



Silver Nanoparticles Mediated-marine Fungal Amylase Improving *Dicentrarchus labrax* Larvae Growth Performance

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ABSTRACT

This study investigates the capability of marine *Aspergillus flavus* amylase (Amy) to synthesize silver nanoparticles (AgNPs) and studies its impact on *Dicentrarchus labrax* (sea bass) larvae growth performance. Characterization of Amy-AgNPs was carried out by FTIR, UV-Vis's spectroscopy, and SEM. The effect of Amy-AgNPs on antimicrobial and antioxidant activities was studied. The colloidal brown color of Amy-AgNPs with FTIR analysis indicated that amylases had the responsibility for capping the produced nanoparticles and making them more stable. Spherical Amy-AgNPs had sizes of 22.88 to 26.35 nm. Significantly, Amy-AgNPs inhibited the growth of fish-tested microbes by a maximum zone of inhibition, 13-20 mm and maximum MICs ranged from 1.6 to 6.3 µg/ml. Also, it acted as scavenged DPPH by 31.46 to 94.0 %. Sea bass larval morphometric measurements including mean total length (TL), width (W), total weight (TW), larvae survival percentage (%) and specific growth rate (SGR) were recorded, compared to the control group using different concentrations (1, 5, 10, 20%) of encapsulated Amy-AgNPs during larval feeding regime for a period of four weeks. The results revealed that 5% Amy-AgNPs was significantly the most effective concentration at $P < 0.05$ and achieved higher survival percentages; $51 \pm 2.8\%$ and increased the specific growth rate by $16.23 \pm 0.62\%$.

INTRODUCTION

Farmed European Sea bass (*Dicentrarchus labrax* L.) is of economic importance worldwide since 96% of the total production originates from aquaculture rather than fisheries (Vandeputte *et al.*, 2019). However, this aquaculture industry is facing several problems encountered in production development, including disease outbreak, high-cost feed, and water pollution, among others (Shaalán *et al.*, 2017) Consequently, aquaculture needs to cope with these problems using modern technologies.

Recently, nanotechnology has become an important field of research involved in the improvement of fish production sector regarding its unique properties, which enable novel applications, including drugs, nutrition, reproduction, and aquatic health (Shah and Mraz, 2020). In more profound, nanoparticles-green synthesis has enormous attention regarding its

advantages such as biocompatibility, eco-friendliness, simplicity and cost-effectiveness (Darwesh and Elshahawy 2021). At this spot, there is a recent rise of the metallic nanoparticles biofabrication using enzymes which have been targeted for several biomedical applications (Ovais *et al.*, 2017). The synthesis of silver nanoparticles AgNPs using enzymes has been reviewed (Darwesh *et al.*, 2019), but scare of research carried out in this area regarding nanoforms.

Silver nanoparticles (AgNPs) are involved in the growing class of nanoproducts (Karimi-Maleh *et al.*, 2020) due to their high electrical and thermal conductivity, excellent catalytic activity, and low manufacturing cost. In aquaculture, AgNPs among other nanoparticles have gained much more interest as a specific tool for controlling many microbial diseases especially with the increasing problem of antibiotic resistant bacteria (Barakat *et al.*, 2016). Silver nanoparticles (AgNPs) are an alternative tool to antibiotics able to overcome the bacterial virulence and their resistance against antibiotics (Abdel-Monem *et al.*, 2020). For instance, AgNPs proved to have bactericidal activity against *Aeromonas* species, which are considered the most common pathogenic bacterial strain threatening the aquaculture industry (Shalan *et al.*, 2017).

Many studies on European seabass (*Dicentrarchus labrax*) were reported to evaluate the effectiveness of nanomaterials to protect and improve fish performance. The potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass is to protect it from fish pathogenic microbes (Kumar *et al.*, 2008). Likewise, Vimal *et al.* (2014) examined the efficacy of DNA vaccine in Asian sea bass against nodavirus through oral route using CS/TPP (chitosan–tripolyphosphate) nanoparticles encapsulation. A new study by Abd El-Kader *et al.* (2020) aimed to evaluate the possible feeding strategies of selenium (Se) nanoparticles on the performances of European seabass. The aim of Fath El-Bab and his co-worker (Fath-El-Bab *et al.* 2020) was investigated the effect of chitosan nanoparticles (CsNPs) to fed European seabass and observe their growth performance, blood hematological and biochemical parameters. Another authors studied the lethal concentrations of silver nanoparticles on Asian sea bass growth performance (Bita *et al.*, 2021) while sensitivity of *Dicentrarchus labrax* primary cultures toward different nanoparticle (TiO₂, polystyrene and silver) was studied after 24 and 48 h of exposure (Jimeno-Romero *et al.*, 2021).

Microbial enzymes can possibly serve as evidence for the fabrication of structurally diverse biological tools to obtain unique nanostructures. Thus, silver nanoparticles synthesized by pure alpha amylase from marine *Aspergillus flavus* was examined. Also, these green synthesized nanoforms were pertained to improve sea bass larval growth performance.

MATERIALS AND METHODS

Fungal amylase

Purified amylase was produced by marine fungal strain, *Aspergillus flavus* AUMC10636 (88.0 U/ml) according to the method described by Beltagy *et al.*, (2022), then maintained at -20°C till further experiment.

Biosynthesis of Amy-AgNPs

Purified fungal amylase was used for synthesis of AgNPs by adding 40 ml of the prepared α -amylase solution (2 mg/ml in Tris-HCl buffer, pH 8.0) and 60 ml of silver nitrate (AgNO_3) (1 mM) freshly prepared solution (**Elegbede et al., 2018**). The solution was kept at ambient temperature ($28 \pm 2^\circ\text{C}$) and after intervals, appropriate aliquots withdrawn and the synthesis of AgNPs occurred. The bio-fabrication of AgNPs was observed by color change (**Sandhu et al., 2017**).

Purification of Amy-AgNPs

Amy-AgNPs were centrifuged at 9,000 rpm for 5 min at 4°C . The clear supernatant was thrown away and the pellet washed five times with distilled water, air dried and used for further chemical characterization.

Characterization of Amy-AgNPs

Characterization of the AgNPs was firstly passed off by measuring the absorbance spectrum (UV-Vis spectral analysis) performed on Mecasys Optizen 3220 UV spectrophotometer at 1 nm resolution. AgNO_3 solution was taken as a control without the enzyme. Fourier transform infrared (FTIR) spectroscopy analysis was done according to method described by **Bhat (2013)** to ascertain the biomolecules acting for the synthesis, capping, and stabilization of Amy-AgNPs. Colloidal Amy-AgNPs dried at 60°C , and analyzed by SEM (JEOL JSM-6360LV), at a speeding up voltage of 15.0 kV under high vacuum.

Antibacterial activity of Amy-AgNPs

Disc diffusion assay

The antibacterial effectiveness of Amy-AgNPs was investigated using fish pathogenic bacteria isolates that kindly provided from Faculty of Veterinary Medicine, Alexandria University, three Gram-negative: *Aeromonas hydrophila*, *Pseudomonas aeruginos*, and *Vibrio anguillarum*, three Gram-positive: *Streptococcus faecium*, *Streptococcus agalactiae*, *Listeria* sp. Disc diffusion method was used for Amy-AgNPs antimicrobial activity against the tested bacteria grown on Muller Hinton (MH) liquid medium. Bacterial cultures were uniformly swabbed onto the MH agar plates; paper discs pre-soaked with 20 μl of Amy-AgNPs. After incubation at 37°C for 24 h, the inhibition zone was detected and recorded using plate triplicates (**Darwesh et al., 2021**).

Minimum Inhibitory Concentration (MIC) Determination

The tested bacterial strains were suspended in MH broth and adjusted to 0.5 McFarland standards. According to the CLSI recommendations, broth micro-dilution test was performed to determine the MICs for amoxicillin as blank antibiotics and AgNPs (**CLSI, 2010**).

Antioxidant activities of Amy-AgNPs

According to **Okawa et al. (2001)**, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was performed to determine the radical scavenging activity of Amy-AgNPs where the absorption of DPPH solution decrease by the addition of an antioxidant using ascorbic

acid as reference. DPPH solution (0.1mM) was prepared by dissolving 4.0 mg of DPPH in 100 ml of ethanol. Different concentration (10 to 100 µg/ml) of Amy-AgNPs and ascorbic acid were used. The reaction mixture was kept in dark condition at 25°C room temperature for 30 min. The mixture absorbance was spectrophotometry read at 517 nm using DPPH solution as control. The percent of radical scavenging activity of the Amy-AgNPs was estimated using the following formula:

$$\text{RSA (\%)} = (\text{Abs control} - \text{Abs sample}) / \text{Abs control} \times 100$$

Where, RSA = Radical Scavenging Activity;

Abs control = absorbance of DPPH radical + ethanol;

Abs sample = absorbance of DPPH radical + Amy-AgNPs.

Larval Rearing and Feeding Regime

Clearwater technique was carried out using the newly hatched larvae (0-4-day post-hatch, dph) produced from the artificially induced spawning for sea bass (*Dicentrarchus labrax*) stock at the National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt. The sea bass hatched larvae were reared till reached the required age for the experiment (one month) in cylindroconical indoor fiber glass tanks (200 L) with stock density of 35 larvae L⁻¹. A continuous flow of filtered aerated sea water (1 L min⁻¹) was used to keep the water at a constant level. For the first 10 days, the larvae were kept in the dark. Once exogenous feeding period was started, the larvae were fed rotifers, *Brachionus plicatilis*, from day 4 to 15 dph (day post-hatch) at a density of 10 to 25 rotifers mL⁻¹, followed by *Artemia nauplii* (A. F. Great Salt Lake, USA) from day 10 to 45 dph following feeding protocol. From day 20 dph feeding with enriched *Artemia metanauplii* were carried out and maintained at 1-5 *Artemia* mL⁻¹ that gradually decreased with increasing age of larvae with substituting it with dry feeding starting from day 45 post-hatch to day 60.

Experimental design

For optimal Amy-AgNPs concentrations assessment and considering safe dose for larval rearing, the study has begun on the 5 experimental groups of sea bass larvae. Four different concentrations of Amy-AgNPs 1, 5, 10 and 20 % represents 0.1, 0.2, 0.5, 1.0 and 2.0 mg mL⁻¹ encapsulated using bioencapsulation technique on live food aiming frugality and co-effectiveness or as recommended by the previous study (**Barakat *et al.*, 2016**). These experimental groups were used for larval rearing period until become post larvae to study their effects on survival percentage. The time-course study was established on the 30th day post-hatch with pre-acclimatization for 15 days in five treatment tanks (each in triplicate), containing sea bass larvae at density 15 larvae L⁻¹. Further tank was hosted by control group (non-treated larvae). This experiment was applied for the most sensitive post larval rearing period to study their effects mainly on survival percentage and development. The experimental period extended till age 60 dph.

Survival and morphometric measurement

Morphometric parameters were measured weekly including: total length of each post-larvae determined by an ocular micrometer with the nearest minimum accuracy of

0.5 mm. Larval weight was recorded by using the mono-pan balance with an accuracy of 0.01mg. On the other hand, growth performance was examined and recorded as survival percentage (%) and specific growth rate (SGR%) using the following equations.:

$$\text{Survival \%} = \frac{\text{Number of larvae at the end of cultivation}}{\text{Starting number of larvae}} \times 100$$

$$(\text{SGR}) (\%/ \text{ day}) = \frac{\ln (\text{Final weight} - \text{initial weight})}{\text{experimental period days}} \times 100$$

RESULTS

Biosynthesis and Characterization of Amy-AgNPs

As shown in **Fig (1)**, it was indicated that crude amylases produced by marine *A. flavus* for the biosynthesized of Amy-AgNPs in 5 min at $30 \pm 2^\circ\text{C}$ as ambient temperature resulting in typical dark brown color solution with gradual deepened over time then stabilized within 15 min.

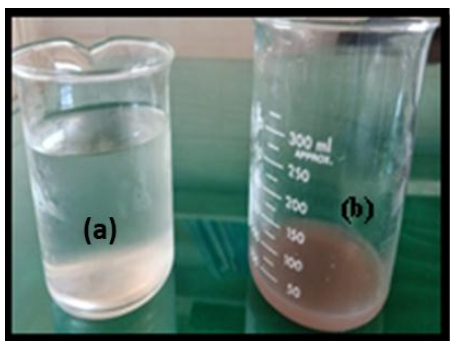


Fig. 1: Biosynthesis of Amy-AgNPs using marine *A. flavus* amylase. Color change during the bioreduction of silver ions, (a) silver nitrate solution, (b) brown color of silver nanoparticles.

Scanning electron microscope (SEM) examination as cleared in **Fig (2)** pointed to the presence of spherical shaped AgNPs with low adhesive level among particles and high uniformity level from 22.88 to 26.35 nm. The obtained results from UV–Vis spectroscopic analysis revealed that the prepared nanofoms were clearly visible and had maximum absorption peak 420 nm as shown in **Fig. (3)**.

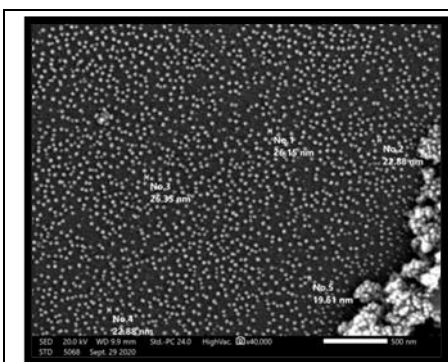


Fig. 2: SEM micrograph of spherical shape amyglase mediated AgNPs.

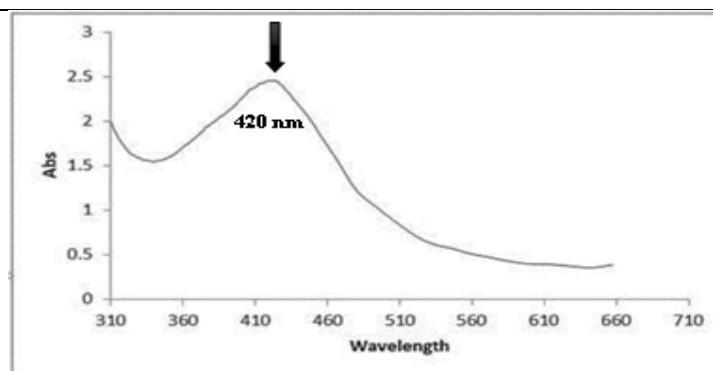


Fig. 3: UV–visible absorption spectrum of the biosynthesized marine fungal amylase mediated AgNPs showing maximum absorption peak 420 nm.

FTIR spectra for the crude of the synthesized fungal Amy-AgNPs showed strong bands at 3697, 3414, 1642 and 1046 cm^{-1} (**Fig. 4**). Other minor peaks were shown at 1421 and 571 cm^{-1} . The broad peaks at 3697-3414 and 1642 cm^{-1} related to N-H bond of amines, and C = O stretch of amides or C = C stretch of alkenes, respectively. An overlap of both N-H and O-H bond stretching of 1° and 2° amines may be took place regarding the broadness of the band. Minor peaks at 1421 and 571 cm^{-1} were attribute to the 3° O-H vibration of alcohols, aromatic nitrogen compounds and C≡C stretch of alkynes, respectively.

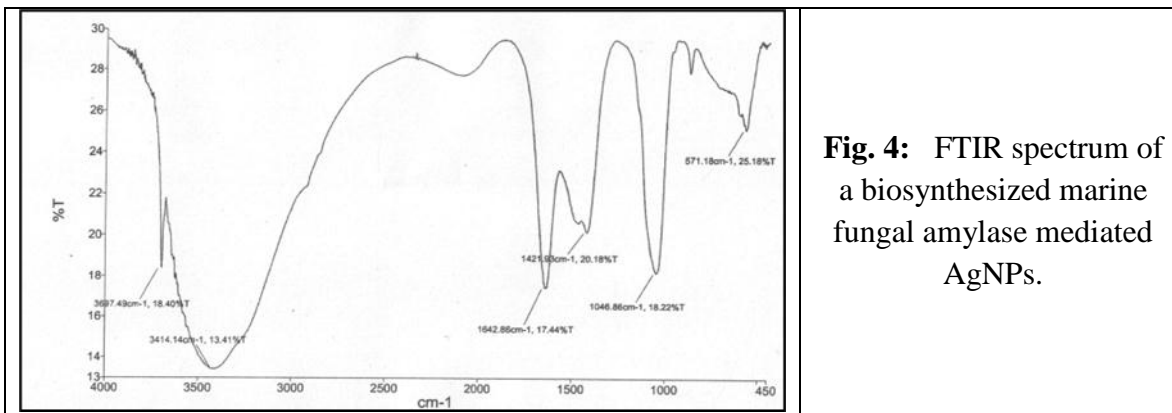


Fig. 4: FTIR spectrum of a biosynthesized marine fungal amylase mediated AgNPs.

Antimicrobial activity

The antibacterial activity of Amy-AgNPs was determined by measuring inhibition zone and confirmed by MICs using amoxicillin as control. The highest activity of this mediated compound was found to be against *A. hydrophila* (20 mm, 1.6 $\mu\text{g/ml}$), followed by 15, 14 & 13 mm and 2.5, 3.2 & 3.6 $\mu\text{g/ml}$ against *St. agalactiae*, *Listeria* sp. and *St. faecium*, respectively (**Table 1 & Fig. 5**).

Table 1: Antibacterial activity and MIC of Amy-AgNPs synthesized by marine *A. flavus*

Bacterial isolates	Inhibition zone in diameter (mm)		MIC ($\mu\text{g/ml}$)	
	Amoxicillin	Amy-AgNPs	Amoxicillin	Amy-AgNPs
<i>A. hydrophila</i>	9	20	50	1.6
<i>P. aeruginosa</i>	16	7	50	33.3
<i>V. anguillarum</i> ,	19	0	34	25
<i>Listeria</i> sp.	25	14	25	3.2
<i>St. agalactiae</i>	25	15	50	2.5
<i>St. faecium</i>	22	13	34	3.6

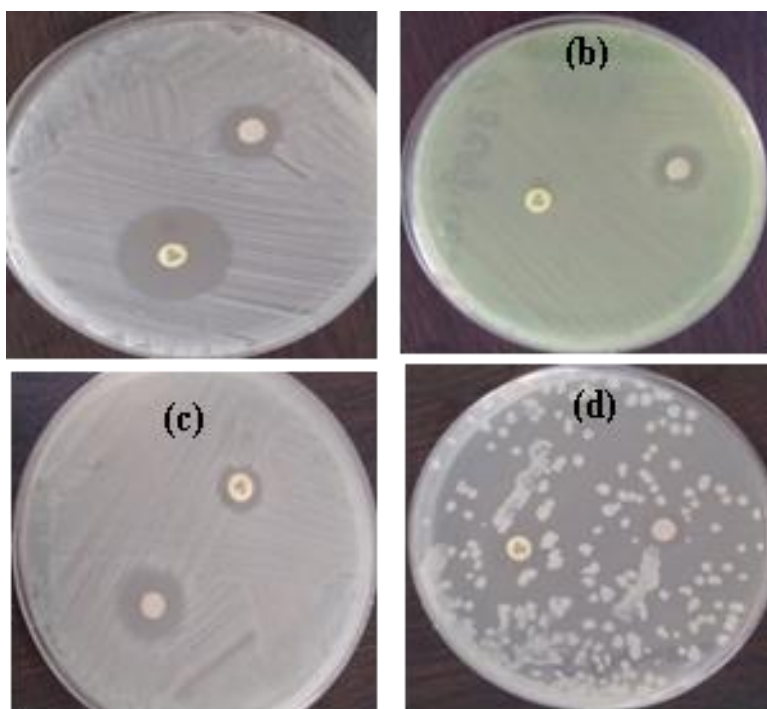


Fig. 5: Agar diffusion assay of Amy-AgNPs synthesized by marine *A. flavus* against *A. hydrophila* (a), *P. aeruginosa* (b), *St. agalactiae* (c) and *St. faecium* (d)

DPPH-free radical scavenging activities

Elevated DPPH radical scavenging levels were displayed using the biosynthesized Amy-AgNPs ranging from 31.46 to 94.0 % at Amy-AgNPs different concentration (10 to 100 $\mu\text{g/ml}$) (**Fig. 6**).

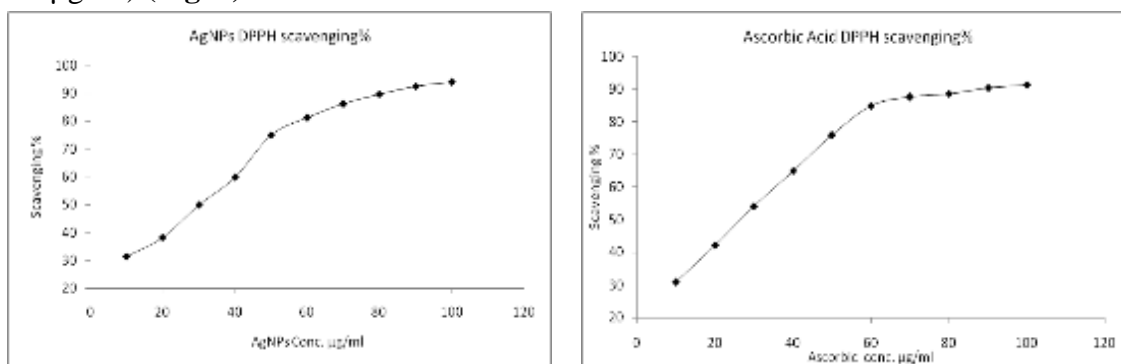


Fig. 6: Antioxidant activities of amylase mediated silver nanoparticles (a) and Ascorbic acid (Standard) (b) using DPPH method expressed as scavenging activities %.

Experimental treatment of *Dicentrarchus labrax*

The comparative growth performance parameters of *Dicentrarchus labrax* in the four 4 experimental groups treated with different concentrations of encapsulated Amy-AgNPs along with control group are shown in **Fig. (7)**. Encapsulation with 5% Amy-

AgNPs group showed a significant reduction in mortality by 24 and 16.23±0.62% SGR, compared with control (no treatment) ($P < 0.05$) after 4 weeks of experiment. The rest of treatments showed poor effect on sea bass survival rate. Growth parameters TL, W and WT showed significant results 2.9±0.1cm, 4.9±0.1mm and 138±1.9 mg, respectively.

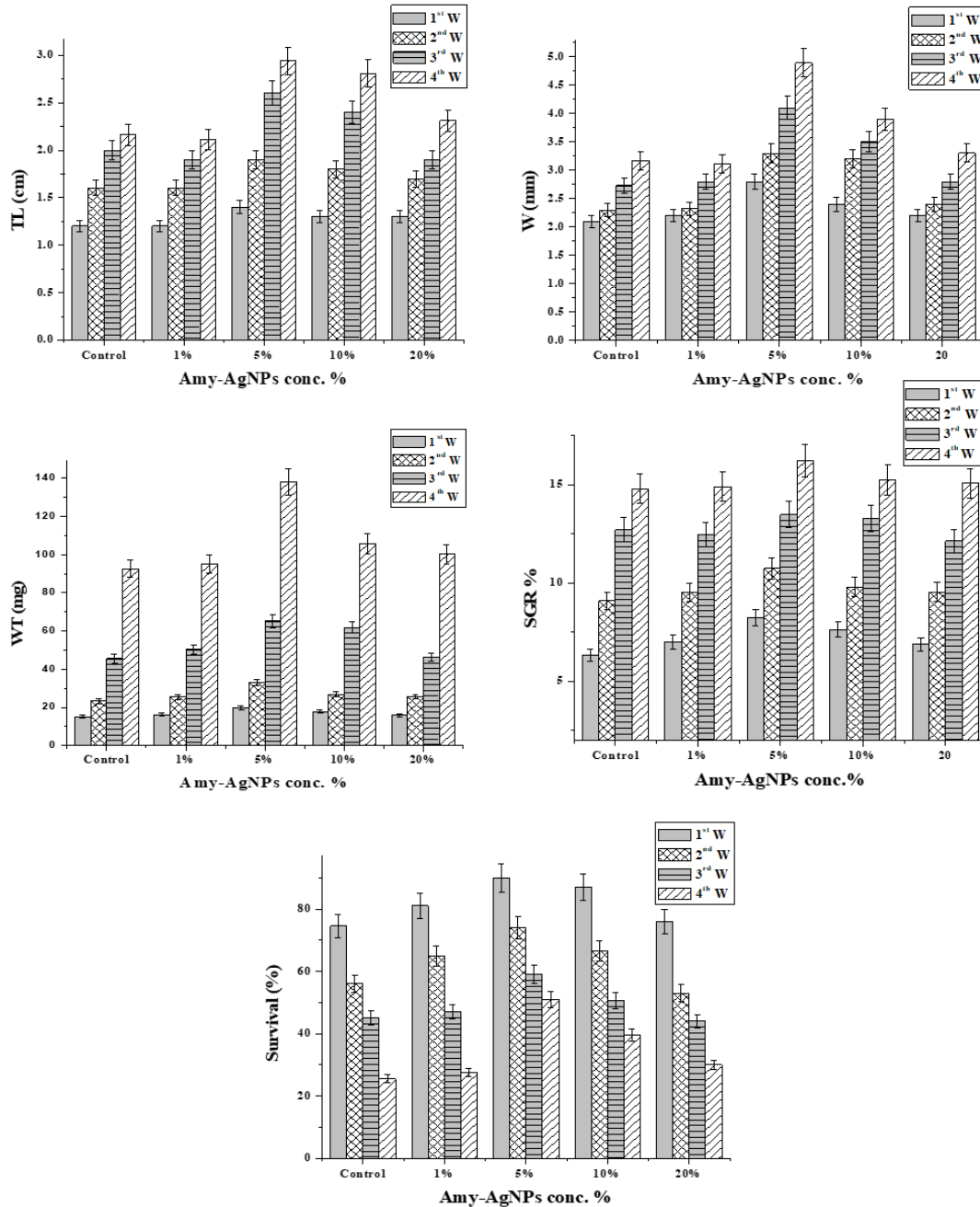


Fig. 7: Morphometric and Growth performance measurements of Sea bass fed on encapsulated Amy-AgNPs during the 4 weeks

DISCUSSION

Biosynthesis and characterization of Amy-AgNPs

In the present study, the biosynthesized Amy-AgNPs by marine *A. flavus* for 5 min at $30 \pm 2^\circ\text{C}$ resulting in typical dark brown color solution with gradual deepened over time then stabilized within 15 min. Formation of Amy-AgNPs dark brown color was done due to the bioreduction of silver ions (Ag^+) using purified amylase. The brown color of biosynthesized Amy-AgNPs have been previously confirmed and reported by **Othman et al. (2019)**

The data concerning SEM examination pointed to the presence of spherical shaped AgNPs with low level of adhesion and uniformity from 22.88 to 26.35 nm. The same spherical amylase-mediated AgNPs by (SEM) indicated the presence of well-defined nanomolecules of ~ 45 and 20 nm size (**Mishra et al., 2012**). Otherwise, the synthesis of elliptical, crystalline and stable enzyme mediated AgNPs with an average size of 38.26 ± 0.4 nm was examined using TEM (**Thapa et al., 2017**). However, SEM confirmed the formation of polydisperse amylase mediated AgNPs that had diameters ranging from 63 to 142 nm of a cubical shape (**Pandey et al., 2016**).

UV–Vis spectroscopic analysis of these nanoforms had maximum absorption peak at 420 nm. Likewise, UV–Vis's analysis of purchased amylase that mediated AgNPs formation indicated the narrow bell-shaped graphs with absorbance at 420 nm (**Mishra et al., 2012**). **Pandey et al. (2016)** studied a bacterium *Pseudomonas* sp. KY548391 producing mediated alpha-amylase showing absorbance at 435 nm. Same spectral analysis was carried out using enzymes mediated AgNPs, exhibited surface plasmon resonance peak at the wavelengths ranged from 402.5 to 410 nm (**Elegbede et al., 2018**) and around at 420 nm (**Debnath et al., 2019**). Alpha-amylase from three different strains of *Bacillus* species were used for the synthesis of AgNPs showed wavelengths range from 400 to 450 nm (**Popescu et al., 2020**). Enzymes mediated AgNPs fabrications within the range of 391–460 nm were previously established (**Widad et al., 2020; El Shanshoury et al., 2020**).

FTIR spectra for the synthesized Amy-AgNPs crude showed strong bands from $3697-1046\text{ cm}^{-1}$ represented by N-H bond of amines, and C=O stretch of amides or C=C stretch of alkenes and minor peaks at 1421 and 571 cm^{-1} attributed to the O-H vibration and C \equiv C stretch of alkynes, respectively. So, FTIR profile could be an evident of these biomolecules on their rich in hydroxyl (O-H) and amine (N-H) groups from the extracted amylases that is responsible for the reduction in Ag^+ , then capping and stabilization AgNPs (**Kumari et al., 2015**). **Pandey et al. (2016)** studied the FTIR analysis of amylase mediated AgNPs peaks that represented by a thiol group (-SH) in cysteine and carbonyl groups (=CO) of amino acid residues and interact with AgNO_3 resulting in stabilization of the nanoforms. Another report by **Debnath et al. (2019)** showed the FTIR of amylase mediated AgNPs revealed that mainly carboxyl, hydroxyl, and amine groups were detected in fungus extract and responsible for Ag^+ to Ag^0

reduction. Many recent reports studied chemical characterization of green synthesized microbial enzymes mediated AgNPs (**Huq, 2020; Mondal *et al.*, 2020**).

Antimicrobial activity

The highest activity of this mediated compound was found to be against *A. hydrophila* (20 mm, 1.6 µg/ml), followed by 15, 14 & 13 mm and 2.5, 3.2 & 3.6 µg/ml against *St. agalactiae*, *Listeria sp* and *St. faecium*, respectively. Biosynthesized silver nanoparticles (AgNPs) considering as safe and effective bioactive agents against two bacterial fish pathogens, showed the lowest MIC values recorded for 2 µg/mL against *A. hydrophila* and 4 µg/mL against *P. fluorescens* (**Essawy *et al.*, 2021**). Likewise, *in vitro* antibacterial activity of green synthesized AgNPs was tested against the two fish pathogens, exhibited clear inhibition zones of 22 and 20 mm with MICs of 1.625µg/ml and 3.25µg/ml against *A. hydrophila* and *P. aeruginosa*, respectively (**Eladawy *et al.*, 2021**). Antimicrobial properties of the biologically synthesized AgNPs were tested against three bacterial fish pathogens (*St. agalactiae*, *A. hydrophila*, and *V. alginolyticus*) using disk diffusion test where the inhibition zones were approximated from 16 to 25 mm (**Ghetas *et al.*, 2021**). **Dahdouh *et al.* (2020)** studied *in vitro* the effect of AgNPs suspension showed maximum activity against *A. hydrophila*, *P. aeruginosa* and *St. agalactiae*. Thereby, AgNPs enzymes mediation is considered as suitable strategies to solve the multidrug resistance bacteria action (**Baptista *et al.*, 2018**).

Three *Bacillus* strains producing alpha-amylase were used for the green synthesis of silver nanoparticles, exhibited their potential antibacterial activity and resulting in cell membrane damage, oxidative stress, and injury to proteins and DNA (**Popescu *et al.*, 2020**). Previous reports showed the synthesized nanoparticles from α -amylase had excellent antibacterial activity (**Mishra *et al.*, 2012; Pandey *et al.*, 2016; Abeleda *et al.*, 2020**).

Antioxidants activities

Elevated DPPH radical scavenging levels were displayed using the biosynthesized Amy-AgNPs ranging from 31.46 to 94.0 % at Amy-AgNPs different concentration (10 to 100 µg/ml). These activities were comparable to those recently reported in aquaculture (**Saleh *et al.*, 2018; Martí-Quijal *et al.*, 2020; Mabrouk *et al.*, 2021**). The free radical scavenging potentials of nanoparticles are credited to the functional groups of bioreductant molecules, whose ability to stick to the surface of the nanoparticles may result in amplified surface areas for activity (**Bhakya *et al.*, 2016**).

Amy-AgNPs treatment for *Dicentrarchus labrax*

After four weeks of experiment, it was found that 5% encapsulated Amy-AgNPs showed mortality reduction by 24% and SGR by 16.23±0.62%, compared with control (no treatment) ($P < 0.05$). TL, W and WT growth parameters were 2.9±0.1cm, 4.9±0.1mm and 138±1.9 mg, respectively. The effect of 10 µg AgNPs L⁻¹ AgNPs aqueous exposure on growth performance of Nile Tilapia, after 8 weeks of treatment was significantly increased weight gain (WG) and SGR (**Mabrouk *et al.*, 2021**). Survival rate

was improved when different supplemented level of astaxanthin was added to sea bass diets where, WG $3.42 \pm 0.18\text{g}$, SGR $3.82 \pm 0.09\%$ and survival rate $98.7 \pm 1.2\%$ increased significantly (Saleh *et al.*, 2018). Survival percentage of sea bass fed on different chitosan supplemented diets were significantly analyzed after 20 days of the experiment, represented at $94.5 \pm 0.5\%$ and 7.22% SGR using 1g Csq (El-Sayed & Barakat, 2016).

CONCLUSION

Fungal amylase produced by *A. flavus* AUMC10636 fabricated spherical shaped Amy-AgNPs with sizes ranging from 22.88 to 26.35 nm. Biosynthesized Amy-AgNPs showed notable antibacterial activities and antioxidant potentials to scavenge DPPH. Furthermore, the potentiality of these nanoparticles to manage sea bass growth performance was confirmed by addition of 5% Amy-AgNPs. Therefore, this study has established the vast nano-biotechnological applications from marine fungal amylase, displaying them as profitable denotation in mariculture biomedical fields.

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