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Synthesis and characterization of novel compounds and determining their antifungal properties against rainbow trout pathogen, *Saprolegnia* sp. *in vitro*

Shokouh Saljoghi Z.[1*]; Farhadian O.[1]; Ramezanian N. [2]; Mehraban Sangatash M.[3]

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Abstract

Saprolegnia growth on trout eggs is a problem frequently encountered in fish hatcheries worldwide. In the present study silver nanoparticles (Ag NPs) were effectively grafted on the zeolite and bentonite framework using the chemical reduction method. Zeolite and bentonite, silver nitrate ($AgNO_3$), and sodium borohydride ($NaBH_4$) were used as inorganic solid supports, a silver precursor, and a chemical reduction agent, respectively. Silver ions were introduced into the porous zeolite through an ion-exchange path, in the external and interlamellar space of bentonite at room temperature. $AgNO_3$ and $NaBH_4$ were used as a silver precursor and reducing agent, respectively. The Ag/zeolite and Ag/bentonite nano compounds were characterized by Field Emission Scanning Electron Microscopy (FESEM), Fourier Transform Infrared (FTIR), X-ray powder diffraction (XRD), Energy-dispersive X-Ray spectroscopy (EDX) analysis. The results showed that Ag NPs form a spherical shape with uniform homogeneity in the particle size. The antifungal activity of Ag NPs in zeolites and bentonite were investigated against *Saprolegnia* sp. with different loads of nano composites and exposure time. All Ag/zeolite NCs and Ag/bentonite NCs were found to have antifungal activities. These results revealed that Ag NPs could be used as effective growth inhibitors in different biological systems, for instance in aquatic animal hatchery facilities.

Keywords: Antifungal properties, Saprolegnia, Nano silver, Zeolite, Bentonite, Saprolegnia growth index

1-Department of Natural Resources, Isfahan University of Technology, Isfahan, Iran

2-Department of Chemistry, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

3-Food Quality and Safety Research Group, Food Science and Technology Research Institute, ACECR, Mashhad, Iran

*Corresponding author's Email: zoheirsaljoghi@gmail.com

Introduction

Fish diseases outbreaks are among the most important problems and challenges of aquaculture development in the world. The main cause of economic losses in aquaculture is diseased fish, and oomycete (water mold) infections rank second to bacterial diseases (Meyer, 1991). Infections by oomycete including Saprolegnia and Aphanomyces species are responsible for infections in aquaculture (Humphrey, 1893). Oomycetes such as Saprolegniales, including the Saprolegnia, Achlya, and Aphanomyces species, have been found responsible for fish infections in aquaculture, fish farms, and hobby fish tanks (Willoughby and Pickering 1977; Baldauf *et al*., 2000). The genus *Saprolegnia* is responsible for significant fungal infections involving both dead and live fish as well as eggs in the aquaculture industry (Pickering and Willoughby, 1982; Noga, 1993; Ke *et al*., 2009; Shahbazian *et al*., 2010). Saprolegnia infections are endemic to all freshwater fish around the world and are partly responsible for the decline of natural populations of salmonids and other freshwater finfish species (Van West, 2006).

The efficacy of several antifungal compounds against growth of Saprolegnia has been studied. Antifungal agents are essential for the maintenance of healthy stocks of fish and their eggs in intensive aquaculture operations. The efficacy of antifungal compounds in the presence of excretory products was noted by Willoughby and Roberts (1992), who found that *Saprolegnia parasitica* zoospores in pure water were sensitive to a combination of penicillin and streptomycin. The search for alternative methods and compounds for the control of oomycete outbreaks has increased, and the effectiveness of many potential oomyceticides has been tested using *in vitro* screening methods.

Bailey and Jeffrey (1989) examined more than 200 compounds to determine their inhibitory activity against potentially pathogenic oomycetes, such as flagellate and *S. hypogyna*. Minimum inhibitory concentrations for each compound were also reported. The use of chemicals against fungal diseases in aquaculture is very common. Malachite green was successfully used for treating all infectious stages of saprolegnia in fish aquaculture facilities (Foster and Woodbury, 1936; Olah and Farkas, 1978; Bailey, 1984; Alderman, 1985; Willoughby and Roberts, 1992). However, since the turn of the century, the use of malachite green has been banned worldwide due to its carcinogenic properties. Several studies revealed the toxicological effect (Bills *et al*., 1977; Meyer and Jorgenson, 1983) and potentially mutagenic properties (Clemmensen *et al*., 1984; Fernandes *et al*., 1991; Srivastava *et al*., 2004) of malachite green. Formalin, aqueous solution containing formaldehyde, has been widely used to control oomycete infection in aquaculture (Cline and Post, 1972; Walser and Phelps, 1993). Similar to malachite green such concerns make it unlikely that formalin will be considered for widespread use in the

short term and, in fact, it is likely to be banned in the future (Abd El-Gawad *et al*., 2015).

Hydrogen peroxide is also effective against a variety of organisms, namely bacteria, yeasts, viruses, fungal and oomycete spores, and is potentially an important oomyceticide for fish culture, with negligible environmental impacts (Schreier *et al*., 1996). Copper containing compounds are usually effective as fungistatic agents when used at high concentrations (Somers, 1967; Richmond, 1977). Other chemical compounds like bronopol (Pyceze©) (Pottinger and Day, 1999; Branson, 2002), iodophores (Muzzarelli *et al*., 2001), ozone (Forneris *et al*., 2003) along with non-chemical treatment methods including ultraviolet irradiation (Rahkonen and Koski, 2002), probiotics (Hatai and Willoughb, 1988; Petersen *et al*., 1994; Hussein and Hatai, 2001; Lategan and Gibson, 2003; Lategan *et al*., 2004), immunization and vaccines (Fregeneda-Grandes and Olesen, 2007) are used.

Various inorganic silver containing antibacterial and antifungal materials have been developed and some products commercially available (Yamamoto *et al*., 1996; Hansel *et al*., 1998; Xu *et al*., 2011). Various antibacterial heavy metals are known to have a wide and relatively safe antibacterial spectrum (Cho *et al*., 2005; Oya, 1996). Silver, copper, zinc, and other antibacterial metals, when bound to inorganic carriers designed for slow release are far superior as inorganic disinfectants in terms of safety, duration of action and resistance to heat when compared with conventional organic agents (Top and Ulkü, 2004). For this reason, the development of inorganic bactericides and disinfectants composed of silver bound to various inorganic carriers for application in domestic and industrial fields is receiving extensive attention (Iwata, 1996). Accordingly, due to the lack of information on the antimicrobial effects of nano silver zeolite and specially nano silver bentonite on fish pathogens, the present study has been designed to examine the *in vitro* inhibitory properties of nano silver inorganic materials against saprolegnia.

Materials and methods

Chemicals

Reagents are of analytical grades and used as received without further purification. $AgNO_3$ (Merck, Germany, 99.98%) was used as the silver precursor. Bentonite, natural clay, was obtained from Tabas, Iran, and used without any further purification. The natural zeolitic tuff (clinoptilolite), provided from Semnan, Iran, $NaBH_4$ (98.5%) and HNO3 (90%) used as reducing and digestion agents were obtained from Merck (Germany). All aqueous solutions were prepared in double distilled water.

Synthesis of Ag zeolite and bentonite nanocomposites by using $NaBH_4$

To synthesis Ag/Bentonite NCs and Ag/Zeolite NCs the silver contents of the samples, 5.0 g Ag $100g^{-1}$ zeolite and bentonite was used. Constant amounts of sodium zeolite and bentonite were suspended in 0.001 M $AgNO_3$ solution

and stirred for 1 week at room temperature to obtain the $AgNO_3$/Bentonite and Zeolite suspension and completed cation exchange. A freshly prepared $NaBH_4$ (4×10^{-2} M) solution was then added to the suspensions under continuous stirring to reach a constant $AgNO_3/NaBH_4$ molar ratio (1:4). Afterwards, stirring continued for an extra hour. The suspensions of Ag/MMT and Ag/ZEO NCs obtained were then centrifuged at 15,000 rpm for 40 minutes, and the precipitates were washed several times using distilled water in order to remove the silver ion residue, and dried overnight at 100°C (Figs. 1 and 2).

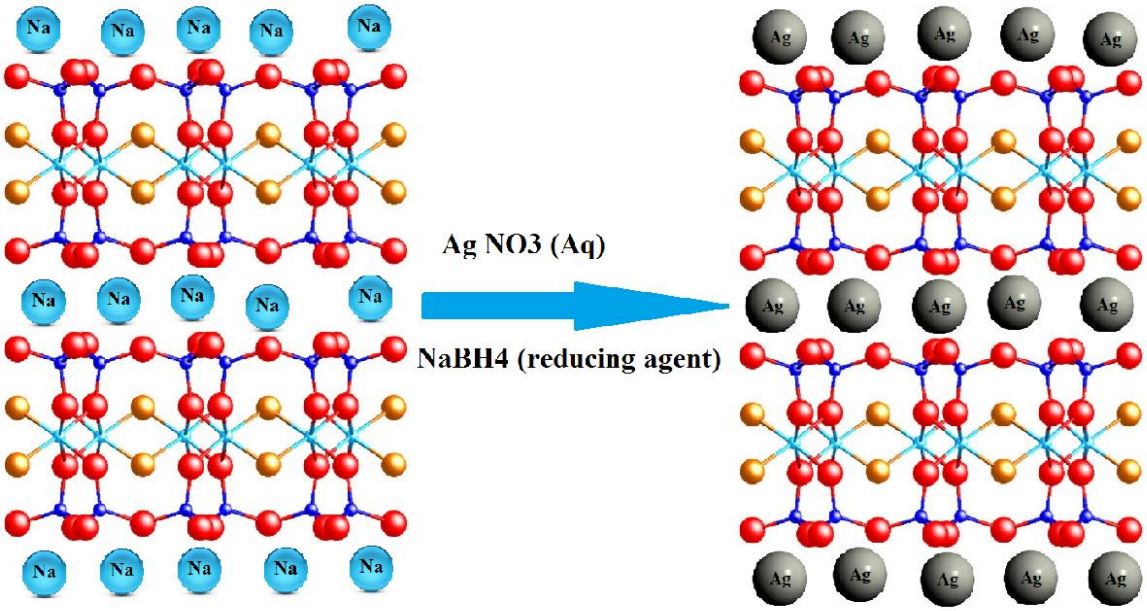

Figure 1: Schematic diagram of synthesis of Ag/bentonite NCs.

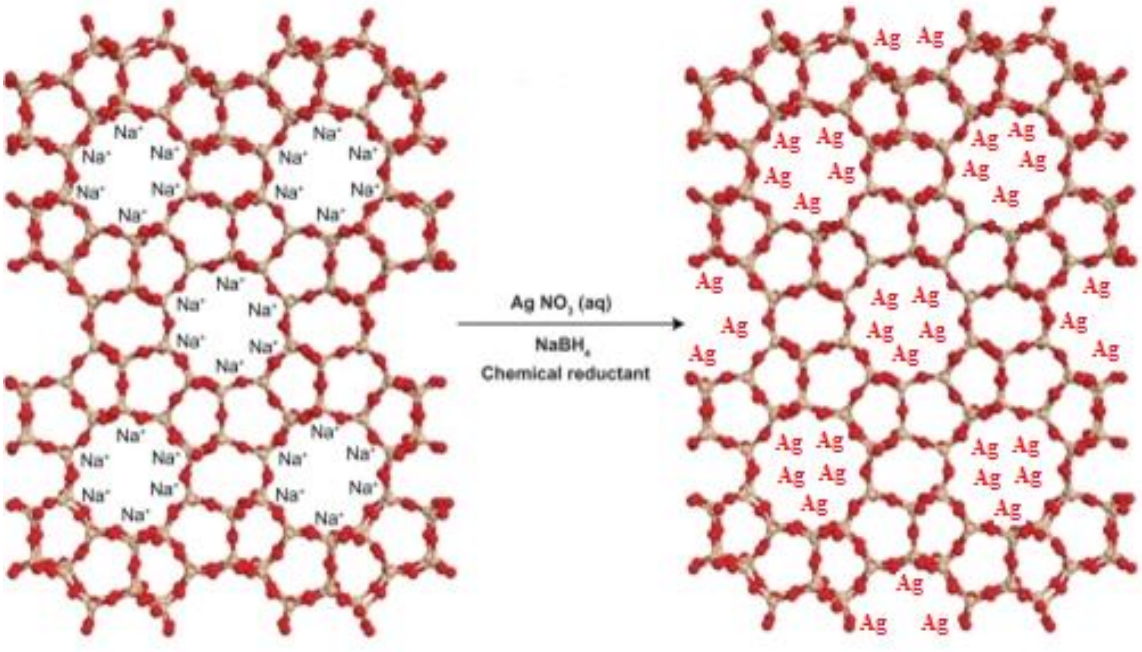

Figure 2: Schematic diagram of Synthesis of Ag/zeolite NCs.

Structural characterization methods and instruments

FTIR spectra of two types of Ag nanocomposites were recorded with KBr discs in the range of 4000–400 cm^{-1} on Nicolet AVATAR360 Fourier Transfer Infrared. FESEM images were taken by MIRA3 TESCAN (Czech).

Antifungal activity assays

A pure stock of fish *Saprolegnia* sp. previously isolated from rainbow trout eggs and characterized by the Department of Aquatic Animal Health, Veterinary Medicine Faculty, University of Tehran was used. The *Saprolegnia* sp. was cultured on a sabouraud dextrose agar (SDA) and stored at 4 °C until use. The antifungal activity of the Ag/zeolite and Ag/bentonite NCs were evaluated by determining the minimum inhibitory concentrations (MICs) using the agar dilution method (Bailey, 1983). The agar dilution method has been recommended as a standard *in vitro* antifungal susceptibility tests by the National Committee for Clinical Laboratory Standards (Dong, 2003). In brief, agar plugs containing fungal hyphae of saprolegnia (5 mm in diameter) removed from the edge of the pure stock were placed in the middle of depression spots on plates containing various concentrations of nanocomposites and incubated at 22 °C. The maximal growth of saprolegnia (colony diameter) was determined after 24, 48, 72, 96 and 120 h, respectively. To determine the inhibitory concentration range, ten test concentrations of two studied nanocomposites 6.66, 13.33, 20, 26.66, 33.33, 40, 46.66, 53.33, 60 and 66.66 mg L^{-1} plus a negative control without nanocomposites were prepared on plates in triplicate. The growth of saprolegnia in the presence of the nanocomposites was compared to that of the control. To evaluate saprolegnia growth, the area over which the saprolegnia hyphae grew in the petri dishes was determined and compared to that of negative control (equation 1). The present study was conducted as a factorial experiment in a completely randomized design. Ten treatments and one control group, consisting of three replications for each group were used. Results were obtained by taking the mean of the three replicates in each group. The data obtained were analyzed by using SPSS software and Microsoft EXCEL. Comparison of mean results was performed using Duncan's multiple range test (DMRT).

$$\text{Saprolegnia growth index (\%)} = 100 \times \frac{\text{Growth of saprolegnia in the presence of nanocomposites}}{\text{Growth of saprolegnia on the control plates}}$$

Results

FTIR analysis of zeolite and Ag/ zeolite NCs

Fig. 3 showed FT-IR spectra of the silicate host structure of zeolite and Ag/zeolite NCs. The FT-IR spectrum of zeolite showed vibration bands at 3450 cm^{-1} for O–H stretching due to the H_2O interporous structure of O–H stretching (H bonding), and at 1629 cm^{-1} for H–

O–H bending. The positions of the vibrational bands at 797–468 cm^{-1} corresponding to Si–O and other interstructure bands remained unchanged; a strong band at 1063 cm^{-1} was associated with the stretching vibration of Si–O, which usually suggests a three-dimensional silica phase. The band at 797 cm^{-1} was assigned to Al–O, and the position bands at 468 cm^{-1} were allocated to the Si–O–Si bending vibration. The FT-IR spectra indicated the rigidity of silicate structure and nonband chemical interaction between the zeolite structure and Ag NPs in Ag/zeolite NCs. The interactions between the zeolite and Ag NPs were associated with the peak at 3448 cm^{-1}. A broad peak was due to the presence of van der Waals interactions between the hydroxyl groups in the zeolite structure related to H_2O and the partial positive charge on the Ag NPs surface.

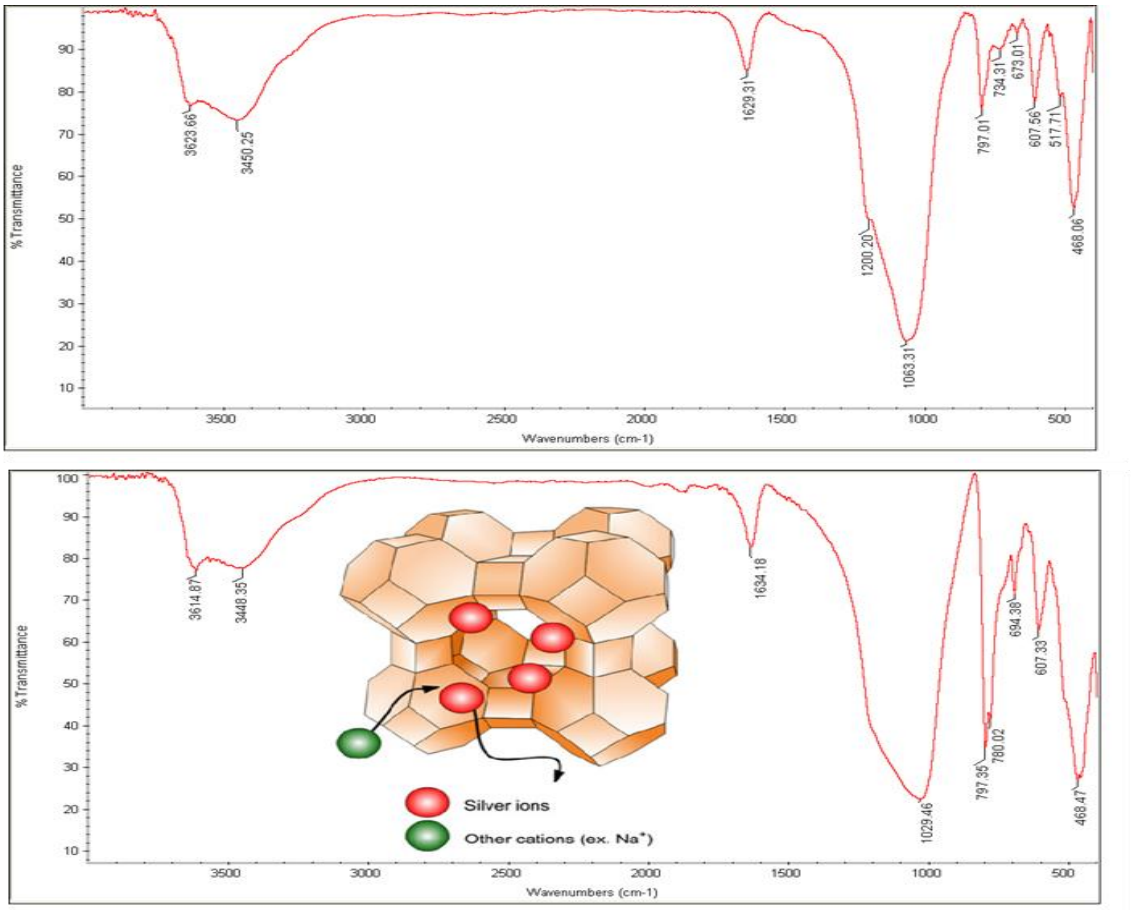

Figure 3: Fourier transform infrared spectra of zeolite and silver/zeolite nanocomposites.

FTIR analysis of zeolite and Ag/ zeolite NCs

The FT-IR spectrum of raw bentonite (Fig. 4) showed the vibration bands at 3630 cm^{-1} for O-H stretching, 3444 cm^{-1} due to the inter-layered O-H stretching (H bonding), at 1646 cm^{-1} for H-O-H bending, 1082, 1039, and 918 cm^{-1} for Si-O stretching, 624 cm^{-1} for Al-OH, 918 cm^{-1} due to (Al, Mg)-OH vibration modes, and 522 and 469 cm^{-1} for Si-O bending (Alemdar *et al*.,

2005). As shown in Fig. 2, there were not many changes in the spectra of Ag/BEN NCs compared with raw bentonite. The FT-IR spectra demonstrated the inflexibility of silicate layers and non-bond chemical interface between the silicate layers and Ag NPs in Ag/ BEN NCs (Shameli *et al*., 2010).

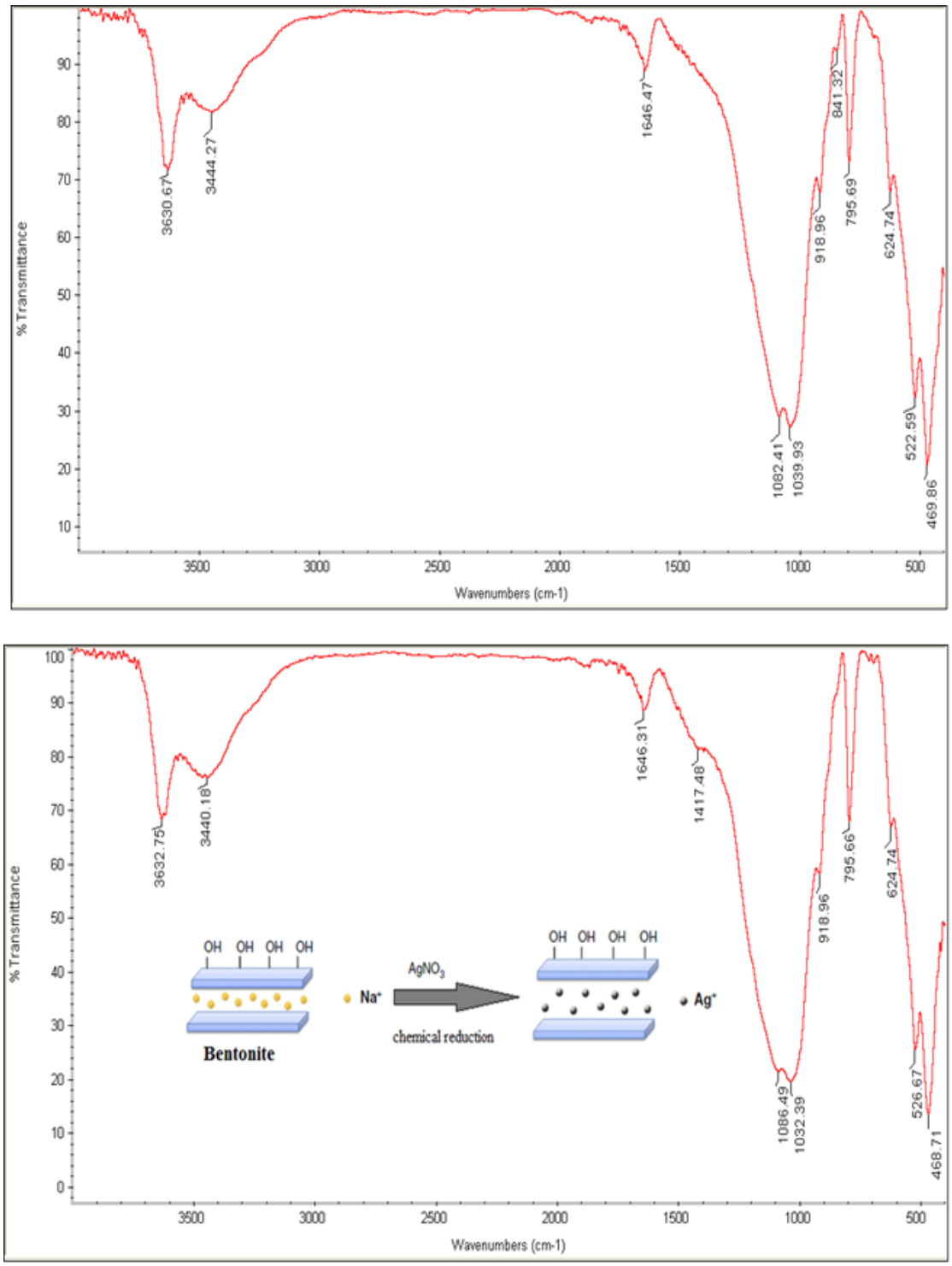

Figure 4: Fourier transform infrared spectra of bentonite and silver/bentonite nanocomposites.

XRD analysis of zeolite and nano Ag zeolite

To determine the crystalline phase of the nanosilver-coated zeolite, X-ray diffraction (XRD) was performed using an Explorer (Italy, GNR company), (Tube: Cu ka, Voltage: 40 kV, Current: 30 mA, detector type: Dectris (fast strip)). An X-ray diffract gram of the AgNP-coated and uncoated natural zeolite was shown in Fig. 5. The XRD results indicated that clinoptilolite was the main phase present in the zeolite samples in this study. As seen in figure. 6, there was distinct difference in the XRD patterns of the AgNP-coated and uncoated zeolite samples.

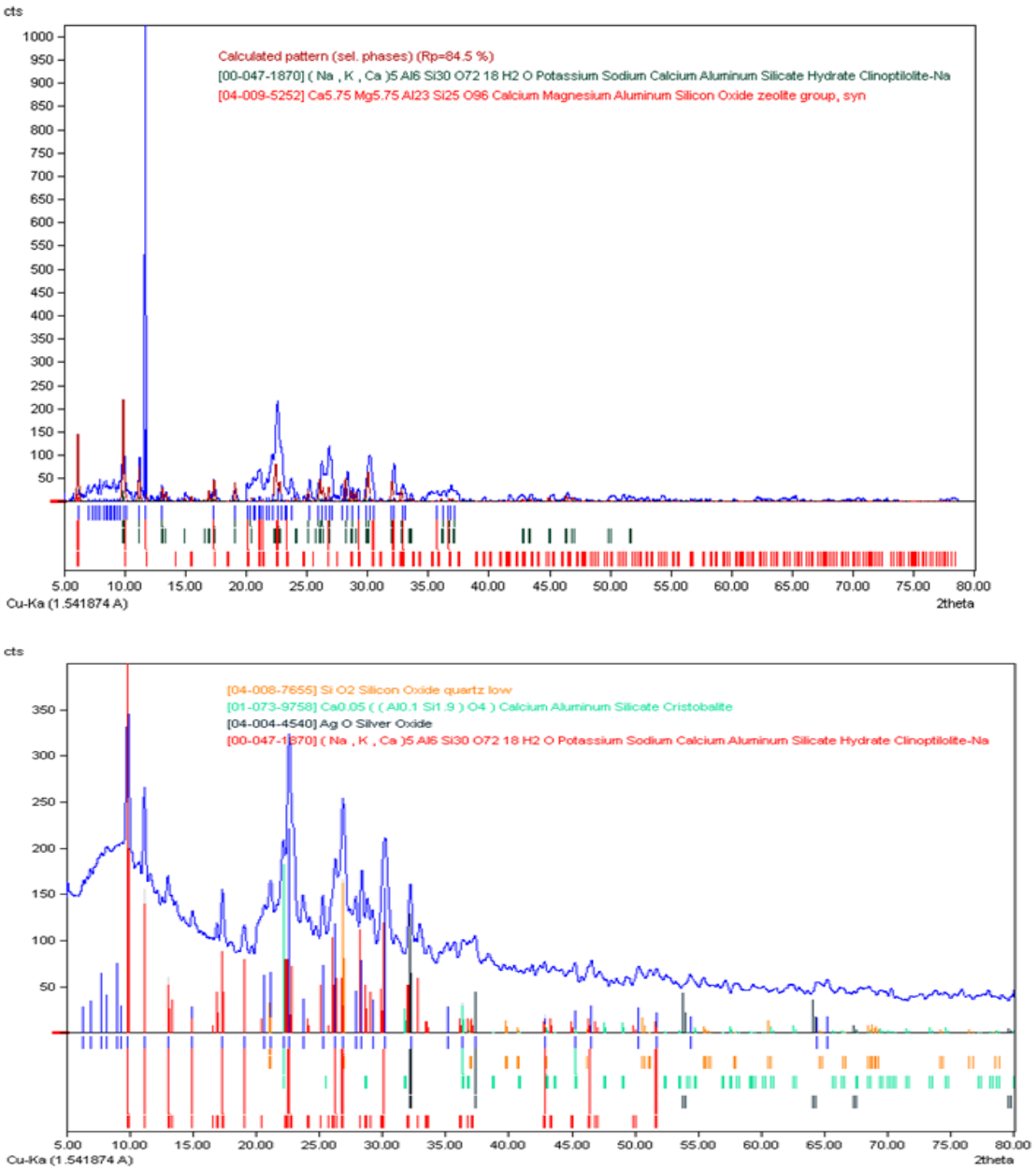

Figure 5: XRD analysis of raw zeolite (above) and silver nanoparticle-coated (below) zeolite.

XRD analysis of bentonite and nano Ag bentonite

The results of the characterization of bentonite by X Ray Diffraction showed that bentonite generally composed of four types of mineral; Sodium aluminum oxid zeolite, Cristobalite Quartz and Beidellite. The major clay phase present was Beidellite. After nanoparticles of Ag were deposited on the clay, new peaks were observed corresponding to the formation Ag in the composite. XRD analysis for nanoAg bentonite showed that nano Ag particles were incorporated in bentonite structure (Fig. 6).

Figure 6: XRD analysis of raw bentonite (above) and silver nanoparticle-coated (below) bentonite.

Morphology of zeolite and Ag/zeolite NCs by FESEM

Fig. 7 presented the FE SEM images of the zeolite and Ag/zeolite NCs. The morphology of zeolite demonstrated a surface with a cubic shape, a typical structure for zeolite (Fig. 7 A). The exterior morphology for Ag/zeolite NCs (7 B), also shows cubic forms without significant morphological differences. Furthermore, the external surfaces of Ag/zeolite NCs gradually become shinier due to the presence and increase of Ag NPs contents (Fig. 7 B).

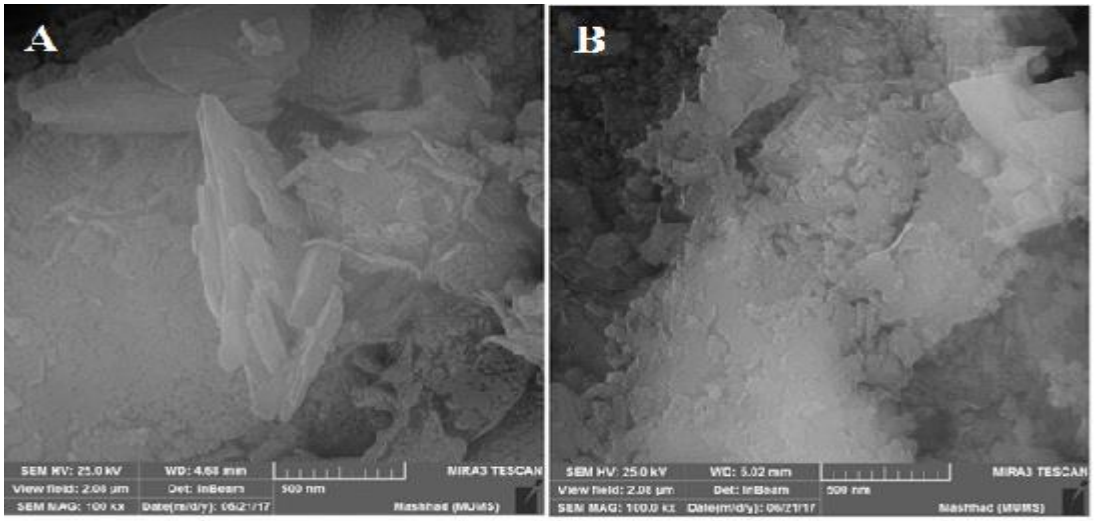

Figure 7: SEM micrographs of zeolite and silver/zeolite nanocomposites.

Morphology of bentonite and Ag/bentonite NCs by FESEM

SEM images of the raw bentonite and Ag/bentonite NCs are presented in Fig. 8. The surface morphology of bentonite demonstrated a layered surface with some large flakes, a typical structure of bentonite (Fig. 8 A). The exterior morphology for Ag/bentonite NCs, showed layered surfaces with large flakes. However, significant morphological differences were observed (Fig. 8 B).

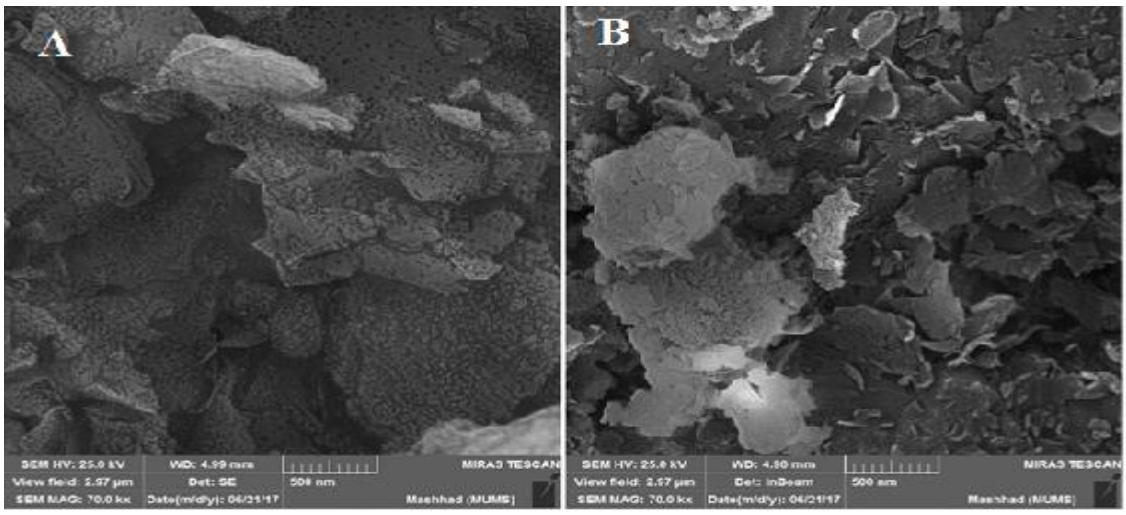

Figure 8: SEM micrographs of bentonite and silver/bentonite nanocomposites.

EDX analysis of raw and nano Ag containing zeolite and bentonite

The successful immobilization of Ag NPs was also confirmed by EDX analysis (Figs. 9 and 10), which showed the presence of Ag along with oxygen, aluminum and silica elements. Concerning the results of XRD and SEM of the Ag/zeolite and Ag/bentonite nanocomposite, Ag NPs were fabricated and immobilized on the zeolite surface and bentonite framework.

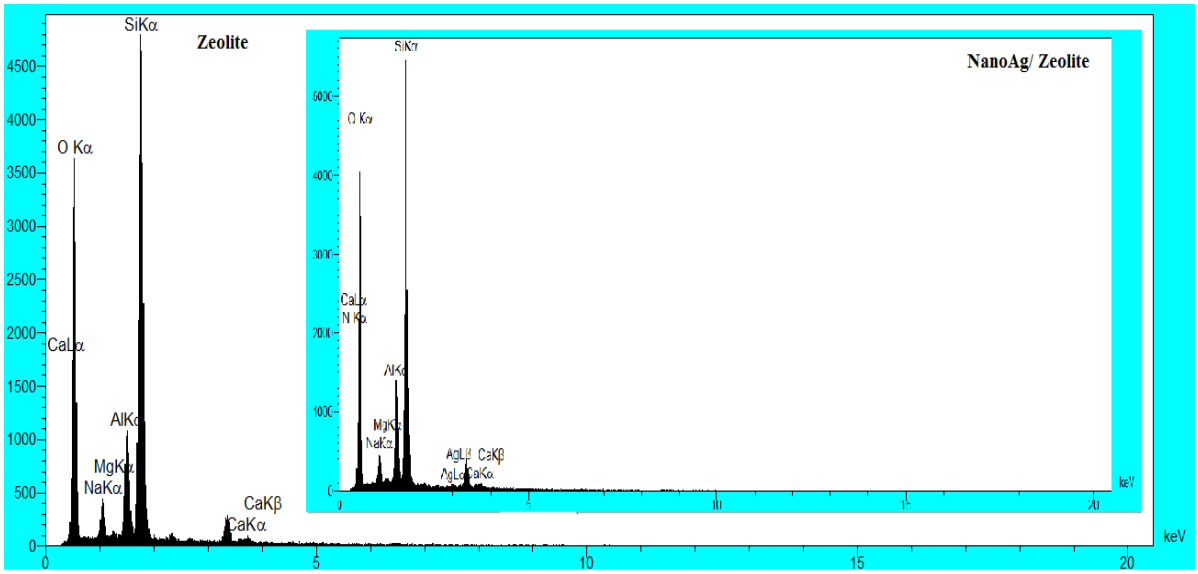

Figure 9: EDX analysis of natural and nano Ag zeolite.

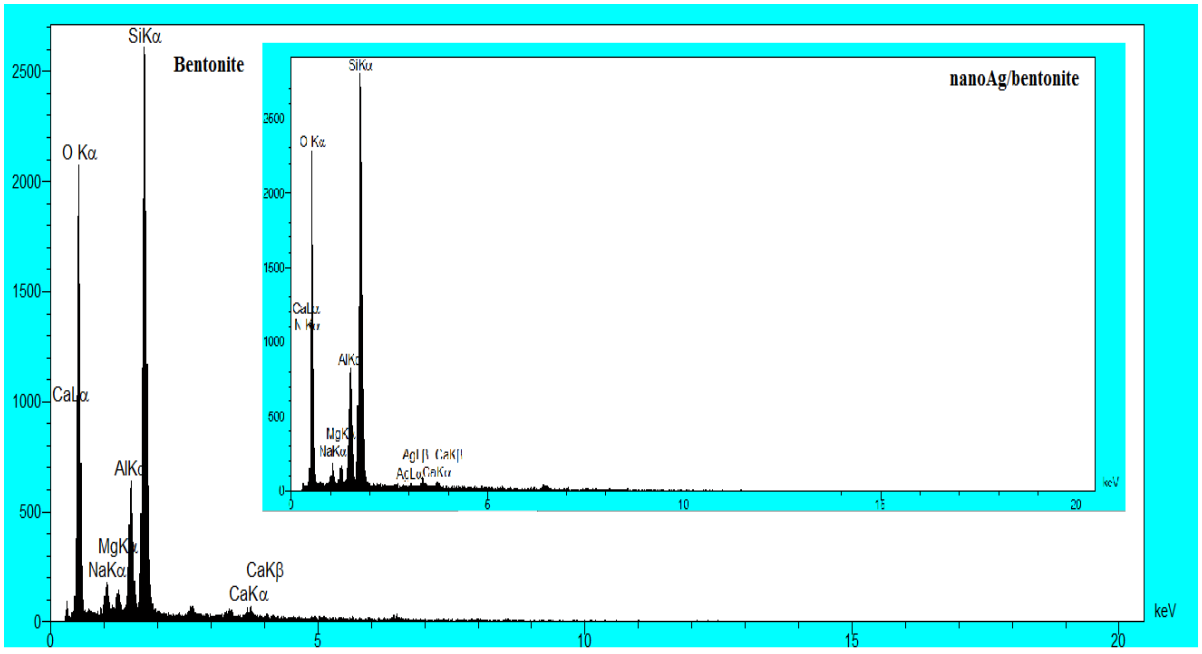

Figure 10. EDX analysis of natural and nano Ag zeolite.

Antifungal activity

The two Ag/zeolite and bentonite composites exhibited dose-dependent activity against *Saprolegnia sp*. The results showed that at 6.66 and 13.33 mg/L of Ag/Zeolite NCS (Figs. 11 and

12) and at 6.66, 13.33, 20, 26.66, 33.33, 40, 46.66 mg L^{-1} of Ag/Bentonite there were not any observable inhibitory effects compared to the negative control (without nano composites) (Figs. 13 and 14). The colonies grew well on the control plates, where after 24 h the whole surface of the media was covered by *Saprolegnia sp*. hyphae. Our results showed that growth inhibition of *Saprolegnia sp*. was significant for Ag/Zeolite NCS.

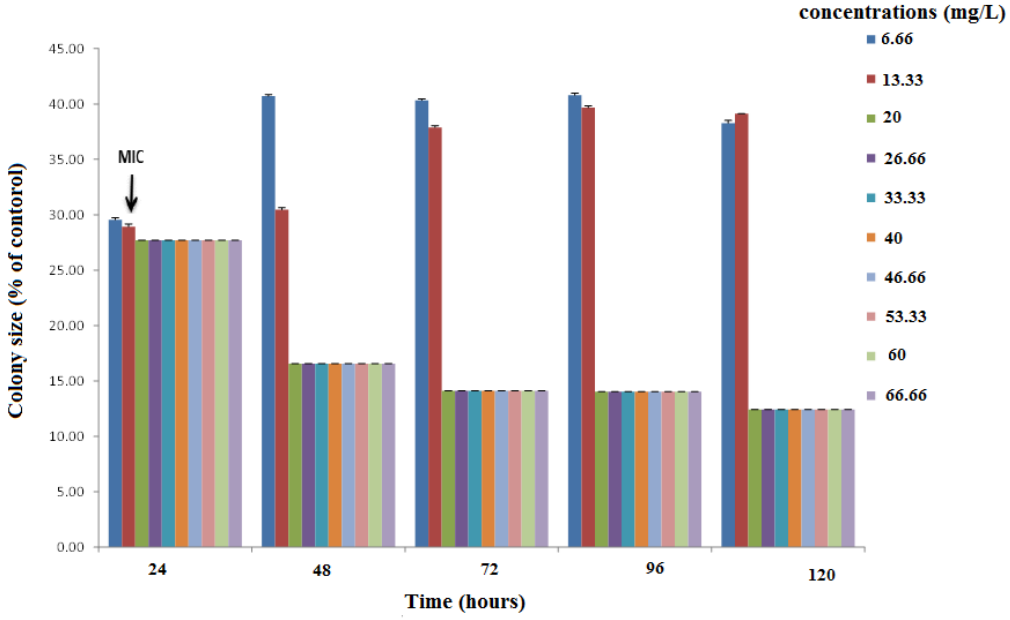

Figure11: Growth of Saprolegnia in different concentrations of Ag/zeolite NCs.

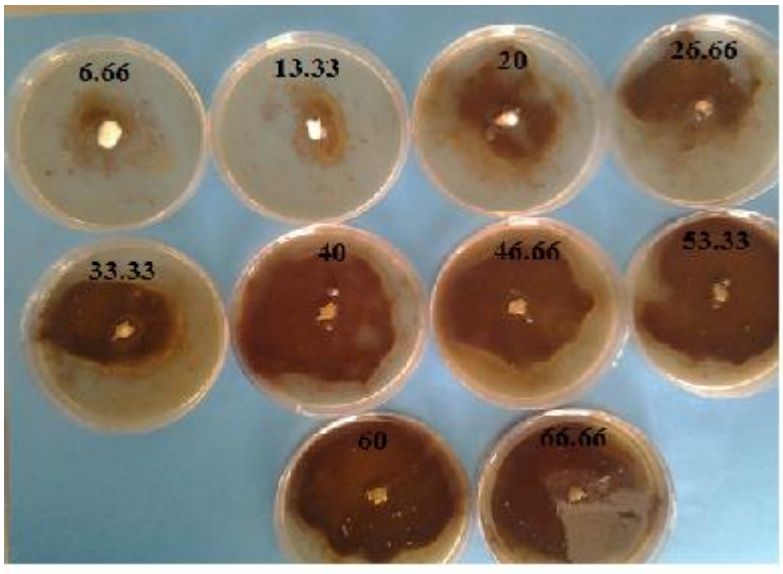

Figure 12: Growth of Saprolegnia on agar plates containing different concentrations of Ag/zeolite NCs.

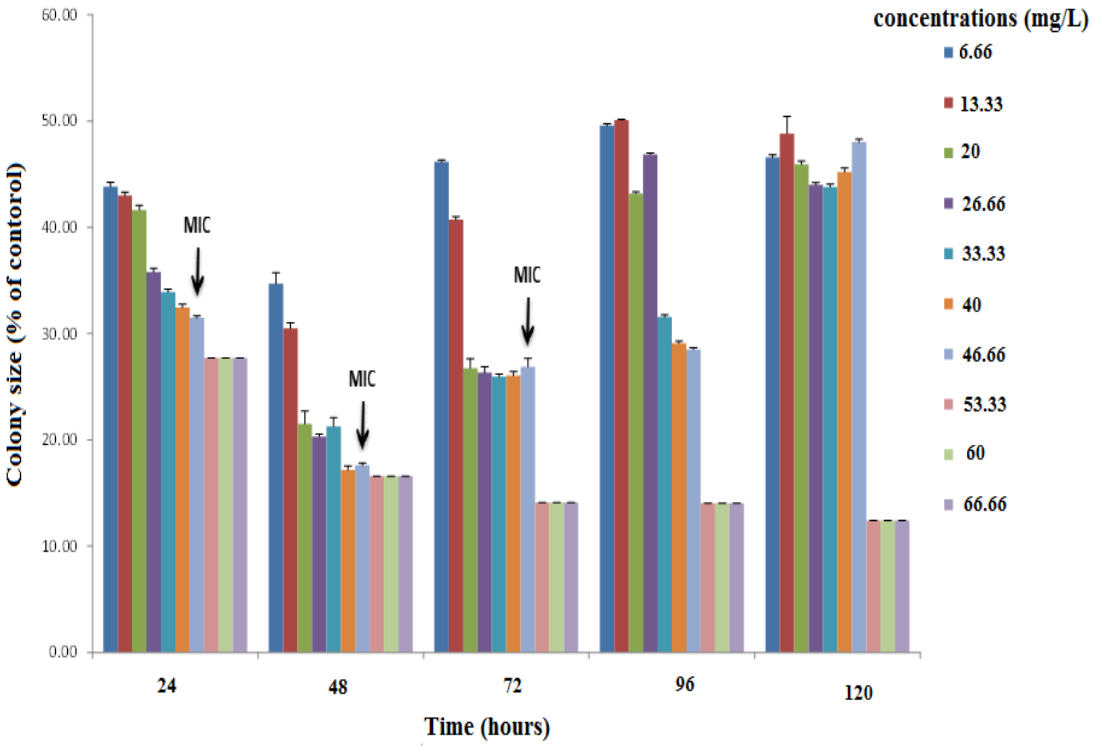

Figure 13: Growth of Saprolegnia in different concentrations of Ag/bentonite NCs.

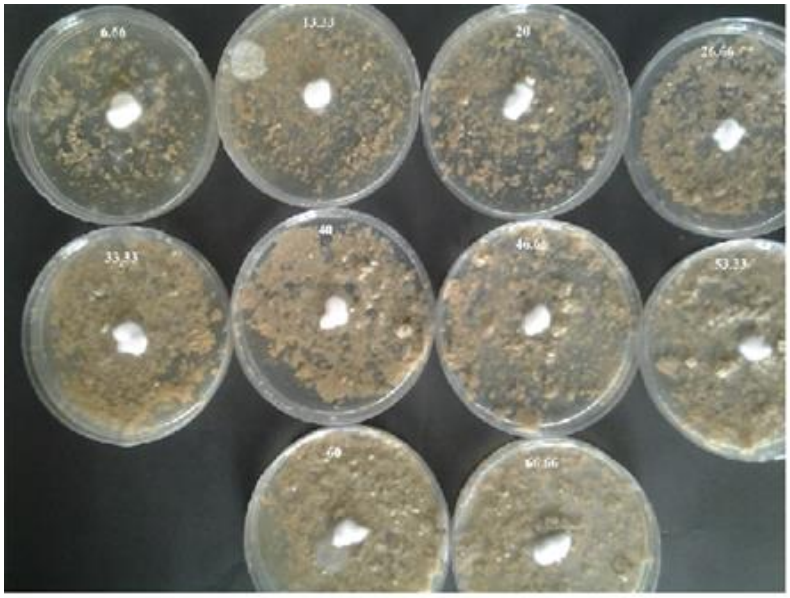

Figure 14: Growth of Saprolegnia on agar plates containing different concentrations of Ag/bentonite NCs.

Discussion

Silver nanoparticles (AgNPs) are known to have bactericidal and fungicidal effects. Since, there is little information available on the interaction of colloidal nanosilver with fish pathogens; the current study investigated the effects of AgNPs on the growth of the *Saprolegnia* sp. *in vitro*. The antifungal activity of AgNPs was then evaluated by determining the minimum inhibitory concentrations (MICs) using two-fold serial dilutions of colloidal nanosilver in sabouraud

dextrose agar at 22 °C. The growth of *Saprolegnia* sp. on the AgNPs agar treatments was compared to that of nanosilver-free agar as controls. In the present study, AgNPs were found to inhibit the growth of the water mould saprolegnia *in vitro*, making AgNPs a good candidate for their indirect use in the aquaculture industry. It seems that AgNPs could be a proper replacement for teratogenic and toxic agents, such as malachite green. In addition, the indirect use of AgNPs could be a useful method for providing new antifungal activity in aquaculture systems.

Metallic silver in the form of silver nanoparticles has made a remarkable comeback as a potential antimicrobial agent. The use of silver nanoparticles is also important, as several pathogenic bacteria have developed resistance against various antibiotics. Silver ions have been known to have powerful antibacterial and antifungal activities (Sadjadi *et al*., 2009). The mechanisms of antimicrobial activity of AgNPs have been extensively reported by several authors (Morones *et al*., 2005; Kim *et al*., 2007; Sanpui *et al*., 2008; Rai *et al*., 2009). AGNPs via changing and damaging the membrane structure of the microorganism (Kim *et al*., 2007), increase the membrane permeability and also interrupt the efflux/influx of materials which subsequently results in microbial cell death. However their interaction with phosphorus and sulfur-containing compounds, such as DNA and proteins, inactivation of certain enzymes, attacking the respiratory chain, generating hydrogen peroxide and free radicals and the release of the silver ions from the nanoparticles have been also reported (Feng *et al*., 2000; Yoshihiro, 2002; Yamanaka *et al*., 2005; Song *et al*., 2006). Contrary to antibiotics resistance arising from irresponsible antibiotic application, silver has been demonstrated to be a consistently effective antimicrobial agent (Marambio-Jones and Hoek, 2010). Several authors have shown the inhibitory effect of silver on bacteria, viruses, and fungi (Prabhu and Poulose 2012; Johari *et al*., 2013; Johari *et al*., 2014). Heavy metals could react with proteins such as different enzymes via their -SH groups and leave them inactivated. Some researchers investigated the inhibition mechanism of silver ions on microorganisms (Prabhu and Poulose, 2012). Silver ions affect DNA molecules, causing the loss of replication abilities of DNA, and interact with thiol groups in protein thereby inactivating bacterial proteins. Many studies have been carried out on the antibacterial and antifungal properties of various natural and inorganic substances such as clinoptilolite, bentonite, montmorillonite and etc. (Top and Ulkü, 2004; Zhou *et al*., 2004; Xu *et al*., 2011).

The Ag NPs were successfully prepared from the $AgNO_3$/ bentonite and $AgNO_3$/ Zeolite suspension of the $AgNO_3$ solution by using sodium borohydride ($NaBH_4$) at room temperature. The surface of zeolite and bentonite fostered the nucleation of Ag NPs during the chemical reduction process. In the present study, the tested nanocomposites were found to inhibit

the growth of the Saprolegnia *in vitro*, making nanosilver zeolite and bentonite a potential candidate for indirect use in aquaculture. Indirect methods can be applicable in aquaculture systems such as fish ponds, hatcheries, and aquarium industries. In conclusion, further investigation of the antibacterial, antiviral, and antifungal activities of nano Ag zeolite and nano Ag bentonite against other fish pathogens is needed in the future.

In conclusion, further investigation of the antimicrobial activities of AgNPs against other fish pathogens is needed.

Acknowledgment

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