(NH_4)_2SO_4$ and water temperature maintained at 34°C using a thermostatic heater. Water was partially exchanged every 72 h by siphoning out two-thirds of the volume, after which the treatment doses were readjusted accordingly.

Sample preparation for different biochemical parameters

Gill, liver, kidney and brain tissues of fish were collected from all experimental groups under aseptic conditions and weighed. The tissues were homogenised (5% w/v) separately in chilled sucrose solution (0.25 M) in a glass test tube using a Teflon-coated mechanical tissue homogeniser (Omni Tissues Master Homogenizer, Kennesaw, GA). The tubes were kept on ice to avoid denaturation of the enzymes during the homogenisation. The homogenates were centrifuged at 5000 rpm for 20 min at 4°C in a cooling centrifuge (Eppendorf AG, 5430 R, Hamburg, Germany). Protein content of the supernatants were quantified following the method of Lowry *et al.* (1951), using bovine serum albumin as standard. The supernatants were collected and stored at -20°C until further analysis.

Antioxidant enzyme activities

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was analysed as per the method of Misra and Fridovich (1972). The assay was based on the oxidation of epinephrine to adrenochrome. by the enzyme. A reaction mixture containing 1.5 ml of carbonate bicarbonate buffer (0.1M; pH-10.2), 50 µl of tissue homogenate and 0.5 ml of freshly prepared epinephrine substrate solution was prepared, mixed well and the absorbance was read at 480 nm over a period of 3 min. Catalase (CAT) (EC 1.11.1.6) activity was determined by the method of Takahara *et al.* (1960). The reaction mixture comprised 2.45 ml of phosphate buffer (50 mM; pH-7), 50 µl of tissue homogenate and 1 ml of hydrogen peroxide substrate solutions (Freshly prepared). The decrease in absorbance was read at 240 nm over 3 min.

Metabolic enzymes

Aspartate aminotransaminase (EC.2.6.1.1) and alanine amino transaminase (EC.2.6.1.2) activities were measured using the Wootton method (1964), which quantifies the release of oxaloacetate and pyruvate, respectively. Lactate dehydrogenase (Lactate NAD1 oxidoreductase; EC.1.1.1.27) was assayed following Wroblewski and LaDue (1955), using 0.2 mM sodium pyruvate as substrate and monitoring the decrease in NADH absorbance of 340 nm. Mate dehydrogenase (NAD + oxidoreductase: EC.1.1.1.37) was estimated as per Ochoa method (1955), employing 1 mg per ml of oxaloacetate in chilled triple distilled water and measuring NADH formation at 340 nm.

Neurotransmitter enzyme activities

Acetylcholinesterase (EC 3.1.1.7) activity was measured by the method of Hestrin (1949). The activity was spectrophotometrically measured by monitoring the increase in absorbance at 540 nm.

Statistical analysis

The data were statistically analysed using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, USA). the data were subjected to one-way ANOVA followed by Duncan's multiple range tests to determine the significant differences among the means. Comparisons were made at a 5% probability level ($p<0.05$)

Results and discussion

Synthesis of Ag-NPs using animal wastes

One Health approach integrates various components across the livestock-human-plant-environment interface to prevent health issues with the objective of achieving food and nutritional security, while also promoting environmental sustainability and social, economic, as well as political well-being (Krishnani *et al.,* 2023). In this context, utilising the by-products of one component to benefit another component offers a promising strategy for advancing the One-Health concept. In the present study, livestock waste was employed for the synthesis of Ag-NPs with potential application in aquaculture. Silver nanoparticles (Ag-NPs) were synthesised using the intestine extracts from swine and sheep, prepared in distilled water, phosphate buffer (PB) and phosphate buffered saline (PBS), with 3 mM silver nitrate as the precursor. The extracts and silver nitrate were mixed in varying ratios ranging from 1:1 to 1:4. A colour change in the suspension, from yellowish-brown to dark brown was observed after 24 h of incubation, indicating the formation of Ag-NPs. Optimal synthesis of Ag NPs was achieved at a 1:1 ratio for sheep extract and 1:4 for swine extract, suggesting that the intestinal extract functioned as a more effective reducing agent as compared to sheep waste extract. Based on these findings, further scale-up for experimental applications was done using these two optimal ratios.

Characterisation of the synthesised particles

The zeta potential of the Ag-NPs synthesised from sheep and swine meat waste, determined by dynamic light scattering (DLS) was found to be -27 mV for nanoparticles synthesised from sheep waste and -32 mV, for those derived from swine waste, indicating good colloidal stability Fig. 1a and b. The UV-Vis absorption spectra of the biologically synthesised Ag-NPs showed characteristic surface plasmon resonance peaks in the range of 400-410 nm (Fig. 2).

The HR-TEM analysis is useful in nanoparticle characterisation studies for determining the shape, size and morphology of Ag-NPs. The size of the particles obtained using HRTEM ranges from 5-100 nm, with a maximum frequency between 5-20 and 20-30 nm and a few days falling within 50-100 nm and more than 100 nm (Figs. 3 and 4). The magnified image of HR-TEM confirmed that the Ag-NPs were capped with biomolecules and uniform; are primarily spherical in shape with the average particle size ranging from 10±5 to 80±20 nm, without significant agglomeration. The lowest-size range of Ag-NPs achieved was synthesised from distilled water and phosphate buffer from the intestine of sheep followed by the swine. The Ag NPs synthesised from the swine intestine had more nanoparticles in the range of 30-50 nm, whereas Ag NPs synthesised from the sheep intestine ranged from 5-20 nm followed by 21-30 nm.

Proteins present in synthesised AgNPs may be responsible for the efficient capping and stabilisation of nanoparticles and this was further confirmed by the FTIR spectrum. In the present study, the FTIR spectrum shows absorption bands at 3271, 2918-2922, 2849, 1579-1632, 1514-1537, 1454, 1397-1403, 1329, 1236 and 1029-1052 cm^{-1} indicating the presence of a capping agent with the nanoparticles (Fig. 5). The broad band at 3271 cm^{-1} in the spectra corresponds to NH amide stretching vibration indicating the presence of amino acids/protein. Bands at 2918-2922 and 2849 cm^{-1} region arose from C-H stretching. The band at 1579-1632 cm^{-1} in the spectra corresponds to C-N and C-C stretching indicating the presence of proteins (Prakash *et al.,* 2013). The weaker band at 1579-1632 cm^{-1} corresponds to amide I (NH) C=O group arising due to carbonyl stretch in proteins. The band at 1454 cm^{-1} was assigned for N-H stretch vibration present in the amide linkages of the proteins. The bands at 1236 and 1029-1052 cm^{-1} were assigned for the C-N (amines) stretch vibration of the proteins.

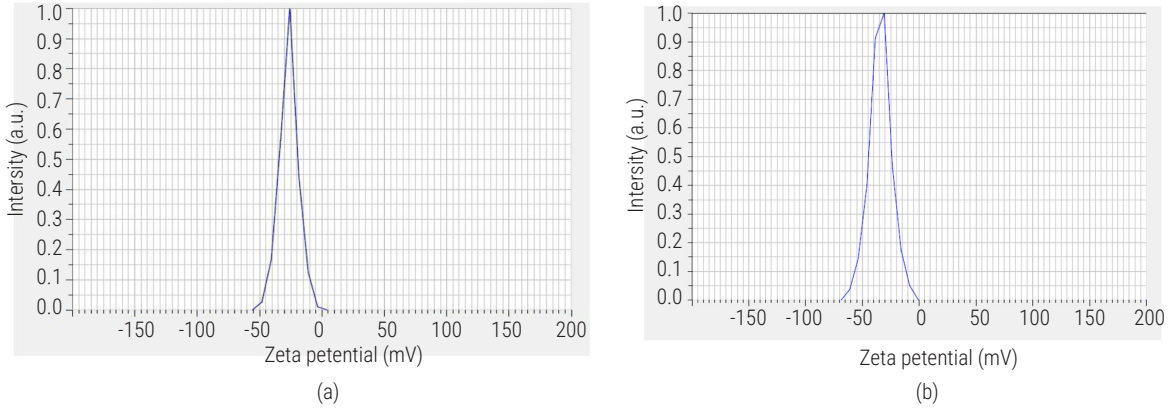


Fig. 1. Characterisation of synthesised Ag-NPs using DLS - Zeta potential. (a) Sheep waste derived Ag NPs (-27 mV); (b) Swine waste derived Ag NPs (-32 mV)

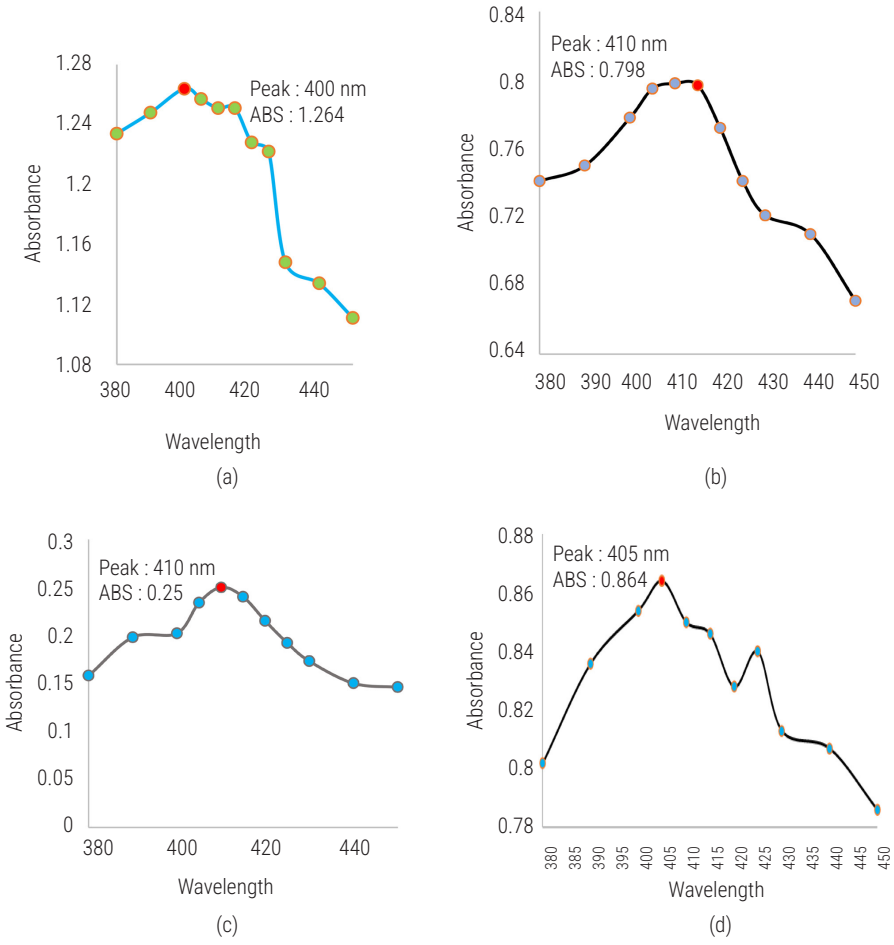


Fig. 2. UV-Visible spectra of Ag-NPs synthesised using sheep and swine wastes. (a) Swine intestine extract in PBS; (b) Swine intestine extract in DW; (c) Sheep intestine extract in PB and (d) Sheep intestine extract in PBS

Antioxidant status

In Fig. 6, the antioxidant status (SOD and catalase) of the gills and liver of *P. hypophthalmus* fingerlings exposed to Ag-NPs individually or concurrently with NH_3 and high-temperature are illustrated. The antioxidant enzyme increases significantly ($p<0.05$) after successful exposure to different toxicity groups as compared to the control. The highest antioxidant enzyme activity was observed in the toxicity group exposed to the combined effect of Ag-NPs, NH_3 and high temperature, followed by the group exposed to the concurrent effect of Ag-NPs and high temperature and then Ag-NPs individually in the case of catalase enzyme. The SOD activity showed no significant difference ($p>0.05$) between the toxicity groups exposed to Ag-NPs individually and combined with NH_3 or high temperature, except for the group exposed concurrently to Ag-NPs, NH_3 and high temperature.

Metabolic enzymes

The metabolic stress enzyme activity in terms of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) of *P. hypophthalmus* fingerling is illustrated in Fig. 7 and 8. The activities of ALT (Fig. 7a) and AST (Fig. 7b) in the liver and kidney of the treatment group increased significantly ($p<0.05$) as compared to the control. The highest AST and ALT activities of the liver and kidney were observed in the group exposed to the combined effect of Ag-NPs, ammonia and high temperature. The groups exposed to the other treatments did not show significant differences ($p>0.05$) between each other in terms of the ALT activity of the liver. In the case of kidney, the most elevated treatment group was group the exposed to Ag-NPs and high temperature, followed by the group exposed to Ag-NPs and NH_3 and then Ag-NPs individually.

The LDH and MDH (Fig. 8a) activities of the liver increased significantly ($p<0.05$) in the toxicity group as compared to the control. The group that was exposed to the combined effect of Ag-NPs, NH_3 and high temperatures had the most elevated activity. The group exposed to Ag-NPs individually or combined with NH_3 or at high temperature showed no significant differences ($p>0.05$) between each other in terms of LDH and MDH activity in the liver.

Neurotransmitter enzyme

The neurotransmitter activity in the form of acetylcholinesterase (AChE) in the brain of *P. hypophthalmus* is illustrated in Fig. 8b. Brain AChE activities were noticeably inhibited ($p<0.05$) in the group exposed to Ag-NPs individually or concurrently with ammonia and

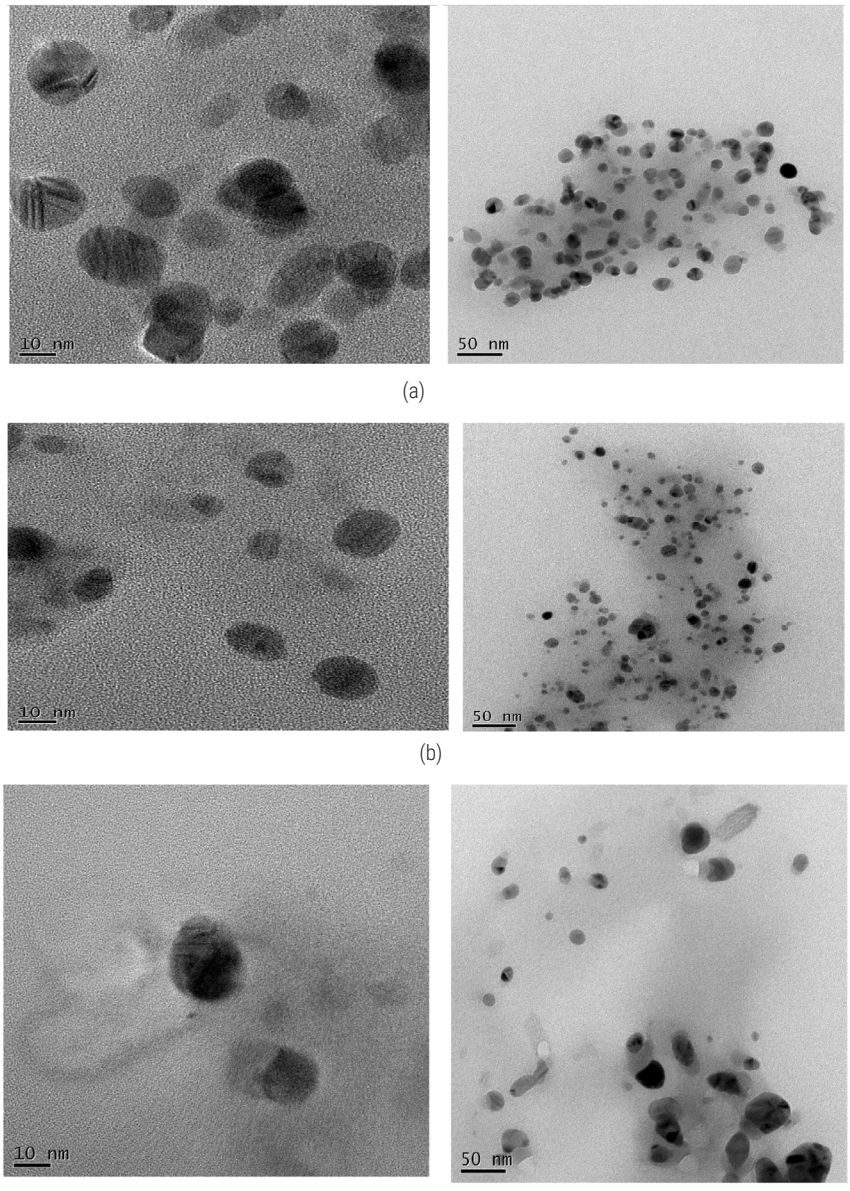


Fig. 3. Characterisation of the synthesised sheep-waste mediated Ag-NPs using HR-TEM. (a) DW, (b) PB and (c) PBS

temperature, or both, as compared to control. The highest inhibition was observed in the group exposed to the concurrent effects of Ag-NPs, NH_3 and high temperatures. The inhibition effect between the groups exposed to Ag-NPs and NH_3 and the group exposed to Ag-NPs and high temperature did not show significant difference ($p>0.05$). The lowest inhibition was observed in the group treated with Ag-NPs individually.

Bactericidal activity

As shown in Fig. 9, Ag-NPs synthesised using sheep intestinal waste in DW, PB and PBS have high bactericidal properties against the tested Gram-negative (*A. hydrophila,* and *E. tarda)* and Gram-positive bacteria (*M. luteus)*, as compared swine waste derived AgNPs. The zone of inhibition (mm) at 50 µl (250 µg) and 100 µl (500 µg) of 5 mg of swine and sheep waste mediated Ag-NPs in DW, PB and PBS per ml of sterile distilled water are given in Table 1. Gram-negative *A. hydrophila* and *E, tarda* have been shown to have the highest sensitivity against the biosynthesised Ag-NPs as compared to the Gram-positive bacteria *M. luteus*.

Swine breeds such as Yorkshire, Hamshire, and Landrace are among the most widely reared globally for human consumption. Swines are euryphagous in nature, consuming a wide variety of foods that may contain various contaminants such as heavy metals and pesticides. As a result, they possess multiple physiological mechanisms to cope with diverse environmental stressors. The biosynthesis of silver nanoparticles (Ag-NPs) from swine intestinal extracts may be facilitated by the presence of metallothionein, a cysteine-rich metal-binding protein found in all eukaryotes. Metallothionein plays

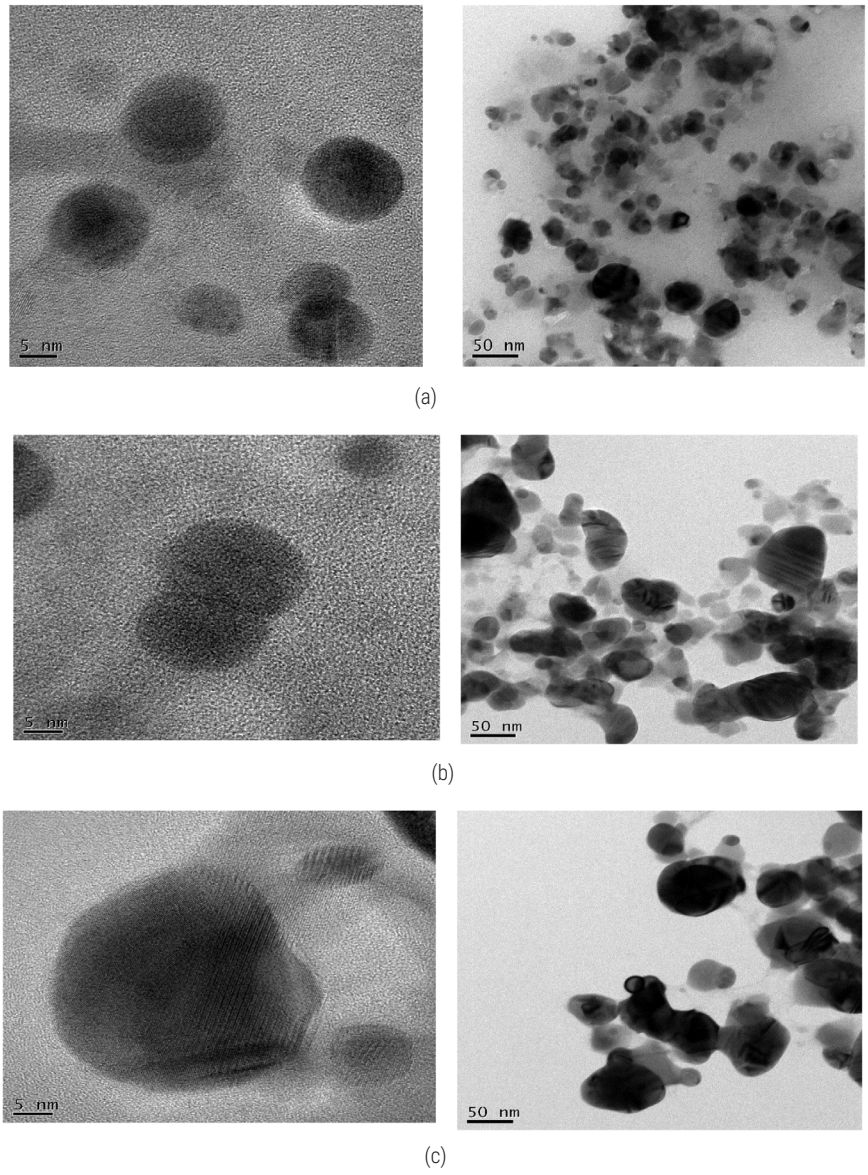


Fig. 4. Characterisation of swine-waste mediated Ag-NPs using HR-TEM. (a) DW, (b) PB and (c) PBS

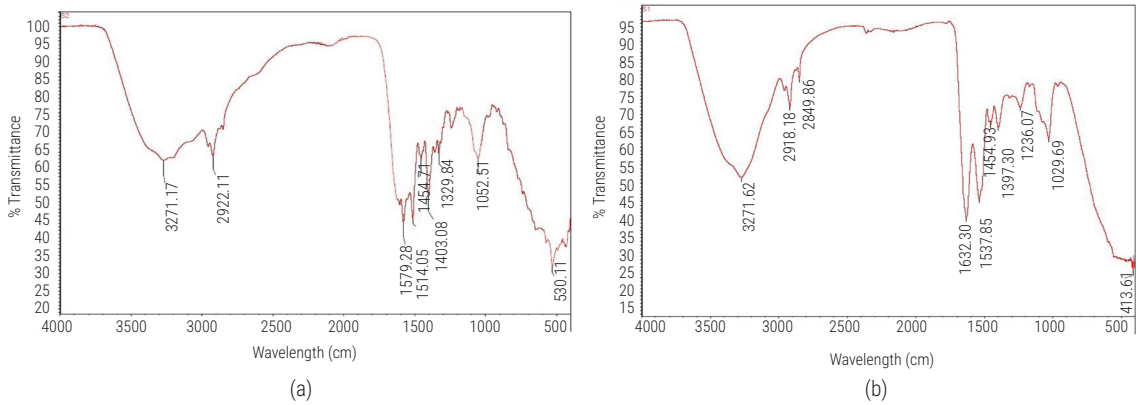


Fig. 5. FTIR spectra of biosynthesised silver nanoparticles stabilised by animal waste proteins. (a) Sheep waste derived Ag NPs and (b) Swine waste derived Ag NPs

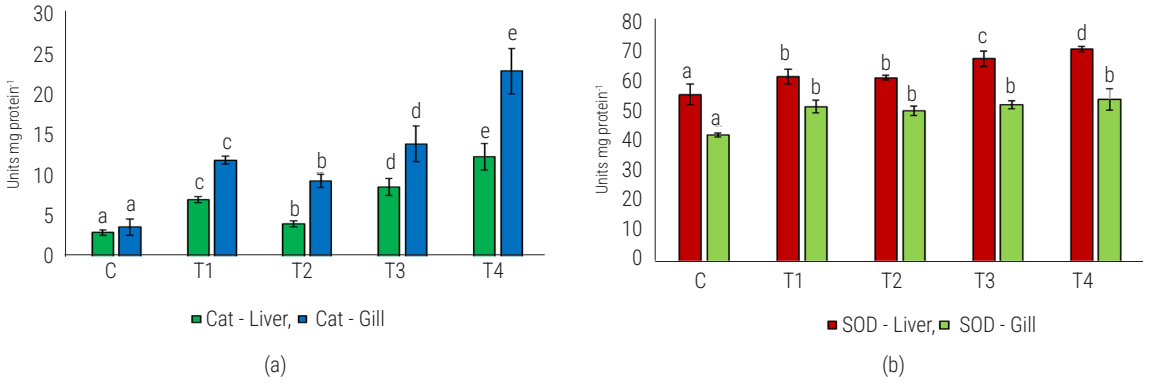


Fig. 6. (a) Catalase activity and (b) SOD activity in the liver and gills of *P. hypophthalmus* exposed to different toxicity groups of Ag-NPs. Different letters above the bar indicate significant differences ($p<0.05$)

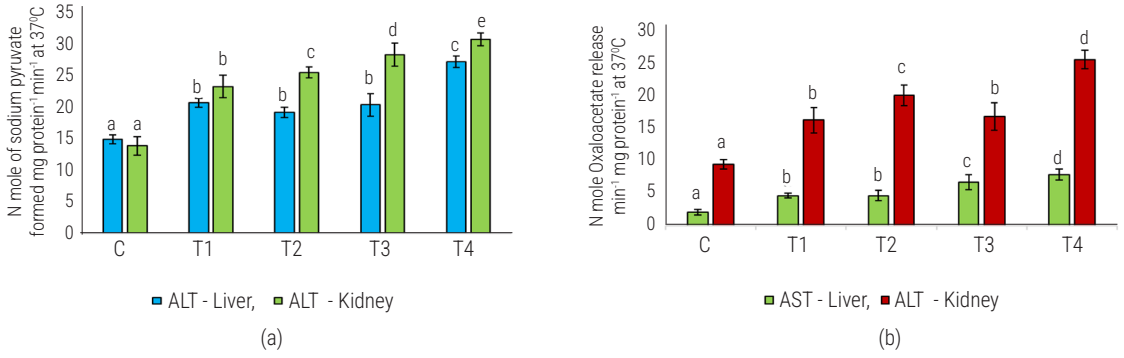


Fig. 7. (a) ALT and (b) AST activity in liver and kidney of *P. hypophthalmus* exposed to different toxicity groups of Ag-NPs. Different letters above the bar indicate significant difference ($p<0.05$).

a crucial role in intracellular metal distribution and accumulation, acting as a reducing functional group capable of reducing metal ions, chelating metals and facilitating the accumulation of metal particles within cells (Yuan *et al.,* 2019). Jha and Prasad (2016) investigated the synthesis of ZnO nanoparticles using goat waste as a reducing and capping agent, and concluded that "from insects to mammals, metallothionein genes are induced in response to heavy metal exposure. They further suggested that even in dead animal tissues, essential biomolecules remain thermodynamically flexible, capable of releasing or absorbing energy. This released energy is likely responsible for driving the phase transformation from microscale to nanoscale structures.

In the present study, the suspension exhibited a yellow-brown colour after 24 h of incubation, which may be attributed to the excitation of surface plasmon resonance (SPR) of the synthesised Ag-NPs. Variations in colour, when compared to other studies on biosynthesised Ag-NPs, could be due to the differences in composition of biomolecules responsible for reducing silver nitrate to silver nanoparticles (Akintayo *et al.,* 2020). The zeta potential of the synthesised Ag-NPs from sheep and swine waste was -27 mV and -32 mV respectively, indicating high colloidal stability. A zeta potential greater than +30 mV or less than -30 mV is generally considered indicative of a stable nanoparticle suspension (Abdelmoteleb *et al.,* 2017). The particle size of Ag-NPs

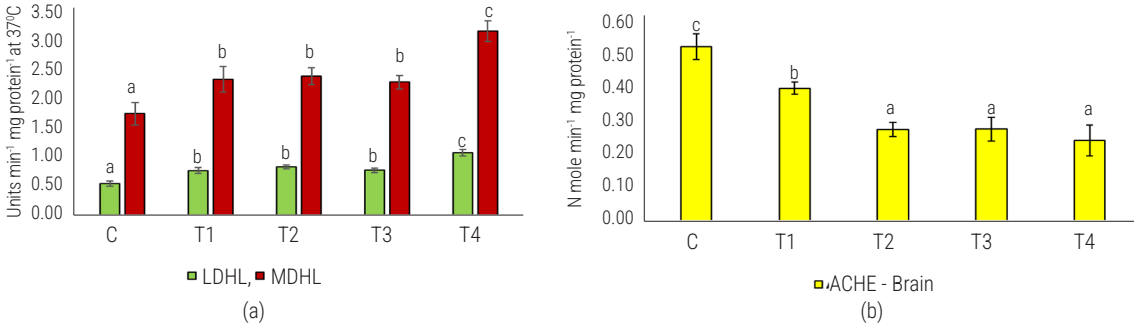


Fig. 8. (a) LDH and MDH activity in the liver and (b) AChE activity in the brain of *P. hypophthalmus* exposed to different toxicity groups of Ag-NPs. Different letters above the bar indicate significant differences ($p<0.05$)

Table 1. Bactericidal properties of Ag-NPs synthesised from swine and sheep intestines

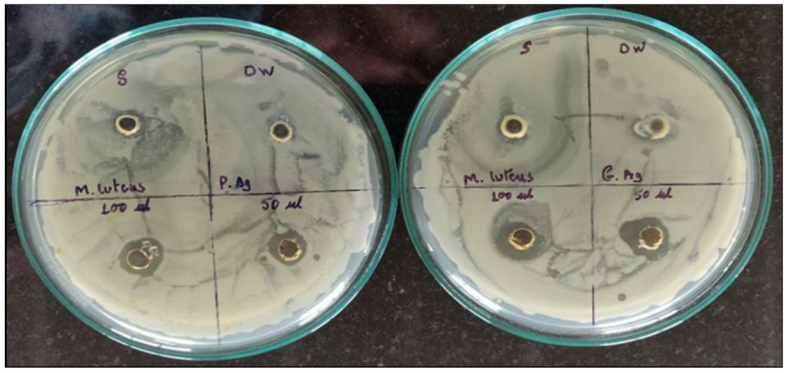
Zone of inhibition (mm)					
Swine intestine mediated AgNPs					
Sl. No	Content		*M. luteus*	*A. hydrophila*	*E. tarda*
1	100 µl Ag-NPs	PBS	11.6	10	12
		PB	10	11	13
		DW	12	15	14
2	50 µl Ag-NPs	PBS	9	6	8
		PB	6	7.6	8.6
		DW	10	13	13
Sheep intestine mediated Ag NPs					
1	100 µl Ag-NPs	PBS	14	15	19
		PB	14	19	16
		DW	16	20	22
2	50 µl Ag-NPs	PBS	9	10	16
		PB	10	15	11
		DW	10	15	18

PBS-Phosphate buffered saline; PB-Phosphate buffer; DW-Distilled water

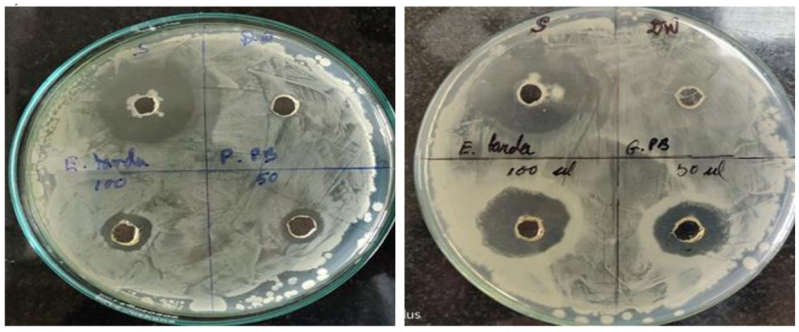
measured by DLS was larger than the size obtained by HRTEM, as DLS measures the hydrodynamic diameter, which includes the nanoparticle core and associated surface-bound molecules (Saha *et al.,* 2017). Biomolecules such as proteins, amino acids, lipids and carbohydrates provide specific functional groups on the surface of nanoparticles, influencing stability and reactivity (Marisca *et al.,* 2019). These biomolecules act as natural capping agents, preventing nanoparticle agglomeration and steric hindrance, while also modulating biological activity and surface chemistry. They help stabilize the nanoparticles within the preparation medium. Various biogenic capping agents, including biomolecules and biological extracts of plants and microorganisms, have been widely reported (Sidhu *et al.,* 2022). The findings of the present study are supported by several previous reports. Kakakhel *et al.* (2020) reported a cheaper and eco-friendly protocol for synthesising silver nanoparticles from animal blood, with a UV absorption peak at 422 nm and particle size ranging from 20-50 nm. Similarly, Kumar *et al.* (2018a) synthesised silver nanoparticles from the gills of *Channa striata* , having average particle size of 297 nm measured by DLS, with a mean zeta potential of -34 mV. Another study supporting the present findings reported the synthesis of silver nanoparticles using *E. coli* transformed with *Candida albicans* metallothionein gene. The transformed bacteria produced a higher yield of silver nanoparticles compared to non-transformed bacteria. Similarly, the biosynthesis of other metallic nanoparticles, such as zinc nanoparticles, has also been demonstrated, where metallothionein was identified as the key biomolecule responsible for the reduction of silver nitrate to silver nanoparticles (Jha and Prasad, 2016).

Chakraborty *et al.* (2024) demonstrated a novel approach of valorisation of nanosilver for detoxification of hexavalent chromium under neutral to alkaline environmental conditions, offering a comprehensive framework for expanding applications in the field of nanobioremediation and circular bioresource utilisation. In a related study, the toxicity of AgNPs on *Anabas testudineus* was evaluated, with a 96-h LC_{50} value determined to be 25.46 mg l^{-1} (Chakraborty *et al.,* 2023). Analysis of physiological parameters and integrated biomarker responses indicated that concentrations of $1/10^{th}$, $1/25^{th}$ and $1/50^{th}$ of the LC_{50} induces measurable stress in fish, whereas exposure to $1/100^{th}$ of the LC_{50} elicited minimal to no stress response. The study concluded that the toxicity of silver nanoparticles increases in the presence of sub-lethal levels of ammonia and elevated temperatures, indicating that their impact is exacerbated under combined abiotic stress conditions. The stress tolerance of aquatic organisms is greatly influenced by contaminants such as heavy metals, ammonia and pesticides (Kumar *et al.,* 2018a; b). The increased concentration of pollutants and rising temperatures induce stress in aquatic organisms, which is reflected in terms of elevated antioxidant enzymes, as well as changes in protein and carbohydrate metabolic enzymes (Kumar *et al.,* 2018a; b). In the present study, the activities of antioxidant enzymea, Catalase (CAT) and superoxide dismutase (SOD) were elevated, following exposure to different toxicity groups. The increase in activity may be attributed to the stress induced by Ag-NPs, either alone or in combination with ammonia (NH_3), elevated temperature, or both. The observed enzymatic response likely represents an adaptive mechanism to counteract oxidative stress resulting from the overproduction of reactive oxygen species (ROS). The stress response in fingerlings may be further intensified by the metallic nature of Ag-NPs, as the presence of transition metals is known to enhance ROS generation, thereby contributing to oxidative stress (Rajkumar *et al.,* 2015).

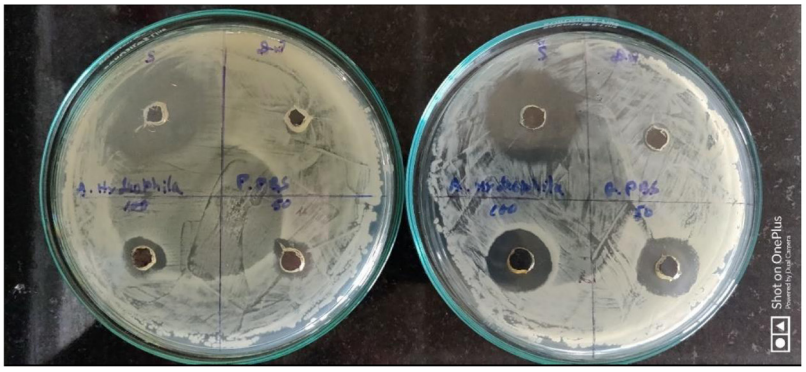
The transaminase enzymes (ALT and AST) in the present study were greatly influenced by the Ag-NPs. ALT and AST are used as biomarkers for stress induced by contaminants (Reddy 2012; Kumar *et al*., 2017a, b; 2018a, b,). In aquatic organisms, transaminase enzyme activity increases during stress conditions. Increase in AST and ALT activities could be attributed to the mobilisation of aspartate and alanine through gluconeogenesis for glucose production to cope with the induced stress. Increased transaminase activity levels could also be attributed to cellular damage, increased plasma membrane permeability, or altered metabolism of enzymes. Increased transaminase activities indicate an adaptive physiological response to combat energy demand (Reddy, 2012).



(a)



(b)



(c)

Fig. 9. Bactericidal activity of synthesised Ag-NPs against (a). *M. luteus,* (b). *A. hydrophila,* and (c) *E. tarda.* S-Streptomycin, DW-Sterile distilled water, 50 µl (250 µg) and 100 µl (500 µg) of 5 mg AgNPs per ml sterile distilled water

LDH and MDH catalyse the oxidation of malate and lactate to pyruvate respectively, functioning at a strategic point between glycolysis and citric acid cycle. These enzymes play a key role in in the terminal steps of glycolysis and energy metabolism (Reddy *et al.,* 2012). In our study, the activities of these carbohydrate metabolic enzymes were elevated following exposure to different toxicity groups, indicating a metabolic response to stress. The increased activity of LDH and MDH, could be attributed to the role of lactate and malate as primary substrates for gluconeogenesis during anaerobic metabolism, supporting glucose production to meet the heightened energy demand under stress conditions. Similar elevations in LDH and MDH activities been previously reported by Reddy (2012); Kumar *et al.* (2018a, b) and Defo *et al.* (2019).

Acetylcholine esterase is an essential enzyme in the modulation of neuromuscular impulse transmission. The primary role of AChE is to separate both acetylcholine and the cholinergic signal molecule from its receptors in the plasma membrane (Myrzakhanova *et al.,* 2013). AChE has been employed as a stress biomarker for pollution or contaminants in the aquatic environment. The activity of AChE is known to be inhibited in the presence of pollutants such as heavy metals (Gupta *et al.,* 2014; Muthappa *et al.,* 2014; Ahmad *et al.,* 2016; Hayat *et al.,* 2017; Kumar *et al.,* 2017, 2018). In the present study, the activity of the neurotransmitter enzyme acetylcholine esterase (AChE) was drastically inhibited following exposure to Ag-NPs, either alone or in combination with ammonia and elevated

temperature. This inhibition of AChE may be attributed to the binding of metal ions to terminal -OH and -SH functional groups on the enzyme. Such interactions likely occur at allosteric sites, inducing conformational changes, that prevent the the substrate from binding to the specific site of the enzymes (Ahmad *et al.,* 2016; Kumar *et al.,* 2016; Hayat *et al.,* 2017).

Functional groups play a crucial role in the capping and stability of AgNPs, as previously reported by Niraimathi *et al.* (2013) and Prakash *et al.* (2013). In the present study, the FTIR spectrum indicated the presence of proteins in the samples of silver nanoparticles, which further confirms that the secondary structure of these proteins remained intact despite their interaction with Ag^+ ions or nanoparticles. Nicholas *et al.* (2010) reported that proteins can bind to nanoparticles either through their free amine groups or cysteine residues. This interaction contributes to the effective capping and stabilisation of AgNPs by proteins.

The biosynthesised silver nanoparticles exhibited strong antibacterial activity at concentrations of 50 µl (250 µg) and 100 µl (500 µg) from a stock solution of 5 mg of Ag-NPs per ml in sterile distilled water, effectively inhibiting both Gram-negative and Gram-positive bacteria. The bactericidal properties of the biosynthesised Ag-NPs have been linked to the interaction with sulfur and phosphorous-containing components of the bacterial cell, disrupting the the respiratory chain and interfering with cell division ultimately leading to cell death (Mahendra *et al.,* 2009). Variations in the inhibition zone observed for Ag-NPs might be due to the cell wall component of bacteria (Chaloupka *et al.,* 2010) and the particle size of the synthesised Ag-NPs (Awwad *et al.,* 2020). The bactericidal activity of AgNPs is influenced several factors including their size, shape, ability to induce oxidative stress and the release of silver ions. These mechanisms can lead to bacterial death or induce a viable but non-culturable (VBNC) state in exposed bacteria (Konigs *et al.,* 2015; de Silva *et al.,* 2021).

Gram-negative bacteria are more susceptible to bacterial cell wall desruption due to the absence of a thick peptidoglycan layer, unlike Gram-positive bacteria, which possess a thicker and more protective peptidoglycan layer (Slavin *et al.,* 2017). Bactericidal activity of a recombinant Elastin-like biopolymer containing a polyhistidine domain complexed with Ag+ ions has been successfully demonstrated against Gram-negative bacteria prevalent in coastal shrimp aquaculture (Krishnani *et al.,* 2014). In the present study, silver nanoparticles (AgNPs) exhibited a larger zone of inhibition against Gram-negative bacteria prevalent in freshwater aquaculture, compared to Gram-positive bacteria. This observation aligns with established antibacterial mechanisms of Ag NPs which include direct physical interaction of their sharp edges of AgNPs with the bacterial cell wall; generation of reactive oxygen species (ROS); entrapment of bacteria within the aggregated AgNPs; induction of oxidative stress; disruption of glycolytic pathways; DNA damage; release of silver ions; and induction of a viable but non-culturable (VBNC) state in pathogenic bacteria (Krishnani *et al.,* 2022).

The present study demonstrated that silver nanoparticles can be uccessfully synthesised using processed wastes from abattoirs, specifically the intestine of sheep and swine. This approach reinforces the concept of circular bioresource utilisation, where waste from one system can serve as a valuable input for another. The biosynthesised silver nanoparticles exhibited strong bactericidal properties against both Gram-positive and Gram-negative bacteria. Further, toxicity analyses provided important insights into the interactive effects of environmental co-stressors, such as sub-lethal ammonia concentrations and elevated temperatures on the toxicity of silver nanoparticles. Chronic exposure studies using stress biomarkers in fish revealed that Ag-NP toxicity increased under combined stress conditions, highlighting the importance of evaluating nanoparticle effects in realistic environmental scenarios Biomolecules present in animal wastes are rich in functional groups that provide natural binding sites, making them excellent modulators nanoparticle surface properties. This study concludes that slaughterhouse waste, particularly sheep and swine intestines, can be effectively utilised for green synthesis of silver nanoparticles. With the growing concern over antibiotic resistance in bacteria, biosynthesised Ag-NPs, when used at optimal concentrations, hold significant potential for application in aquaculture health management. These nanoparticles demonstrated potent antibacterial properties without any adverse effects on fish, positioning them as a viable alternative to synthetic antibiotics in efforts to combat antimicrobial resistance. Further research into the scalable production of Ag-NPs from animal waste extracts is warranted particularly to address climate change-induced stressors and to promote climate resilience in aquaculture. This approach aligns with the principles of the One Health framework, with potential benefits extending across fisheries and other sectors of agriculture.

Acknowledgments

The authors are grateful to the Director, ICAR-CIFE, Mumbai and the Director, ICAR-NIASM, Baramati for extending encouragement and experimental facilities to carry out the research work.. ICAR, New Delhi is gratefully acknowledged for awarding a fellowship to the student and providing an Institute project to the corresponding author. The use of animals conforms to the existing laws in India. The care and treatment of animals used in this study were in accordance with the guidelines for care and use of animals in scientific research, laid down by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate Change (Animal Welfare Division), Government of India.

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