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Original Article

The efficiency of biosynthesized zinc oxide nanoparticles by *Fusarium* sp. against *Saprolegnia parasitica* isolated from common carp eggs in fish hatchery

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Abstract: *Saprolegnia* spp. infect common carp (*Cyprinus carpio* L.) eggs in hatcheries. Therefore, this study aimed to evaluate the antifungal effect of biosynthesized zinc oxide nanoparticles (ZnNPs) against *Saprolegnia* spp. as an eco-friendly treatment. Biosynthesized ZnNPs were characterized using atomic force microscope (AFM), size distributor and Ultra Violate-Visible spectrometer (Uv-Vis), and Scanning Electron Microscope (SEM). Biosynthesized (ZnNPs) had a spherical shape with diameters ranging 10-70 nm. Antifungal activity was tested by fungal radial growth inhibition on corn meal agar. The highest concentration of 100 ppm of ZnNPs showed a remarkable inhibition rate of 79% against *Saprolegnia* spp., demonstrating similar efficiency as the positive control i.e. malachite green in the inhibition percentage rate of fungal growth. This study showed that biosynthesized zinc oxide nanoparticles had a significant antifungal effect ($P<0.05$) and can be used as an alternative option to control *Saprolegnia* spp. in fish hatcheries.

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Introduction

One of the concerns in fish breeding is fungal infections with *Saprolegnia* spp.. To control this, malachite green is used as an effective traditional method (West, 2006). This fungus attaches to the dead eggs and transfers to the live eggs; therefore, it is considered a crucial issue in aquaculture (Tedesco et al., 2021). In aquaculture, malachite green and sodium chloride are traditionally used due to their fungicidal effects (Hashimoto et al., 2011), but adverse effects of malachite green e.g. its carcinogenicity and negative environmental impacts, have limited its application in many countries (Al-Mahmood et al., 2017; Al-Mahmood et la., 2021). Hence, it is important to replace malachite green with an effective antifungal agent with fewer side effects (Fajardo et al., 2022).

The rapid development of nanotechnology and its successful applications in the agri-food sector make it a promising candidate for this purpose (Taha et al., 2021; Al-Ardi, 2022). Nanotechnology has many applications in detecting and controlling pathogens, water treatment, and sterilizing ponds (Bhattacharyya et al., 2015; Luis et al., 2019). Various studies have confirmed zinc oxide nanoparticles' antifungal and antibacterial effects (Swain et al., 2014). Aquaculture fungal infections can be controlled using nanoparticles with antibacterial and antifungal properties (Shammari, 2016). Studies have confirmed their effectiveness as an antifungal agent. Zinc oxide nanoparticles (Zn-NPs) have antimicrobial activity (Mendes et al., 2022). Biosynthesized nanoparticles represent an eco-friendly alternative. In addition, a more effective synergetic effect is possible through a combination of nanoparticles and natural extracts. For these reasons, this study aimed to characterize and evaluate a biosynthesized ZnNPs against

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Saprolegnia spp. by an *in vitro* assay.

Materials and Methods

Isolation of *Saprolegnia*: *Saprolegnia* spp. was isolated from the infected eggs of common carp from Al-Wahda fish hatchery, Baghdad, and identified based on their morphological characteristics. For the pure culture of *Saprolegnia parasitica*. *Saprolegnia* fragments from the eggs were removed and directly inoculated in Corn Meal agar (CMA) medium with antibiotics (chloramphenicol). After the fungus (white, cotton-like mycelia), a disk of the colony (6 mm) was placed on a plate with sterilized distilled water and baits of 5-10 sesame seeds were distributed around it. The disk was kept at 20°C for 24 hours. The colonized seeds were placed on another plate with sterile distilled water and kept at 20°C for 5 days. Subsequently, the baits were examined under a light microscope (Lenovo, LX400) to verify identification by the hyphae, zoosporangium, zoospores, and sexual structures (Sandoval-Sierra and Dieguez-Uribeondo, 2015).

Biosynthesis of Zn-NPs by aqueous *Fusarium* sp. extract: The fungus *Fusarium* sp. was obtained from the Biology Department, College of Science, Mustansiriyah University. Preparation of aqueous *Fusarium* sp. extract cultured in liquid medium for 14 days at 25°C with a shaking rate of 150 rpm. Then fungal biomass was homogenized and filtrated (100 ml). The extract was filtered using a millipore filter (0.2 μm), and stored at -4°C before use. The flask containing 5 g of zinc acetate with 50 ml of deionized and sterile distilled water mixed with 50 mL of the aqueous *Fusarium* extract for 30 min under continuous stirring at 35°C and then allowed to keep at room temperature for 48 hrs. The initial PH of the solution was about 5.5, and the color of the aqueous solution changed to a dark brown solid. The product was collected by centrifugation at 10000 rpm for 10 min and careful washing with distilled water. The final products were obtained by drying overnight. The resulting dried sample was crushed into powder and stored in an airtight container for further analysis.

Preparation of zinc oxide nanoparticle and malachite green stock: One mM of Zinc nitrate hexahydrate in a 1:2 ratio, Zn(NO3)26H2O was added to 50% fungal extract solution while stirring continuously. Following complete dissolution, the mixture was stirred at 100°C for 5 hours, and the supernatant was discarded after cooling.

After centrifuging twice at 6000 rpm for 15 minutes, washing, and drying at 85°C for 5 hours, the solid product (pale white) was collected. Further studies were conducted using the dried powder, which was kept at room temperature until it changed color. In preparation for the malachite green stock, 10 parts per million were used (Ahmad et al., 2019).

Characterization of biosynthesized Zn-NPs:

Nanoparticles were evaluated for their morphology and stability using a FEMTO UV-VIS photometer (Ultraviolet-visible spectroscopy) within a week and a year after synthesis (800xi) at a wavelength of 400 to 800 nm (Ghozali et al., 2015). For atomic force microscopy (AFM), a thin sample layer was put on a glass slide by dropping 150 μl of the sample and drying for 10 mins. The slides were then scanned with the AFM. Scanning Electron Microscope (SEM) photographs of the biosynthesized ZnNPs were performed using a Philips XL30 SEM (Netherlands).

Antifungal activity of biosynthesized Zn-NPs: The antifungal effect of the synthesized Zn-NPs was tested against *Saprolegnia* spp. using CMA medium prepared with different concentrations of nanoparticles (10, 25, 50 and 100 ppm) prepared by adding a volume of 0.5 ml of each 0.05, 0.10, 0.25, and 1 mg/ml aseptically into the plates loaded with 9.5 ml of CMA medium compared with negative control of distilled water and positive control malachite green. All experiments were done in three replicates. Petri dishes were inoculated in the center with 4 mm fungal plugs and incubated at 20±2°C for 10 days. The radial growth of the colonies was measured and the percentage of inhibition of mycelial growth was calculated using the equation of Inhibition% = [(G1- G2)/G1] x100; where G1 is the radius of normal growth in control plates and G2 is

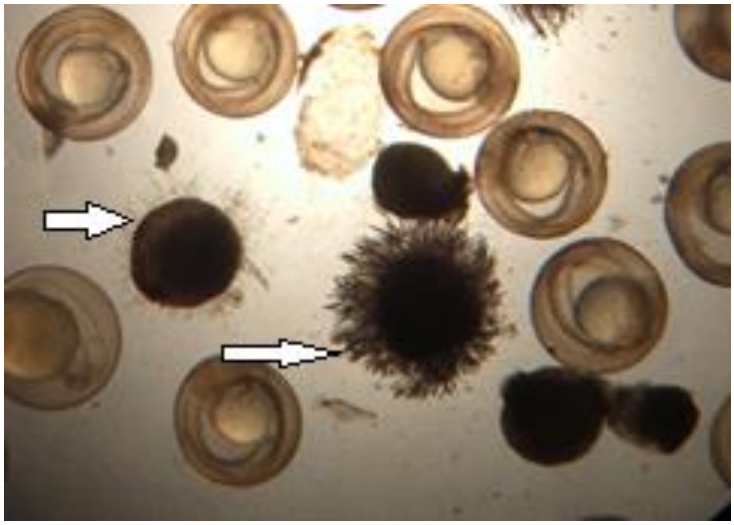

Figure 1. Infected common carp eggs labeled with arrows during incubation period after 48 hours, (10X).

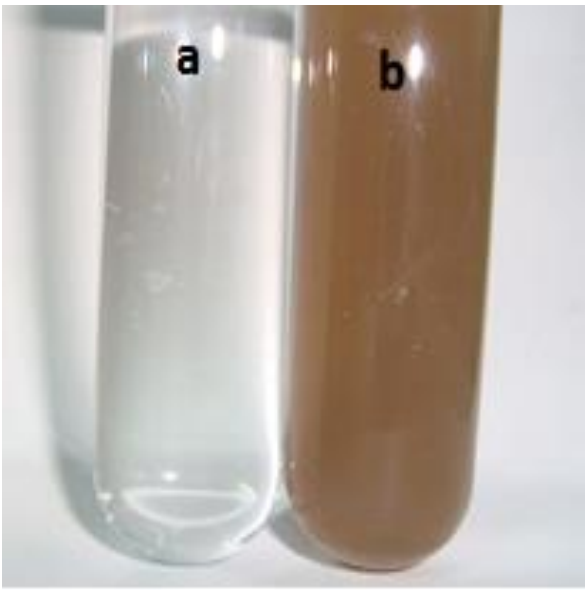

Figure 2. Color changes in aqueous solution (a) zinc oxide before adding *Fusarium* sp. extract (b) after one hour of adding fungus extract at 25°C.

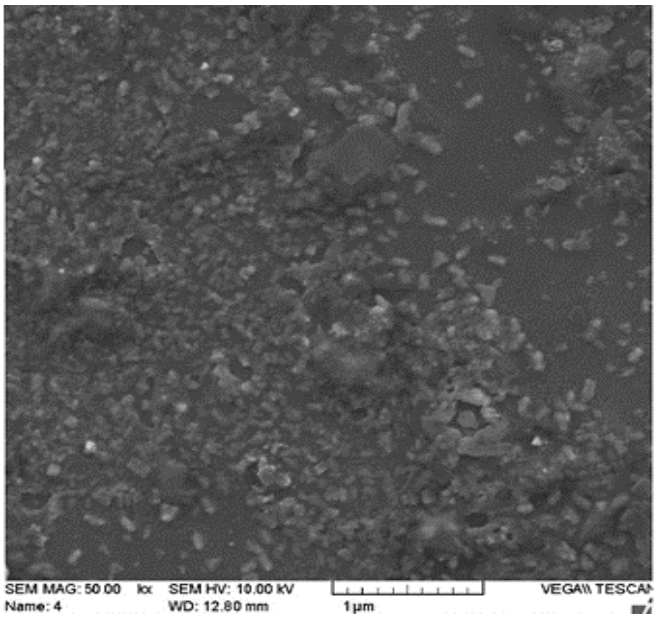

Figure 3. SEM of biosynthesized ZnNPs by *Fusarium* sp.

the radius of inhibited growth.

Statistical analysis: The significant difference between values of the incubation conditions was determined using a two-way ANOVA. In addition, the Least Significant Difference (LSD) and correlation were performed to test whether group variance was significant or not ($P \leq 0.05$) in SPSS program version 26.

Results

Saprolegnia spp. was isolated from the infected eggs of common carp that were covered with white or grey threads of cotton-like mycelia (Fig. 1).

ZnNPs were synthesized using *Fusarium* extract, which changed the colour from colourless aqueous extract to red-brown as an indicator of the formation of ZnNPs (Fig. 2). Biosynthesized (ZnNPs) had a spherical shape with diameters ranging 10-70 nm. Studies show that other metals where the colour change shows the synthesis of the respective nanoparticles; however, it was characterized by UV–visible spectroscopy using *Fusarium* extracts at 360 nm that recorded an absorption peak, which is attributed to the formation of ZnNPs.

Scanning electron microscopic images of ZnoNPs synthesis using *Fusarium* extract are presented

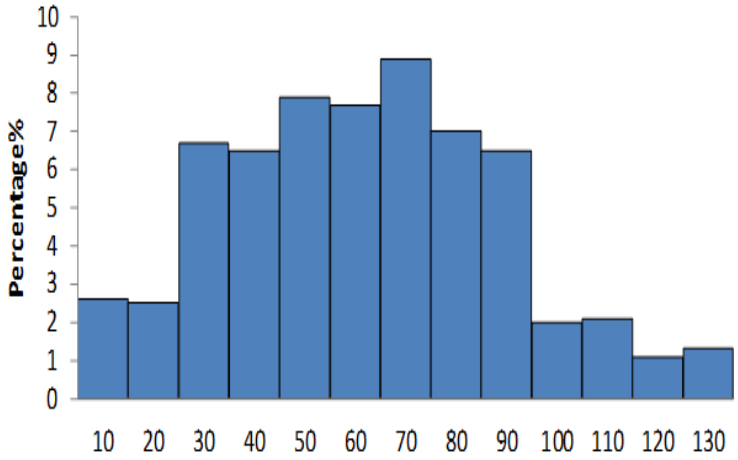

Figure 4. Diameters, amount of biosynthesized ZnNPs by *Fusarium*.

Table 1. Inhibitory rate (%) of *Saprolegnia parasitica* fungi using different concentrations of Zn nanoparticles compared with malachite green.

Test Fungi	Zn-NPs concentrations (ppm)				Malachite green 10 (ppm)
	10	25	50	100	
Inhibitory rate (%)	21.2±0.2^{c}	47.2±1.2^{b}	75±0.4^{b}	79±0.5^{a}	80.2±1.2^{a}

The inhibition rates in (%) mean± standard error (SE). different letter(s) are significantly different.

in Figure 3, showing the accumulations of particles. The size and shape of synthesized ZnNPs were reported by AFM microscopy. The biosynthesized Zno were spherical with a few cylindrical particles with variations in particle size ranging from 10-70 nm (Fig. 4).

Biosynthesized ZnNPs at 100 ppm showed a remarkable inhibition rate of 79% against *S. parasitica* followed by 75 and 47.2% for the lowest concentrations (Table 1). There was no significant difference ($P \leq 0.05$) between inhibition rates at 100 ppm ZnNPs with malachite green. Mycelia growth of *S. parasitica* was inhibited in all the tested concentrations.

Discussion

Saprolegniaceae species such as *Achlya* and *Saprolegnia* can infect fish eggs of different species in aquaculture and nature (Lone and Manohar, 2018), considering crucial problems in their incubation (Ali et al., 2020). Malachite green is widely used as an antifungal agent, but it is teratogenic and mutagenic, causing abnormalities in eggs and fish, which since 1991, it was banned (Jogaiah et al., 2019). Therefore, other methods are used to treat saprolegniasis. It has been suggested application of nanoparticles in aquaculture. Zinc oxide nanoparticle is an inorganic compound soluble in water and its antifungal properties are proven. It is connected to the membrane of microorganisms in the phase of the growth cycle, prolonged the time of germination of organisms (Ahmad et al., 2018).

The changes in color as an indicator of ZnNPs formation approved the formation of the respective nanoparticles as reported in other works (Jogaiah et al., 2019; Mahmood et al., 2022). UV-Vis spectroscopy is a suitable technique to detect the biological reaction compared to other methods, such as physical, chemical and hybrid methods, where an additional force is required, which may carry toxic bi-products that lose their stability (Jeevanandam et al., 2018). The results showed that malachite green and zinc oxide nanoparticles have significant antifungal effects.

Based on the results, with an increase in the concentration of biosynthesized ZnNPs, the antifungal activity was increased, similar to the results of (Ahmad et al., 2018; Johari et al., 2019) regarding the antifungal properties of silver oxide nanoparticles against *Saprolegnia*. The antifungal activity of ZnNPs was dose-dependent, and the best

inhibition concentration was 25 ppm in the current study. A significant increase in the inhibition of *Saprolegnia* was recorded at higher concentrations of 50 and 100 ppm, respectively, which agrees with the findings of Ahmad et al. (2018). The fungus can be reduced using zinc oxide nanoparticles *in vitro*; therefore, it can be possible to control saprolegniasis disease in fish hatcheries fishes instead of malachite green and NaCl. M

In conclusion, all concentrations of biosynthesized ZnNPs showed a remarkable inhibition rate against *Saprolegnia* showing similar efficiency compared to the positive control (malachite green), and a 100-ppm concentration of ZnNPs is suggested efficient for use against saprolegniasis in fish hatcheries.

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