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New technique for improving fish packaging hygiene and prolonged shelf life

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Zinc oxide nanoparticles (ZnO NPs) have received great attention due to their optical, physical, and antimicrobial properties. They have toxic effect against microbes without any effect on mammalians cells. They are used in several applications including extending the shelf life of food. The study aims to determine the minimum inhibitory concentrations of ZnO NPs against different aquaculture fish fungus species and their storage period. A total of 160 samples were collected from different types of aquaculture fish samples as follows: rabbitfish, bream, red mullet, saddle grouper, spangled emperor, gilthead seabream, mackerel fish, and Asian seabass. ZnO NPs activity against the isolated fungus species was evaluated by estimating minimum fungicidal inhibitory concentration and inhibition of fungal enzymes (amylase, protease, and lipase). The storage period of the fish in a package containing ZnO NPs was determined by estimating the sensory characteristics of the treated fish. The results obtained recorded the following fungus species from aquaculture fish samples: *Aspergillus niger* (gi: JX112703), *Aspergillus oryzae*, *Aspergillus awamori*, *Penicillium* species, *Aspergillus tubingensis*, *Trichosporon montevideense*, *A. niger* (gi: MG889596), and *Byssochlamys spectabilis*, respectively. This study is the first to apply ZnO NPs for fish preservation which have a powerful antifungal effect against all the isolated fungi. The study recommends using 3% ZnO NPs in fish packaging film; it inhibited most of the fungus species, extending the shelf life of most of the fish species to more than 15 days.

Key words: Zinc oxide nanoparticles, shelf life, fish preservation, minimum inhibitory concentration (MIC), antifungal, aquaculture fish fungus.

INTRODUCTION

Fish is an essential source of many necessary elements to human health such as protein, vitamins, and different nutrients (Khan et al., 2018). However, the average consumption of fish in Saudi Arabia is low, equivalent to 9 kg per person per year; while in Japan, a person consumes 60 kg per year. Saudi citizens have increased awareness about the importance of seafood and its reflection on human health. Saudi Arabia's aquaculture projects produce nearly 70,000 tons of fish, and the government is seeking to raise production to 600,000

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tons by 2030 (Rahman et al., 2017). Most researches indicated that fish reared in a polluted aquatic environment have a high susceptibility to different illnesses and contamination than those reared in non-polluted marine environments (Ngo et al., 2021). Depletion of aquatic oxygen, pH changes and the unusual increase of the aquatic organic content increase the microbial loads of fish (Cannas et al., 2020). Aquaculturing on polluted aquatic environment by chemical and/or biological contaminations affects the quality of harvested fish; it causes decreased metabolism, liver damage, ulceration, neoplasia, immunosuppression, hyperplasia in fish. It damages the epithelia, tail, fins and gills of fish. This decreases aquaculture production and/or results in production of low quality fish due to its high biological load. It also affects adversely the shelf life of fish (Javed et al., 2016; Ayalew and Fufa, 2018). The improper management, treatment and storage of fish lead to waste of about 50% of fish harvested annually (Chavan et al., 2011). The high distance between landing sites, markets, and consumption areas may cause a high economic loss. To reduce the wastage that occurs by oversupply and to prolong the storage life of fish, an effective novel technique for fish storage is required. The main cause of harvesting fish spoilage is fungal contamination, which appeared as discoloration, off-flavor, rotting, and textural quality of fish. This leads to loss of nutrient quality which causes huge economic loss and hazard to consumers' health (Magwaza et al., 2017; Rico-Munoz et al., 2018). The most encountered fungal genuses are *Aspergillus*, *Trichosporon*, *Penicillium*, and *Aspergillus* species especially *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus tubingensis*. Meanwhile, the presence of pathogenic fungi may cause immunocompromised handlers and consumers to have aspergillosis. This has encouraged scientists to develop a technique to control food borne microorganisms (CODEX, 2009; Derrick, 2009). The contamination of fish by fungi decreases its shelf life which leads to waste of about 60% of the fish aquaculturing cost (Tacon, 2020). It also has environmental and financial cost impact due to the difficulty of disposing the spoiled fish (White, 2013).

Zinc oxide is used recently in food packaging and processing due to its safety, thermal stability and mechanical resistance. It improves the physical character of packaging materials (Duncan, 2011; Rajeshkumar, 2019). The antimicrobial properties of this metal are exaggerated when used as nanoparticles especially in food technology (Qasim, 2011). Zinc oxide nanoparticles (ZnO-NPs) are one of the most effective food packaging substances due to their binding patterns and interactions properties, moisture absorption, monolayer moisture, and solubility (Crona et al., 2020). They also have excellent physical properties such as permeability, desorption, degree elongation, tensile strength, and mechanical properties as food packaging substances (Swain et al., 2014; Paul et al., 2019). Few research works have been done on ZnO nanoparticles that spread through food. Although, the toxicological side effect of ZnO NPs needs more studies to determine their effects on food safety (Paul et al., 2019). Most scientists have studied the toxicity of ZnO NPCs. They found that they are non-toxic materials and have vital mineral supplement for the human body. They have selective toxicity against wide range of microbes that encourage their use as food additives (Stoimenov et al., 2002; Zhang et al., 2007). The US Food and Drug Administration (21CFR182.8991) reported ZnO as one of five safe Zn compounds (Bradley et al., 2011).

There was a surveillance shortage in fish spoilage fungus species in aquaculture fish generally and marketed fish species in Saudi Arabia. This encourages us to perform this study, which aimed to find additional knowledge to enhance proper control of the storage life of fish and fish product by determining the antifungal effect of different concentrations of ZnO NPs on different fish fungal contamination, the minimum inhibitory concentrations (MICs) of nanoparticles against fungal contamination of aquaculture fish sold in Jeddah, Saudi Arabia markets, and the storage period of fish using packages containing ZnO NPs.

MATERIALS AND METHODS

Sample collection

A total of 160 samples were collected from 8 types of aquaculture fish (20 fish from each type): rabbitfish "*Siganus rivulatus*", bream "*Pagrus pagrus*", red mullet "*Mullus surmuletus*", saddle grouper "*Najil P. pessuliferus*", spangled emperor "*Lethrinus nebulosus*", gilthead seabream "*Sparus aurata*", mackerel fish "*Scomberomorous commerson*", and Asian seabass "*Lates calcarifer*". The samples were freshly purchased and stored in ice box and refrigerated. All fish samples were collected from January to July 2021 and transferred to the laboratories of Collage of Science, University of Jeddah.

Fungal Isolations and Identification

The fish surface was disinfected by 1% formaldehyde; the fish was dipped into it from 1 to 5 min. Then, it was transferred to 70% alcohol, and washed with sterilized distilled water. About 1 g of the inner fish tissues were directly spread onto the Petri plates of potato dextrose agar (PDA) medium. Penicillin and streptomycin (50 mg/L) were added to the medium to avoid bacterial contamination. The medium was aseptically dispensed into sterile Petri dishes (Koh et al., 2000; Cao et al., 2015). Identification was done by observing the colony color and texture. It was stained with 0.05% trypan blue in lactophenol. Then, molecular identification was done by PCR detection (Javadi et al., 2012; Rico-Munzo et al., 2018).

Nanoparticle substances

Zinc Oxide NPs (70 ± 15 nm) were bought from Nano Gate (Creating New Scientific Horizons, Egypt). Original suspension of ZnO NP (12 mmol L^{-1}) was then diluted using PDA to make different concentrations of ZnO NPs: 1, 2, 3, and 5% and NP-free

solution [control] (He et al., 2011).

Antifungal test

Autoclaved PDA media with ZnO NPs at different concentrations "1, 2, 3, and 5%" were incubated at 25°C. Then, the antifungal activity was evaluated at the time intervals of 2, 4, 6, 9, and 12 days. The diameter of the fungus colonies was detected in triplicate plates as described previously (Fraternale et al., 2003).

Microbial culture

This was obtained by isolating pure fungus containing identified *Aspergillus* spp., *Penicillium* spp. and *Byssochlamys spectabilis.* It was tested for amylase production by starch plate method (Ross, 1976), protease and lipase by skim milk agar (Zaitz et al., 2004).

Amylase assay

About 25 g of starch agar medium was suspended in 1000 ml distilled water. About 4 mm of fungal culture was cut on a labeled plate and then incubated at 25°C/48 h with a drop of iodine solution for 30 s. The color of the medium changed because amylase is a starch hydrolyzing enzyme (Ross, 1976).

Protease assay

About 51.5 g of skim milk agar plate was suspended in 1000 ml of distilled water. The fungi were inoculated separately. Hydrolysis results were obtained in the clear zones (zone of hydrolysis) around the fungal colonies at the end of the incubation at 25°C for 48 h (Ali, 1992).

Lipase assay

Agar (2.5%) was added to 2% Tween 20-80 and 0.01% Victoria Blue B (or other indicators). About 1 cm of circular well around the different isolated fungi was grown at 30°C. Lipolytic microorganisms were picked out from the culture plates (Samad et al., 1989).

Determination of the storage period of fish using packages containing NPs

Red Sea fish (about 300 g) was purchased and transferred into a sterile glass container under sanitized conditions. It was packaged with polyethylene (PE) films previously prepared with different concentrations of ZnO (1, 2, 3, and 5%), which were sprayed on the PE surface ("10×150 cm"). It was left to dry at room temperature (26°C). Each 100 g of fish was wrapped by each package film concentration, stored under dark and cool conditions (4°C) and compared to the fish wrapped by uncoated PE film package. The shelf life of each fish type was observed and the fungal growth was tested by using PDA. The fish were incubated between 28±2°C/examined daily for 7, 14, and 18 days and 18±2°C/examined daily for 18 days. The experiments were repeated at least 5 times using each concentration for results confirmation (Ebrahimiasl and Rajabpour, 2015; Al-Naamani et al., 2016).

Sensory evaluation of treated fish

Four samples of different fish treatments were covered in small dishes. The samples were evaluated for the following parameters: acceptability by odor intensity, appearance, flavor and after cooking taste, juiciness, tenderness, off-flavor, and off-odor. An eight-point of scoring scale (8 = extremely intense/tender/juicy, 7 = very intense/tender/juicy, 6 = moderately intense/tender/juicy, 5 = slightly intense/ tender/juicy, 4 = slightly bland/tough/dry, 3 = moderately bland/tough/dry, 2 = very bland/tough/dry, 1 = extremely bland/tough/dry) was applied for odor and flavor intensity, tenderness, and juiciness, respectively (Sallam, 2007).

Statistical analysis

The statistical program, SPSS version 16 for window was used for the determination of means, standard error, and analysis of variance (ANOVA) using the one way (mean at the significance level of ($P<0.05$). Statistical significance was tested at the 5% level of significance in this study (SPSS 16, 2007).

RESULTS

Prevalence of different fungal genera in the examined fish samples

The total positive result was 147/160 (92%) from the total examined samples, while the negative result was about 13/160 (8%). The positive prevalence results were found in (Table 1) *Aspergillus niger* (gi:JX112703) followed by *A. oryzae, Aspergillus awamori* and *Penicillium* spp*., A. tubingensis, Trichosporon montevideense,* and *A. niger* (gi:GM889596). The fungus with the lowest incidence was *B. spectabilis*. The different types of fungi found in the different fish species are arranged as follows: Asian seabass "*L. calcarifer*" and *A. oryzae*. In the case of Red Sea bream "*P. pagrus*", the fungus species with the highest incidence was *A. niger* (gi:JX112703). Rabbitfish "*S. rivulatus*" had *A. niger* (gi:JX112703). Spangled emperor "*L. nebulosus*" reported the highest incidence of *Penicillium* spp. Gilthead seabream "*S. aurata*" samples reported *A. oryzae*. Mackerel fish "*Scomberomorous commerson*" samples had *A. niger* (gi:JX112703). Red mullet "*M. surmuletus*" samples reported *T. montevideense*. Saddle grouper "*Najil P. pessuliferus*" samples reported *A. niger* (gi:JX112703).

Identification of isolated fungal genera

Figure 1 describes the isolated fungal species with their phylogenetic molecular tree and most related genera. *Aspergillus niger* (gi:JX112703) colonies were spherical, had thick walls, densely black to dark brown conidia, and white mycelia. Microscopically, *A. niger* (gi:JX112703) spores appeared as dark brown/carbon black. It was grouped into 5 groups with 100% genetic similarity*. A. niger* (gi:MG889596) colonies had compact white base; they have condensed black conidial heads which enlarge and roughen with maturity. *A. niger* (gi:MG889596) is a filamentous fungus that looks like a plant structure. It was

Table 1. Prevalence of different fungal Spoilage species isolated from fish samples.

Fish type	Fungus Types							
	Aspergillus species					*Penicillium* spp.	*Byssochlamys spectabilis*	*Trichosporon montevideense*
	A. niger (gi:JX112703)	*A. niger (gi:MG889596)*	*A. awamori*	*A. oryzae*	*A. tubingensis*			
Asian seabass "*Lates calcarifer*"	4 (20)	2 (10)	4 (20)	6 (30)	4 (20)	2 (10)	3 (15)	2 (10)
Bream "*Pagrus pagrus*"	10 (50)	3 (15)	5 (25)	1 (05)	0 (00)	2 (10)	0 (00)	5 (25)
Rabbitfish "*Siganus rivulatus*"	13 (65)	0 (00)	4 (20)	2 (10)	4 (20)	0 (00)	2 (10)	0 (00)
Spangled emperor "*Lethrinus nebulosus*"	5 (25)	4 (20)	3 (15)	2 (10)	4 (20)	6 (30)	0 (00)	0 (00)
Gilthead seabream "*Sparus aurata*"	0 (00)	0 (00)	0 (00)	13 (65)	2 (10)	6 (30)	0 (00)	0 (00)
Mackerel fish "*Scomberomorous commerson*"	4 (20)	0 (00)	1 (05)	0 (00)	1 (05)	2 (10)	0 (00)	0 (00)
Red mullet "*Mullussurmuletus*"	2 (10)	1 (05)	1 (05)	0 (00)	0 (00)	0 (00)	0 (00)	5 (25)
Saddle Grouper "*Najil P. pessuliferus*"	3 (15)	1 (05)	0 (00)	3 (15)	0 (00)	0 (00)	0 (00)	0 (00)
Total	41/147 (28.0)	11/147 (8.0)	18/147 (12.3)	27/147 (18.4)	15/147 (9.4)	18/147 (12.3)	5/147 (3.4)	12/147 (8.2)

grouped into 5 groups with 100% genetic similarity.

A. awamori colonies appeared as visible peripheral growth, and had smooth surface (light-yellow); they had several conidia which are black, whitish-yellow, and dark brown with wavy slight surface. Microscopically, *A. awamori* appeared as single and aggregated colonies that resemble plant-like filamentous fungi. It was grouped into 7 groups with 99% genetic similarity. *A. oryzae* colony surface appeared as olive-green or green; it has white conidia and white mycelia. Microscopically, *A. oryzae* colonies appeared as pale grey to black; they have conidial heads with a coarse wall and short column. *A. oryzae* was grouped into 6 groups with 100% genetic similarity.

Macroscopically, *A. tubingensis* colonies appeared as yellow at the beginning and became flat, granular, and bright to dark yellow-green with radial grooves. They are finely wrinkled, globular, and have warty conidia. Microscopically, *A. tubingensis* appeared as single aggregated pale yellowish-green colonies; they have radiated conidia heads with coarse roughened wall and loose columns. *A. tubingensis* was grouped into 4 groups with 99% genetic similarity. Macroscopically, *Penicillium* spp. colonies have woolly texture. Initially, their color is white then turns to yellowish or pinkish; they have olive-gray or white conidia. Microscopically, *Penicillium* spp. has branched hyaline or simple conidia with cup-shaped phialides; they have brush-like clusters at the tips, which are known as "penicilli". *Penicillium* spp. were grouped into 4 groups with 99% genetic similarity. Macroscopically, *B. spectabilis* appeared as wheat-colored conidia with wrinkled yellowish to light brown; it has wooly to downy texture, and brown to pale surface. Microscopically, the individual aggregate of *B. spectabilis* has branched hyaline with brush like tip; it is ovoid with elongated, solitary chains. *B. spectabilis* was grouped into 8 groups with 99% genetic similarity. *T. montevideense* macroscopically appeared as dense pure white mycelia and conidia. From the PDA plate it appeared as light-yellow wrinkled reverse with globose vesicle and radiated conidia head. Microscopically, *T. montevideense* appeared as yeast-like colonies with septate hyphae, arthroconidia, and budding cells. *T. montevideense* was grouped into 4 groups with 99% genetic similarity.

Effect of ZnO nanoparticles on the different types of isolated fungi (*in vitro*)

The effect of different concentrations of ZnO NPs (1, 2, 3, and 5%) on the isolated fungi (*in vitro*) was compared to that of antifungal drug on the fungal species (Table 2 and Figure 2). The results revealed that 5% concentration of ZnO NPs was more effective than the antifungal drug followed by 3, 2, and 1%, respectively. 5% ZnO NPs inhibited 2.90 cm *A. niger* (gi:JX112703), which was the highest. This is followed by 3% ZnO NPs which inhibited 2.50 cm *A. niger* (gi:JX112703). The antifungal drug inhibited about 2.30 cm *A. niger* (gi:JX112703). 2% ZnO NPs inhibited 2.20 cm *A. niger* (gi:JX112703). 1% ZnO NPs inhibited

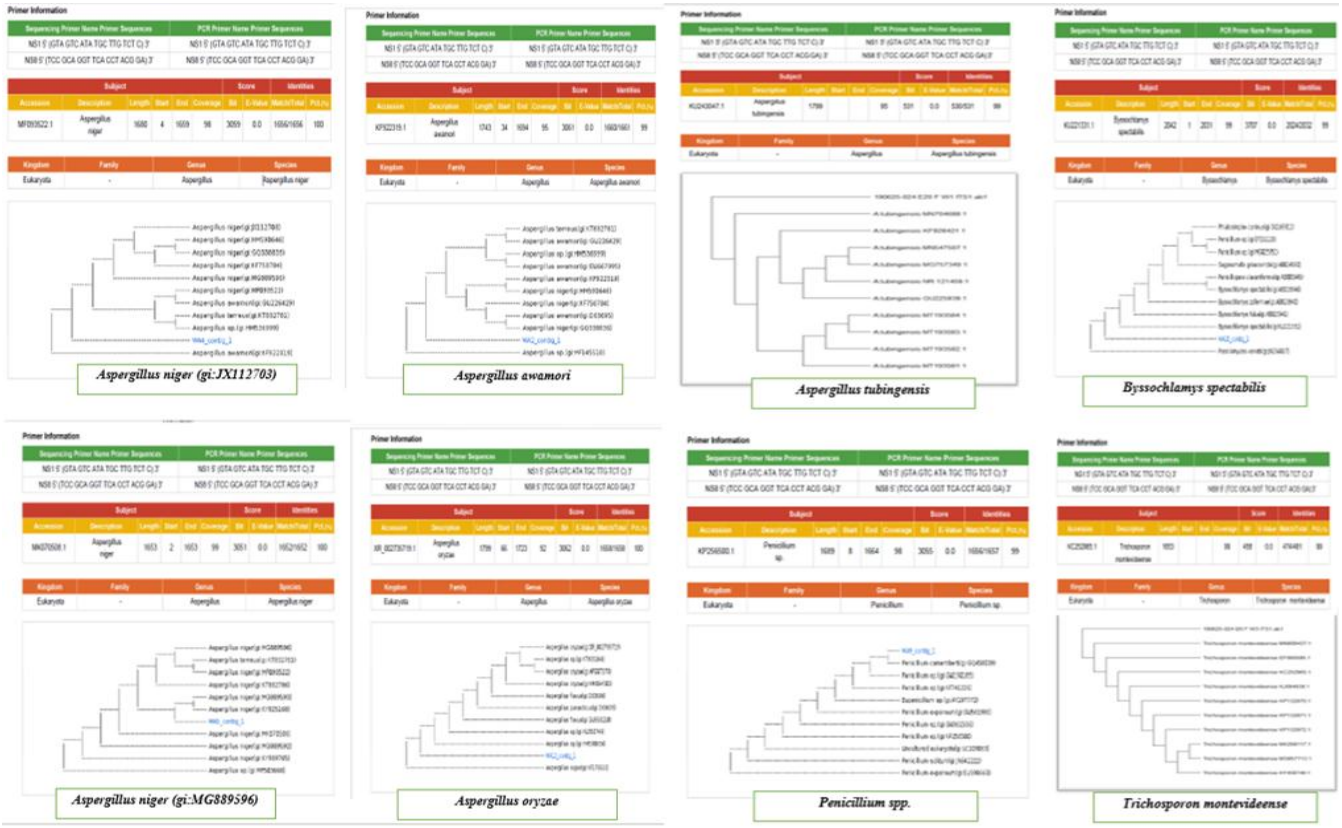

Figure 1. Phylogenetic molecular tree of the selected isolate and the most related gene isolate and the most related gene.

about 1.90 cm *A. niger* (gi:JX112703), which was the lowest inhibition effect. Nearly similar effect was detected against *A. niger* (gi:MG889596); the inhibition effect began gradually with 5% ZnO NPs inhibiting about 2.80 cm *A. niger* (gi:MG889596), 3% ZnO NPs inhibited 2.20 cm *A. niger* (gi:MG889596). 2% ZnO NPs and the antifungal drug had similar inhibition against the fungus (about 2.00 cm). The lower inhibition zone was measured with 1% ZnO NPs, which inhibited 1.70 cm *A. niger* (gi:MG889596).

About 2.30 cm *A. awamori* was inhibited by 5% ZnO NPs, which was the highest inhibition effect followed by 3% ZnO NPs and the antifungal drug which inhibited 2.00 cm of the fungus. 2% ZnO NPs inhibited about 1.90 cm of the fungus. The lowest inhibition zone was measured with 1% concentration of ZnO NPs, which inhibited 1.7 cm of the fungus. *A. oryzae* was one of the most resistant fungi although1.90 cm was inhibited by 5% ZnO NPs; while the inhibition zone was 1.80 cm in the case of 3% ZnO NPs and the antifungal drug. Lower inhibition zone measured by 2 and 1% was as follows: 1.50 and 1.30 cm, respectively. *A. tubingensis* was one of the lower resistant fungi. The inhibition effect was about 1.90, 1.70, 1.60, 1.40 and 1.30 cm in case of 5, 3, 2 and 1% ZnO NPs, and antifungal drug, respectively.

Penicillium spp. has the highest inhibition effect: 5 and 3% ZnO NPs inhibited it by 2.80 and 2.50 cm. The inhibition zone was equal in the case of the antifungal and 2% ZnO NPs concentration, which inhibited 1.90 cm of the fungus. The lowest inhibition effect was recorded in 1% ZnO NPs which inhibited 1.80 cm of the fungus. 5, 3, and 2% ZnO NPs had the highest inhibition effect against *B. spectabilis* (3.20, 2.50, and 2.20 respectively). Antifungal drugs recorded the same inhibition zone (1.50 cm) with 1% ZnO NPs. 5, 3, and 2% ZnO NPs inhibited *T. montevideense* by 2.80, 2.50, and 2.00 cm; while 1.90 and 1.50 cm was inhibited by the antifungal drug and 1% ZnO NPs, respectively.

Effect of ZnO NPs on isolated fungal enzymes

ZnO NPs affect the growth of fungi by attacking their cell structure and/or fungal enzymes. Table 3 shows the effect of adding ZnO NPs on amylase enzyme secreted by different fungal species in the case of *Aspergillus* spp. The fungal growth was about 14 mm in the control samples, while the growth decreased to 4 mm after the antifungal drug (1, 2, 3 and 5%) was added. The amylase activity decreased by about 19 mm in *Aspergillus* spp. plates, while it decreased to 8 mm only in the antifungal drug. 7 mm amylase was inhibited after adding 1 and 2%

Table 2. Effect of ZnO nanoparticles on different types of isolated fungi (*in vitro*).

Fungus	1%				2%				3%				5%				A. F			
	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±	Minimum (cm)	Maximum (cm)	Mean (cm)	S.E. ±
Aspergillus niger (gi:JX112703)	0.0	3.0	1.9^{a}	1.1	1.8	4.00	2.2^{a}	1.0	0.0	4.5	2.5^{b}	1.2	0.5	4.6	2.9^{b}	1.3	2.0	3.0	2.3^{a}	1.1
Aspergillus niger (gi:MG889596	0.0	2.8	1.7^{a}	0.9	0.3	3.4	2.0^{a}	0.9	0.0	4.1	2.2^{b}	1.1	0.2	4.5	2.8^{c}	1.3	0.5	2.0	2.0^{a}	0.8
Aspergillus awamori	0.0	3.1	1.7^{a}	0.9	0.0	3.0	1.9^{a}	0.9	0.3	3.2	2.0^{b}	1.0	0.6	3.7	2.3^{b}	1.1	1.8	2.1	2.0^{b}	0.9
Aspergillus oryzae	0.0	3.0	1.3^{a}	0.8	0.0	2.6	1.5^{a}	0.6	0.0	2.7	1.8^{b}	0.9	0.0	3.6	1.9^{b}	0.8	0.8	3.0	1.8^{b}	1.0
Aspergillus tubingensis	0.0	2.3	1.3^{a}	0.7	0.0	2.5	1.4^{a}	0.5	0.0	2.3	1.7^{a}	0.6	0.0	3.5	1.9^{b}	0.8	0.4	3.0	1.6^{b}	0.7
Penicillium spp.	0.0	2.5	1.8^{a}	0.8	0.0	2.7	1.9^{a}	0.8	0.7	3.8	2.5^{b}	1.3	1.0	4.1	2.8^{b}	1.3	1.6	2.5	1.9^{a}	0.9
Byssochlamys spectabilis	0.0	2.7	1.5^{a}	0.5	0.0	3.5	2.2^{b}	1.0	1.0	3.7	2.5^{c}	1.3	0.0	4.2	3.2^{d}	1.7	1.4	3.0	1.5^{a}	0.5
Trichosporon montevideense	0.0	2.0	1.5^{a}	0.6	0.0	2.8	2.0^{b}	0.9	0.0	3.0	2.5^{c}	1.2	0.0	3.5	2.8^{c}	1.2	1.7	2.2	1.9^{b}	0.8

Means followed by a different letter in the line are significantly different ($p>0.05$).

ZnO NPs; it was completely inhibited after adding 3 and 5% ZnO NPs. *Penicillium* spp. growth decreased by about 11 mm in the control case, while it decreased to about 4 mm after adding ZnO NPs. The amylase activity decreased from 15 mm in the control and to 9 mm after adding 1 and 2% ZnO NPs. 3 and 5% ZnO NPs reduced the amylase activity by about 8 and 4 mm, respectively. *B. spectabilis decreased to* about 15 mm in the control plates, which it decreased to 4 mm in all other treatments. Amylase activity of *B. spectabilis* was 20 mm in the control and antifungal case. It was about 25 mm after adding 1 and 2% ZnO NPs, while it decreased to 19 and 12 mm, respectively after adding 3 and 5% ZnO NPs.

Table 3 reported the effect of adding ZnO NPs on protease enzyme secreted by different fungal species (*Aspergillus* spp.). The fungal growth was about 9 mm in the control, 1, 2 and 3% ZnO NPs samples, while the growth decreased to 8 mm after adding the antifungal drug and 5% ZnO NPs. The protease activity was not detected in *Aspergillus* spp. plates. The growth of *Penicillium* spp. was about 12 mm in the control case, while it was about 4 mm in the case of antifungal drug and all concentrations of ZnO NPs. The protease activity decreased from 16 mm in the control case to 6 mm in 1 and 2% ZnO NPs; while the antifungal, 3 and 5% ZnO NPs completely inhibited it. *B. spectabilis* was about 10 in the control plates, which decreased to 4 mm in all other treatments. Protease activity of *Byssochlamys spectabilis* was 13 mm in the control, while it decreased to 8, 7, 6 and 0 mm in the antifungal drug, 1, 2, 3 and 5% ZnO NPs, respectively.

Table 3 shows the effect of adding ZnO NPs on lipase enzyme secreted by different fungal species (*Aspergillus* spp.). The fungal growth was about 13 mm in the control and 3% samples; while the growth decreased to 12 mm after adding antifungal drug, 1 and 2% samples. The lowest growth recorded was about 11 mm in 5% ZnO NPs concentration. The lipase activity was not detected at all in *Aspergillus* spp. plates. The growth of *Penicillium* spp. was about 14 mm in the control case, while it was about 4 mm in the case of antifungal drugs and all concentrations of ZnO NPs. The lipase activity decreased from 21 mm in the control case to 11, 10, 10, 9 and 6 mm in case of the antifungal drug, 1, 2, 3 and 5% ZnO NPs, respectively. *B. spectabilis* recorded about 16 mm in the control plates, which decreased to 4 mm in all other treatments. Lipase activity of *B. spectabilis* was 22 mm in the control and 20 mm with antifungal case; it was about 18, 18, 17 and 16 mm after adding 1, 2, 3 and 5% ZnO NPs, respectively.

Effect of adding ZnO NPs to different fish types packages on their shelf life

Table 4 and Figures 3 to 10 show the effect of adding different concentrations of ZnO NPs on the shelf life of different fish species samples in comparison with the control samples under refrigeration temperature. In the case of Asian seabass "*L. calcarifer*", bream "*P. pagrus*", rabbitfish "*S. rivulatus*", their shelf life extended from 3 days in the control sample to about 4, 5, 10, and 18 days after adding 1, 2, 3 and 5% ZnO NPs concentrations, respectively. The shelf life of saddle grouper "*Najil P. pessuliferus*" was extended

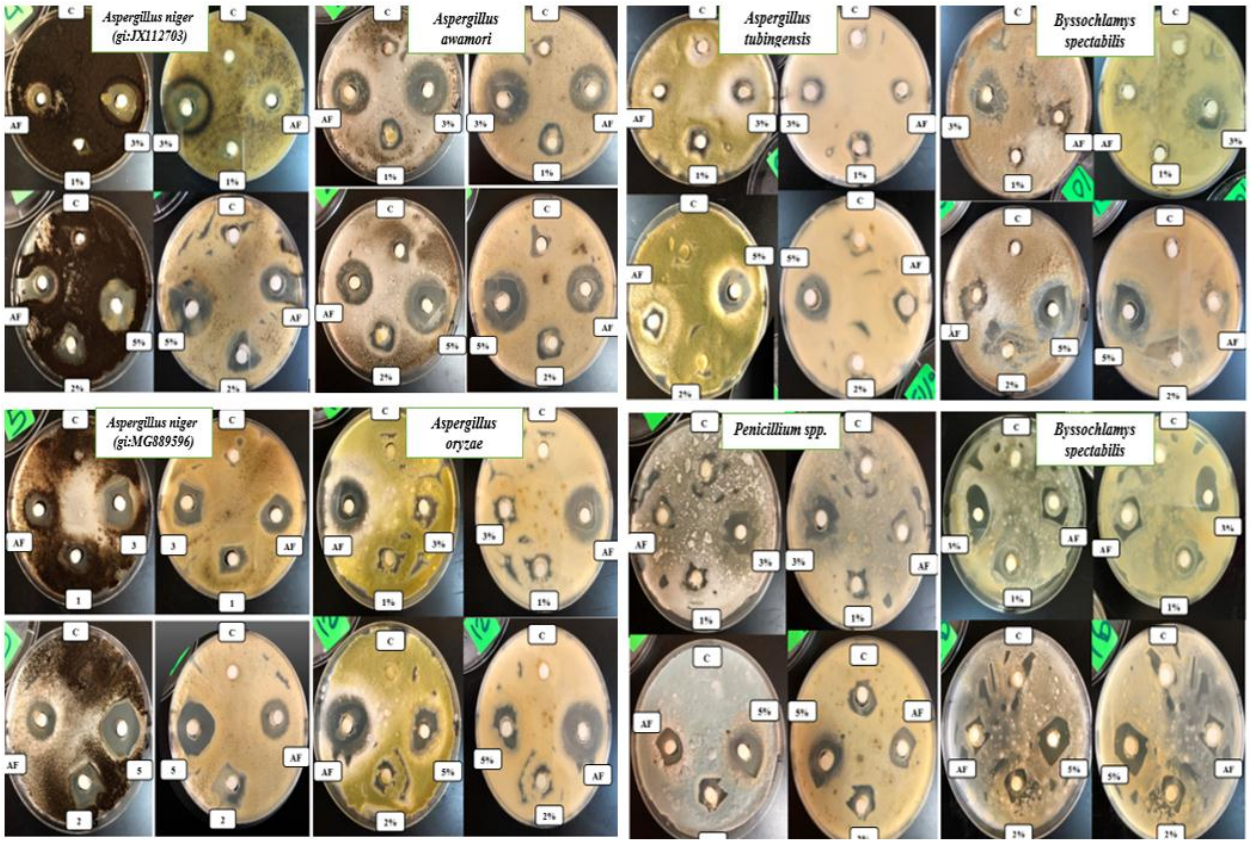

Figure 2. Antifungal effect of different ZnO NPs concentration against isolated fungus. C = Control, AF = Antifungal Concentration, 1% = 1% ZnO NPs Concentration, 2% = 2% ZnO NPs Concentration, 3% = 3% ZnO NPs Concentration, 5% = 5% ZnO NPs Concentration.

to about 2 days in the control and 1% samples, 3 days in 2% ZnO NPs concentration, 9 days and 15 days in 3 and 5% ZnO NPs, respectively.

It was observed that the saddle grouper "*Najil P. pessuliferus*" samples were the least affected by adding different concentrations of ZnO NPs, which got to 2 weeks in 5% concentration. The shelf life of almost all the fish types was affected positively by the addition of different concentrations of ZnO NPs.

DISCUSSION

The different fungus species are arranged as follows: 28% *A. niger* (gi:JX112703), 18.4% *A. oryzae,* 12.3% *A. awamori* and *Penicillium* spp., 9.4% *. tubingensis,* 8.2% *T. montevideense*, 8.0% *A. niger* (gi:MG889596), and 3.4% *B. spectabilis*. Most other studies reported that *A. niger* as the primary spoilage fungi affected different fish species. Park et al. (2014) found about 95.21% of fish fungal spoilage caused mainly by *A. niger*; about 33.3% *A. niger*, which is considered the most predominant fungal isolates (Odu and Ameweiye, 2003). On the other hand, lower results were reported by Samaha et al. (2015) who found fungal fish spoilage as follows: 24% *A. niger* and 48% *Penicillium* spp. Greco et al. (2015) found that *A. niger* predominated (57%) followed by *Penicillium* spp. (12.84%) in fish samples. While, Iqbal and Saleemi (2013) found fungal spoilage of fish in Punjab by *Aspergillus* spp. (78.5%) and *Penicillium* spp. (3.5%). Akwuobu et al. (2019) recorded also that the main fungal genera that contaminated fish sold in Makurdi were *Aspergillus* (28.6%), and *Penicillium* spp. (18.2%).

Aspergillus spp. causes a disease known as "aspergillosis", which appeared as cough, fever, breathlessness, or chest pain. The incidence of infection can be more common between immunosuppressed patients or those who suffer another pulmonary condition. Several species of *Aspergillus* spp., which often contaminate food, are *A. niger*, *A. oryzae*, *A. awamori*, and *A. tubingensis* (Singapurwa et al., 2018; Akwuobu et al., 2019). *Byssochlamys* spp. mostly occurs in compost, air, and different food items. Generally, this fungus accommodates heat above 85°C and microaerophilic condition results in mycotoxins production such as deoxynivalenol and vomitoxin (Casas-Junco et al., 2017).

Polluted aquaculturing has effect on the immunity of fish. This results in the rapid death of fish and makes

Table 3. The mean value of the effect of addition of ZnO NPs on different fungus species enzymes activity.

ZnO nano. Concentration	Amylase Enzyme Activity (mm)						Protease Enzyme Activity (mm)						Lipase Enzyme Activity (mm)					
	Aspergillus spp.		*Penicillium* spp.		*Byssochlamys spectabilis*		*Aspergillus* spp.		*Penicillium* spp.		*Byssochlamys spectabilis*		*Aspergillus* spp.		*Penicillium* spp.		*Byssochlamys spectabilis*	
	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z	G	C.Z
Control	14	19	11	15	15	20	9	0.0	12	16	10	13	13	0.0	14	21	16	22
Antifungal	4	8	4	9	4	20	8	0.0	4	0.0	4	8	12	0.0	4	11	4	20
1%	4	7	4	9	4	25	9	0.0	4	6	4	7	12	0.0	4	10	4	18
2%	4	7	4	9	4	25	9	0.0	4	6	4	7	12	0.0	4	10	4	18
3%	4	0.0	4	8	4	19	9	0.0	4	0.0	4	6	13	0.0	4	9	4	17
5%	4	0.0	4	4	4	12	8	0.0	4	0.0	4	0.0	11	0.0	4	6	4	16

Inoculums disc 4 mm; G: Growth, C.Z: Clear zone.

fish get spoiled rapidly after catch. This is due to high fungal and bacterial opportunist including the lower nutritive value of fish caused by stress syndrome from the different aquatic pollutants (Bukola and Zaid, 2015).

ZnO NPs have antimicrobial effect by disintegrating the cell wall of microbes via lysis. The morphology of the micro fungus changes after treatment. ZnO NPs have potent antifungal effect (Shen et al., 2015). The high fungicidal effect of ZnO NPs in this study may be due to their small size (Rajiv et al., 2013; Jeong et al., 2014). ZnO NPs may affect the permeability membrane of the microbial cells, releasing the membrane proteins and lipids. This results in the death of the microbial cell (Padalia and Chanda, 2017). The properties of ZnO NPs used for the development of fungicides have become an urgent issue in medicine and microbial food control (Kairyte et al., 2013). Padalia and Chanda (2017) reported that ZnO NPs have very effective antifungal activity; they have better effect than standard antibiotic amphotericin B. Rajeshkumar (2019) had similar results that fungal inhibition effect correlated inversely with size and concentration of ZnO NPs and concluded that ZnO NPs acted very impressively against the fungal pathogens.

Amylases are the most vital extracellular enzymes which hydrolyze the molecules of starch resulting in diverse products such as dextrin and composed of glucose unit (Gupta et al., 2003). Amylase can be obtained from several fungi. Several studies reported that fungal origin amylases are more stable (Sanghvi et al. 2011). Malaikozhundan et al. (2020) reported almost similar results that ZnO NPs greatly inhibited the microbial amylase activity to about 25 and 25 $\mu g/mL^{-1}$; protease activity was inhibited and lipases activity was inhibited to 25 $\mu g/mL^{-1}$. Lower results were reported by Namasivayam et al. (2016) where microbial enzyme activity was inhibited by metal nanoparticles, which revealed a broad surface plasmon peak presented at 430 nm. Nanoparticles are extremely stable for many months after the reaction. The enzyme was not inhibited at all by the tested concentrations. As in the control samples, there was no significant difference in the enzyme activity ($P>0.05$), which revealed 6.02, 6.18, 6.23, 6.53, 6.88 µ/ml and 4.52, 4.50, 4.68, 4.72 µ/ml, respectively. Also, Verma and Verma, (2018) recorded amylase produced by the fungi as follows: *Penicillium* spp. had the highest amylase production 1(0.93 cm) followed by *Aspergillus* spp. (0.6 cm); Kathiresan and Manivannan (2006) recorded *Penicillium* spp. produced maximum amylase (136 U/ml). Sharma and Shukla (2008) reported that maximum amylase was produced by *Aspergillus* spp. (185 U/ml).

Freshness is one of the parameters used to judge the quality of fish and can be determined by using the sensory analysis method. Sensory analysis is simple, fast, and provides immediate quality information about the tested fish products. The sensory characteristics of fish are obvious to the fish consumers and are essential for the consumption of fish and its products (Reineccius, 1991). Results observed that the saddle grouper "*Najil P. pessuliferus*" samples were the least affected after adding ZnO NPs (different concentrations); it got to 2 weeks after adding 5% ZnO NPs. The shelf life of almost all the types of fish was affected positively by the addition of different concentrations of ZnO NPs.

Researchers reported that ZnO NPs molecules must penetrate or be in contact with microbial cells to perform their inhibitory activity (Mohd et al., 2019). Similar results of ZnO NPs effect on the cell

Table 4. Effect of addition of ZnO NPs to different fish types package on their shelf life.

Types of fish	Control (day)	1% (day)	2% (day)	3% (day)	5% (day)
Asian seabass "*Lates calcarifer*"	3	4	5	10	18
Bream "*Pagrus pagrus*"	3	4	5	10	18
Rabbitfish "*Siganus rivulatus*"	3	4	5	10	18
Spangled emperor "*Lethrinus nebulosus*"	2	3	5	10	18
Gilthead seabream "*Sparus aurata*"	2	3	4	10	18
Mackerel fish "*Scomberomorous commerson*"	2	3	4	10	18
Red mullet "*Mullussurmuletus*"	2	3	4	10	18
Saddle Grouper "*Najil P. pessuliferus*"	2	2	3	9	15

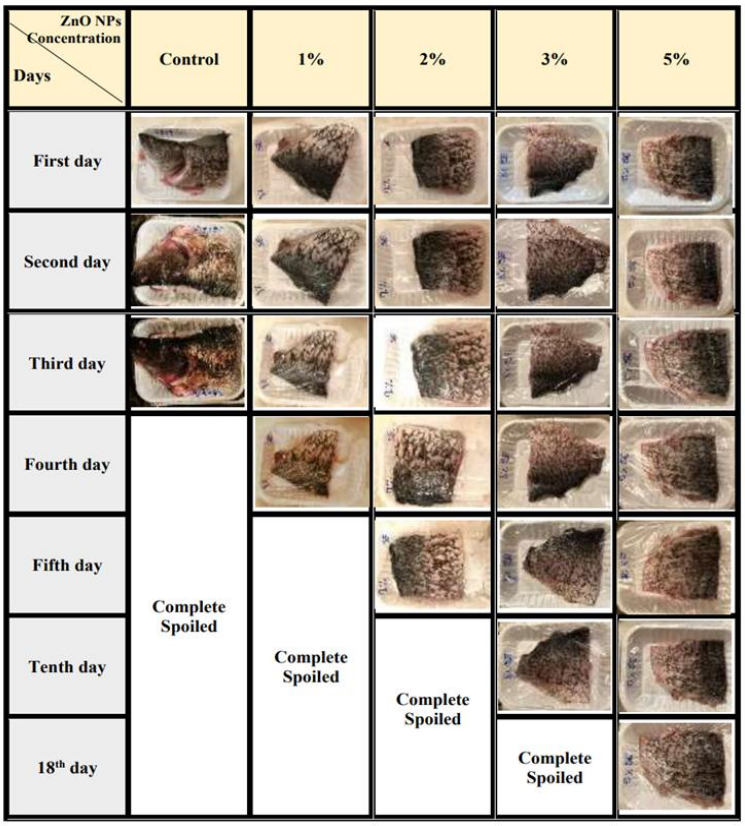

Figure 3. Effect of addition of ZnO NPs on "Asian seabass" fish sensory characters.

wall of microbes were given by Shawky et al. (2014). They noticed that the antimicrobial effect of ZnO NPs occurred in 2 ways: firstly, H_2O_2 was formed on ZnO NPs surface due to the hydrogen bond between the hydroxyl group of fungi cellulose molecules and the atom of oxygen of ZnO NPs, resulting in the inhibition of the fungal growth; secondly Zn^{2+} was released leading to cell membrane damage and interaction with intracellular contents (Moraru et al., 2003).

Similar antifungal activities of ZnO NPs inhibited different fungal growth. They increased with higher contraction of ZnO NPs, especially 200 and 300 ug/ml; they had about 7 to 15 mm inhibition diameter (Hassan et al., 2014). This inhibition effect was clearer against *Aspergillus* spp. (1.013296 µg/ml) and fluconazole which were 0.001-0.56 and 0.062-128 µg/ml, respectively. ZnO NPs changed the microbial cell structure of the fungi including the cell membrane, leading to leakage of the cytoplasm and distribution of the fungal cells. ZnO NPs can inhibit conidial development and damage conidiophores or hyphae (He et al., 2011).

The daily dietary intake of zinc for adults is about

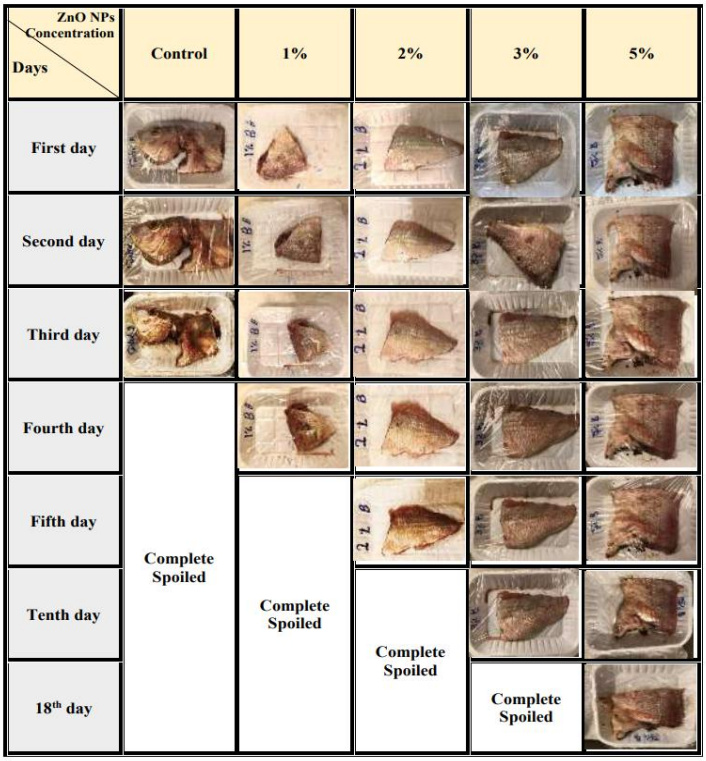

Figure 4. Effect of addition of ZnO NPs on “Bream” fish sensory characters.

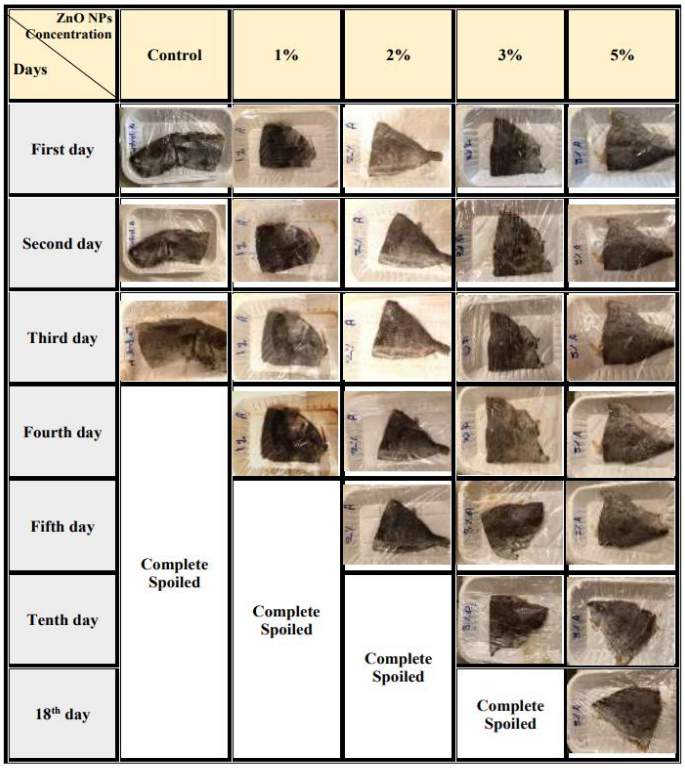

Figure 5. Effect of addition of ZnO NPs on “Rabbitfish” fish sensory characters.

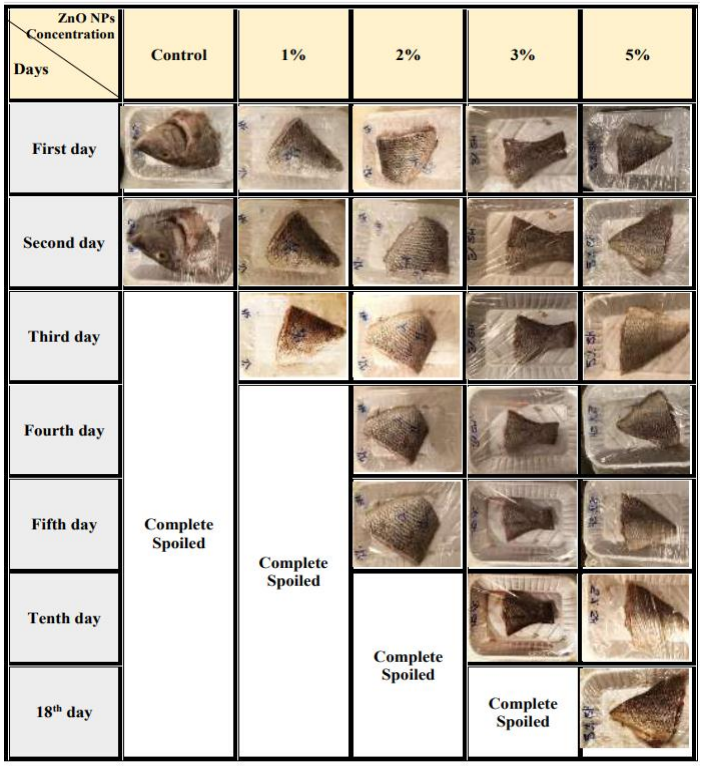

Figure 6. Effect of addition of ZnO NPs on "Spangled emperor" fish sensory characters.

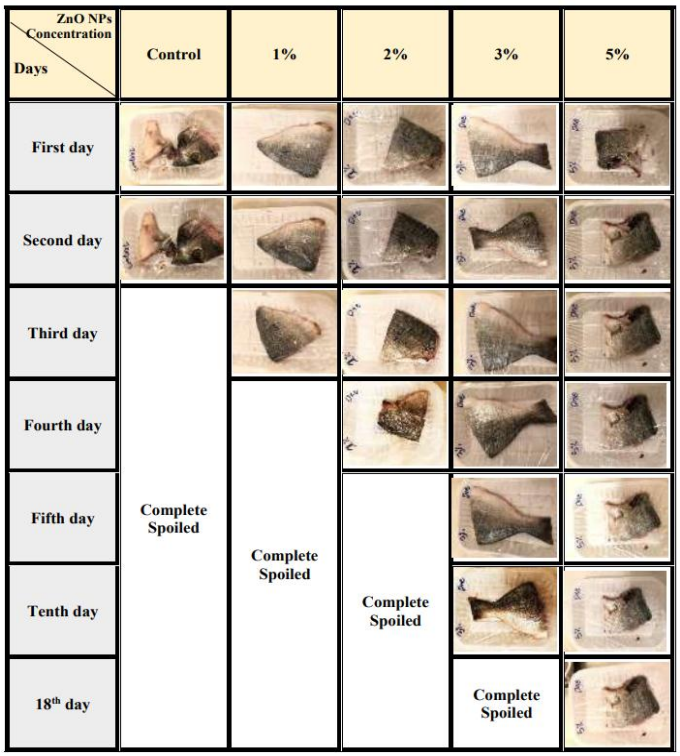

Figure 7. Effect of addition of ZnO NPs on "Gilthead Seabream" fish sensory characters.

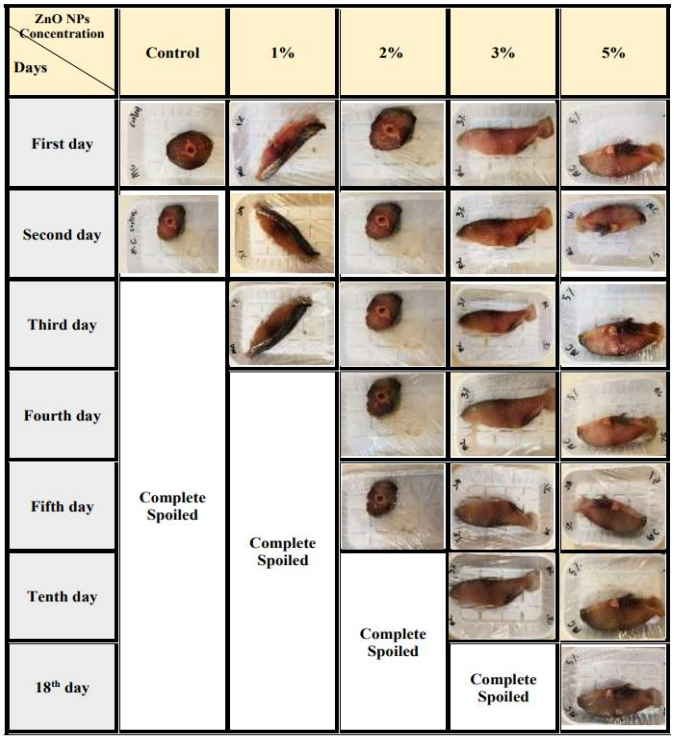

Figure 8. Effect of addition of ZnO NPs on “Mackerel” fish sensory characters.

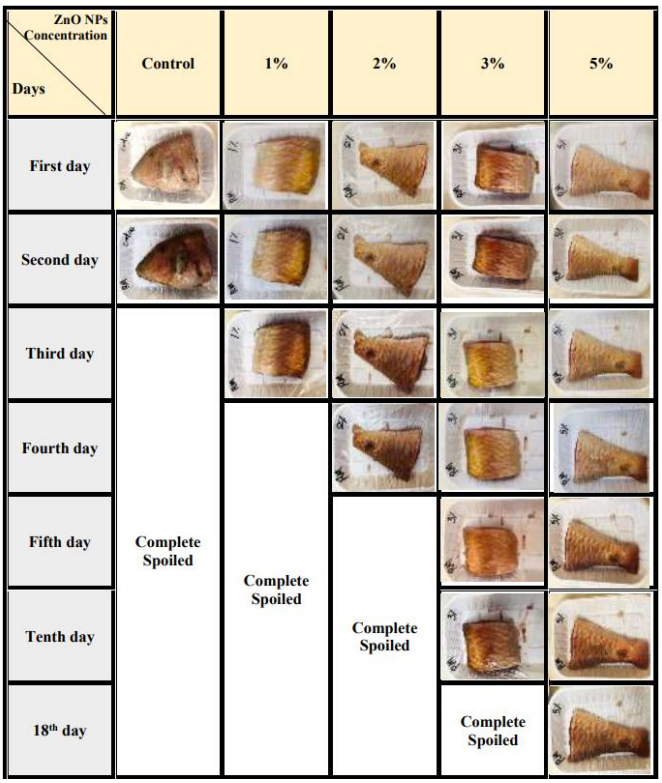

Figure 9. Effect of addition of ZnO NPs on “Red mullet” fish sensory characters.

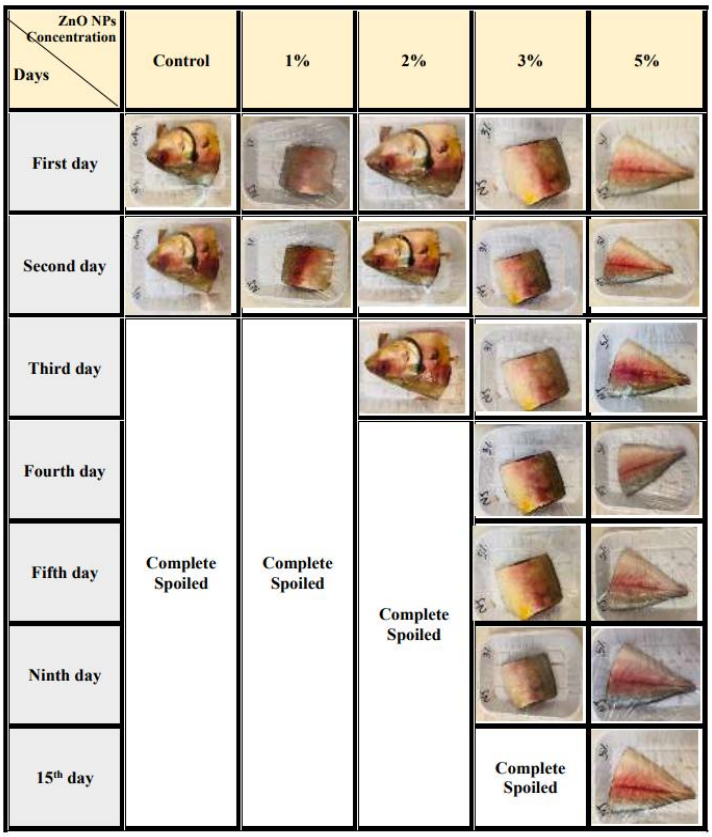

Figure 10. Effect of addition of ZnO NPs on "Saddle Grouper" fish sensory characters.

40 mg, which is equivalent to 143 ml of liquid egg daily intake of 0.28 mg of ZnO per ml (Hassan et al., 2014). According to the National Research Council, the recommended dietary allowances (RDA) for humans are about 15,000 and 12,000 mg/different for healthy men and women (Yilmaz and Aksoy, 2006). Sensory attributes of fish including their juiciness, appearance or tenderness, odor, flavor, aftertaste, and acceptability scores were significantly decreased ($P<0.05$) with prolonged storage time (Sallam, 2007).

Conclusion

It is concluded that there is a gradual increase in the fungal growth with increased ZnO NPs concentration in all tested fish species, especially at 3 and 5% concentration. It is recommended to use 3% concentration of ZnO NPs to improve the safety of fish and prolong their shelf life up to 15 days.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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