



BIOSYNTHESIS OF ZINC NANOPARTICLES USING CULTURE FILTRATES OF *Aspergillus*, *Fusarium* AND *Penicillium* FUNGAL SPECIES AND THEIR ANTIBACTERIAL PROPERTIES AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

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ABSTRACT: The present study demonstrates a positive correlation between zinc metal tolerance ability of an isolated fungi and their potential for the synthesis of zinc oxide nanoparticles (ZnO-NPs). A total of 5 fungal cultures were isolated from the rhizospheric soils of plants naturally growing at Sharkia Governorate in Egypt and identified based on morphological characteristics. These isolates are belongs to *Aspergillus niger* (An), *Aspergillus tubulin* (At), *Aspergillus fumigatus* (Af), *Penicillium citrinum* (Pc) and *Fusarium oxysporum* (Fo). These isolates were used in the synthesis of zinc-oxide nanoparticles (An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs) using Zinc sulfate as the precursor compared to the references strains of *A. tubingensis* Mosseray AUMC No.6915, *A. fumigatus* Fresenius AUMC No.48 and *A. terreus* Thom AUMC No.75. *Aspergillus* and *Fusarium* isolates have been shown to have a high zinc metal tolerance ability and a potential for extracellular synthesis of ZnO nanoparticles under ambient conditions. The synthesized ZnO-NPs were tested by the detection of a notable absorption peak at 285 to 296 nm, appearing in UV-Vis spectra due to surface-plasmon-resonance. Transmission electron microscope (TEM) results revealed that An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs exhibited a crystalline structure with hexagonal wurtzite shape (30–100 nm size). ZnO nanoparticles exhibited excellent antibacterial activity against tested Gram-positive and Gram-negative bacteria. The ZnO nanoparticles showed better antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* compared to *Salmonella enterica*, *Escherichia coli* and *Pseudomonas aerogenosa*. The effectiveness of inhibition of the microbial biofilms formation of *S.aureus*, *L. monocytogenes* and *B. cereus* compared to *S. enterica*, *E. coli* and *P. aerogenosa* was analyzed at a concentration of 100 µg/ml.

Key words: *Fusarium oxysporum*, *Aspergillus fumigatus*, Zinc sulfate, ZnO nanoparticle characterization, antimicrobial activity; bacterial pathogens.

INTRODUCTION

Synthesis of nanoparticles employing microorganisms has attracted much due to their usual optical, chemical, photoelectron chemical and electronic properties and many biological organisms, such as bacteria, fungus, yeasts and plants either intra or extracellular (Castro-Longoria *et al.*, 2010). Eukaryotic organisms such as fungi are extremely good candidate for the synthesis of metal nanoparticles. The fungi

give nanoparticles with good monodispersity and well-defined dimensions. Because of their tolerance and metal bioaccumulation ability, fungi are taking the center stage of studies on biological generation of metallic nanoparticles (Sastry *et al.*, 2003). A distinct advantage of using fungi in nanoparticle synthesis is the ease in their scale-up (*e.g.*, using a thin solid substrate fermentation method). Given that fungi are extremely efficient secretors of extracellular enzymes, it is thus possible to easily obtain

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large-scale production of enzymes. Further advantages of using a fungal-mediated green approach for synthesis of metallic nanoparticles include economic viability and ease in handling biomass. However, a significant drawback of using these bio-entities in nanoparticles synthesis is that the genetic manipulation of eukaryotic organisms as a means of overexpressing specific enzymes (*e.g.*, the ones identified in synthesis of metallic nanoparticles) is relatively much more difficult than that in prokaryotes. The biological method of the ZnO NPs synthesis is gaining importance due to its simplicity, eco-friendliness and extensive antimicrobial activity (**Gunalan *et al.*, 2012**). The main factor for the increase of the resistance pathogenic bacteria is overuse of antibiotics and this has led to the emergence and spread of resistant pathogens and resistant genes in them (**van den Bogaard and Stobberingh, 2000**). Nanoparticles as antimicrobial agents are better efficiency on resistant bacteria, less toxicity and heat resistance. Among metal oxide Nanoparticles, ZnO have many significant features such as chemical and physical stability, high catalysis and effective antibacterial activity (**Kalyani *et al.*, 2006**). Recently, ZnO NPs have been used in food packaging materials and various matrices and methods for incorporation of ZnO into those matrices have been reported. ZnO is incorporated into the packaging matrix, free to interact with the food materials offering preservative effects (**Espitia *et al.*, 2012**). Presently, ZnO NPs have found applications in sunscreens, paints and coatings as they are transparent to visible light and offer high UV absorption (**Franklin *et al.*, 2007**) and are also being used as an ingredient in antibacterial creams, ointments and lotions, self cleaning glass, ceramics and deodorants (**Li *et al.*, 2008**). ZnO nanoparticles have been lately tested for their antimicrobial potential and seem to possess both antibacterial and antifungal potential. They are active against both Gram-positive and Gram-negative bacteria and also show considerable activity against more resistant bacterial spores (**Azam *et al.*, 2011**). It was also observed that doping of ZnO NPs with other metals such as gold, silver, chromium *etc.* improved the antimicrobial activity of ZnO NPs (**Shah *et al.*, 2014**; **Jime'nez *et al.*, 2015**). Also, inhibitory

effects of ZnO nanosuspension are correlated with their size and concentration, with smaller particles offering better inhibitions in higher concentrations (**Buzea *et al.*, 2007**; **Padmavathy and Vijayaraghavan, 2008**). ZnO NPs, in particular, are environment friendly, offer easy fabrication and are non-toxic, biosafe and biocompatible making them an ideal candidate for biological applications (**Rosi and Mirkin, 2005**; **Mohammad *et al.*, 2010**). Additionally, as per the US Food and Drug Administration, zinc sulphate with other three zinc compounds (Zinc Oxide, Zinc Nitrate, Zinc Chloride) have been listed as generally recognized as safe (GRAS) material (**FDA, 2015**). Various chemical methods have been proposed for the synthesis of ZnO NPs, such as reaction of zinc with alcohol, vapor transport, hydrothermal synthesis, precipitation method *etc.* However, these methods suffer various disadvantages due to the involvement of high temperature and pressure conditions and the use of toxic chemicals. The advantages of using inorganic oxides nanoparticles as antimicrobial agents are their greater effectiveness on resistant strains of microbial pathogens, less toxicity and heat resistance (**Zhang *et al.*, 2010**). ZnO has biocidal action and strong antibacterial agent due to its physiochemical properties and biocompatibility (**Mirzaei and Darroudi, 2017**). Plus, the crystallite size and the nanoparticle shape have an effect on the antibacterial activity which smaller ZnO nanoparticles have higher antibacterial activity. Several reports have addressed the harmful impact of nanomaterials on living cells, but relatively low concentrations of ZnO are nontoxic to eukaryotic cells (**Zaveri, *et al.*, 2010**; **Krishna *et al.*, 2011**) stated that ZnO nanoparticles significantly inhibit the growth of a wide range of pathogenic bacteria under normal visible lighting condition. Thus, the current study aims to isolate fungi from soil contaminated with heavy metals for biosynthesizing of zinc-oxide nanoparticles using zinc sulfite as the precursor compared to the references strains of *A. tubingensis* Mosseray AUMC No.6915, *A. fumigatus* Fresenius AUMC No.48 and *A. terreus* Thom AUMC No.75. The second aim of this study was to evaluate the zinc-oxide nanoparticles efficiency

against Gram-positive and Gram-negative bacteria as antibacterial agents.

MATERIALS AND METHODS

Preparation of Stock Solution of Zinc Sulphate

The stock solution of zinc sulphate was prepared by dissolving 1.61 g/l of zinc sulfate in 20 ml of sterilized distilled water to get the 10 mM concentration and then stored for use in the experimentations.

Sampling and Isolation of Fungi

The soil samples (n=10) were collected from the rhizospheric soils of plants naturally growing at Sharkia Governorate, Egypt. These samples were used for isolation of metal-tolerant fungi. Potato Dextrose Agar (PDA: potato extract 1000 ml, sucrose 20 g and agar 15g) were used for isolation of fungi. This media was containing 1mM of zinc sulfate (**Jain et al., 2013**). Plates were incubated at 28°C for 72 hr. The tolerant colonies were picked up and transferred to Czapek Dox Agar slants and preserved at 4°C in a refrigerator.

Strains Used

Fungal strains of *Aspergillus tubingensis* Mosseray AUMC No.6915, *A. fumigatus* Fresenius AUMC No.48 and *A. terreus* Thom AUMC No.75 were obtained from The Center of Fungi Science, Assiut University, Egypt. These standard of fungi strains were used as a references strains in the current study. While bacterial strains of *Staphylococcus aureus* ATCC 6538, *Listeria monocytogenes* Scott A, *Salmonella enterica* serovar Enteritidis PT4, *Pseudomonas aeruginosa* and *Escherichia coli* ATCC 8739 were obtained from Egyptian Culture Collection (MERCIN), Ain Shams University, Cairo, Egypt.

Metal Tolerance Profiles of Fungal Isolates

A maximum tolerable concentration (MTC) assay was performed to determine the zinc metal tolerance ability of fungal isolates according to the method of **Jain et al. (2013)**. The experimental plates were prepared by supplementing PDA medium with varying amounts of zinc sulfate to obtain final concentrations of Zn⁺² ions in the ranges of 200, 400, 800, 1600, 2000, 2500, 3000

and 3200 µg ml⁻¹. Plates without Zn⁺² ions were used as a control. Each plate was subdivided into four equal sectors and an inoculum of test fungi (10⁶ fungal propagates ml⁻¹) was spotted on the media surface. After inoculation, the plates were incubated at 28°C for 4 days under dark conditions to examine the fungal growth. The experiment was done in triplicate. The maximum concentration of Zn⁺² ions in the medium which allowed the growth of a fungus was taken as MTC.

Extracellular Synthesis of (ZnO-NPs) Nanoparticles by Fungal Strains and Isolates

Biomass was produced by cultivation of isolated and strains fungi in Malt Glucose Yeast Peptone (MGYP) broth composed of yeast extract and malt extract 0.3% each, glucose 1%, peptone 0.5%. Biomass preparation of *Aspergillus niger*, *Aspergillus tubulin*, *Aspergillus fumigatus*, *Penicillium citrinum* and *Fusarium oxysporum* used in this study have originally been isolated from soil contaminated with heavy metals compared to the standard strains of *A. fumigatus*, *A. tubulin*, *Fusarium oxysporum* and *Penicillium citrinum*. Fungi were grown in 250 ml Erlenmeyer flasks each containing 50 ml liquid medium containing (g/l): KH₂PO₄ 7.0 g; K₂HPO₄ 2.0 g; MgSO₄·7H₂O 0.1 g; (NH₄)₂SO₄ 1.0 g; yeast extract 1.0 g and glucose 15.0 g. Inoculated media were incubated at 28 ± 2 °C and 180 rpm for 5 days, after which each fungal biomass was separated using Whatman filter paper No. 1 and extensively washed by deionized water. The collected fungal biomass was transferred to 100 ml of deionized water in the 250 ml Erlenmeyer flask and further incubated at 140 rpm for 72 hr., in an orbital shaker. Each fungal biomass was filtered again with Whatman filter paper No. 2 and the collected cell-free filtrate was subjected to biosynthesis of zinc oxide nanoparticles (**Jain et al., 2011**). The treatments have been named An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs. 10 ml of 3.0 mM ZnSO₄ salt were added to 10 ml filtrate of each fungus (1;1) and adjusting the pH to 6.5 and incubated in orbital shaker for 150 rpm at 32°C for 72 hrs., in adark condition. White precipitate deposition at the bottom of the flask indicated the formation of nanoparticles. White aggregate

formed at the bottom of the flask was separated from the filtrate by centrifugation at 10,000 rpm for 10 min. (Baskar *et al.*, 2013). Simultaneously, a positive control and negative control were maintained by incubating the each fungus mycelium with de-ionized water and Zinc sulphate solution (Dhoble and Kulkarni 2015).

Characterization of ZnO-NPs

UV-Vis spectra analysis

Each sample (An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs) was measured for its maximum absorbance using UV-Vis spectrophotometry (Jasco Corporation, Tokyo, Japan). The optical property of ZnO nanoparticles was analyzed *via* ultraviolet and visible absorption spectroscopy in the range of 200–800 nm. This techniques were carried out at The Center of Fungi in Faculty of Science, Al-Azhar University, Cairo, Egypt.

Transmission electron microscopy (TEM)

TEM analysis was performed to determine the morphology, size and shape of the ZnO nanoparticles. TEM measurements were done by HITACHI H-800, operating at 200 kV. The TEM grid was prepared by placing a drop of the bio-reduced diluted solution on a carbon-coated copper grid and later drying it under a lamp. The size distribution and stability of ZnO NPs were registered. This techniques were carried out at The Center of Fungi in Faculty of Science, Al-Azher University, Cairo.

Antibacterial Assay

Inhibition Zone

The technique was performed using agar well diffusion (Magaldi *et al.*, 2004). Six different pathogenic bacterial strains were used in the current study. Gram positive bacteria *i.e.* *Bacillus cereus*, *Staphylococcus aureus* and *Listeria monocytogenes* as well as Gram negative bacteria *i.e.* *Escherichia coli*, *Salmonella enterica* and *Pseudomonas aeruginosa* were used as test organisms and initially cultured in nutrient broth. All the pathogenic microbes were grown overnight in Mueller-Hinton broth (MHB). 100 µl culture of each strain was spread evenly on a Mueller-

Hinton agar (MHA) plate. Then the wells were made into agar at 4 mm diameter using sterile cork-borer with the distance between well and another more than 22 mm. 100 µL of each An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo-ZnO-NPs was placed over the MHA plates. The plates were incubated at 35°C for 24 hr. β-lactams group (AMP: ampicillin), fluoroquinolones group (OFX: ofloxacin) and aminoglycosides group (AK: amikacin) were used as the control. The diameters of inhibition zone against the tested bacteria (Yuvaraja *et al.*, 2017) were recorded in millimeter using metric ruler.

Growth-reduction

To examine bacterial growth, overnight cultures of approximately 1×10^9 CFU/ml were diluted 100-fold into 50 ml of Tryptone Soya Broth in 250 ml flasks. 100 µl of each An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs suspensions were added to the respective flasks. Cultures were then grown for up to 48 hr., at 200 rpm, 37°C. Bacterial growth was measured by optical density at 600 nm (OD_{600}).

Antibiofilm formation by Microtiter Plate (MTP) Method

All bacterial strains were grown overnight at 37°C as pure cultures on Tryptone Soya agar. The single colonies were inoculated in 3 ml Tryptone Soya Broth (TSB). Suspensions were incubated for 24 hr., at 37°C and then diluted at 1 : 40 in a fresh TSB (2.4×10^7 CFU/ml) using 0.5 MacFarland standard tube. This dilution was used as the inoculum in the microtiter plate test. Microtiter plate test was performed according to Dubravka *et al.* (2010) and Stepanović *et al.* (2000). For each strain, 100 µl aliquots of prepared suspension culture were inoculated into four wells of the 96-well tissue culture plates containing fresh TSB (Nuncclon Delta, Nunc, Roskilde, Denmark), then each treatment was received 100 µl of ZnO nanoparticles. Each culture plate included a negative control, four wells with TSB were used. The plates were incubated at 37°C for 24 hr. Afterwards, content of each well was removed by aspiration and the wells were rinsed three times with 250 µl sterile physiological saline. The plates were dried in inverted position. The attached bacteria were

fixed for 15 minutes at room temperature by adding 200 μl volumes of methanol into each well. The plates were stained with 200 μl aqueous solution of crystal violet 0.5% (Crystal Violet, Fluka) for 15 minutes at room temperature. Following staining, the plates were rinsed under running water until there was no visible trace of stain. The stain bound to bacteria was dissolved by adding 200 μl of 95% ethanol. Then microplates were washed, air dried and adherent biofilm was dissolved in 33% (V/V) glacial acetic acid. To determine the potential of bacterial pathogens treated with An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs to form biofilm, the microtiter plate-based assay and the biofilm formation on abiotic surfaces was quantified as described by **Stepanović *et al.* (2007)**. Biofilm formation was measured at 570 nm in an ELISA reader set (Thermo Scientific Multiskan FC, USA), and expressed in optical density (OD) values. The mean OD value of negative controls (OD_{NC}) was 0.070 ± 0.005 . The treatments were considered biofilm producers when their OD values were three times greater than the standard deviation of the mean OD_{NC} . Additionally, the bacterial strains with An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs showing ability to produce biofilm were classified as weak ($\text{OD}_{\text{NC}} < \text{OD} \leq 2 \times \text{OD}_{\text{NC}}$), moderate ($2 \times \text{OD}_{\text{NC}} < \text{OD} \leq 4 \times \text{OD}_{\text{NC}}$) or strong ($\text{OD} > 4 \times \text{OD}_{\text{NC}}$) biofilm producers (**Li *et al.*, 2012**). Biofilm production by each strain was determined from four independent experiments and each independent experiment included triplicates for each bacterial strain. The mean of the twelve absorbance values of each isolate was recorded as a result. Absorbance values $> \pm 4 \times$ the standard deviation were rejected.

Statistical Analysis

Data from microbiological analyses were entered into Excel 2010 and calculated for standard division ($\pm\text{SD}$) for all experiments.

RESULTS AND DISCUSSION

Identification of Fungal Isolates

Preliminary identification of fungi was performed on the basis of morphological parameters such as color, spore shape,

arrangement, and hyphal branching pattern after staining with cotton blue. Identification of fungi is still heavily dependent upon microscopic observation of the physical appearance of both spores and spore-bearing structures, usually achieved by first obtaining the fungi in pure culture. Examination of just the mycelium rarely enables an identification, nor are spores alone sufficient as there are many species producing very similar spores. The UK has an organised network, the UK National Culture Collection (www.ukncc.co.uk), through which it is possible to obtain appropriate taxonomic advice and services on many microorganisms, including fungi. Also, the further identifications were carried out in the Department of Plant Pathology, Faculty of Agriculture, Zagazig University, Zagazig, Egypt compared to the references strains of fungi. All the isolates were belong to *Aspergillus niger*, *Aspergillus tubulin*, *Aspergillus fumigatus*, *Penicillium citrinum*, and *Fusarium oxysporum*. All these isolates were used in biosynthesis of zinc oxide nanoparticles.

Metal Tolerance Profile of Fungal Isolates

All the five fungal isolates were subjected to a screening for metal tolerance towards zinc, and the results were expressed in terms of MTC. A higher proportion (100%) of fungal isolates showed significant tolerance with a varying degree of magnitude. The genus *Aspergillus* exhibited a more prominent level of zinc metal tolerance followed by *Fuzarium* then *Penicillium*. It is evident from the results that *A. niger*, *A. tubulin*, *A. fumigatus*, *F. oxysporum* and *P. citrinum* isolates have tremendous zinc metal tolerances with a MTC value of 3000 and 2500 $\mu\text{g ml}^{-1}$, respectively (Table 1). Due to its maximum MTC value, *A. niger*, *A. tubulin*, *A. fumigatus*, *P. citrinum* and *F. oxysporum* isolates were selected for further studies on extracellular synthesis of ZnO nanoparticles compared to the references strains. The current study approaches to establish the relationship between metal tolerance ability of a soil fungus and its potential for synthesis of ZnO nanoparticles. **Jain *et al.* (2013)** found that *Aspergillus aeneus* isolate NJP12 has been shown to have a high zinc metal tolerance ability and a potential for extracellular synthesis of ZnO nanoparticles under ambient conditions.

Table 1. Zinc metal tolerance profile for ZnSO₄ of fungal isolates after 96 hr.

Isolate	A maximum tolerable concentrations (MTC) ($\mu\text{g ml}^{-1}$)							
	200	400	800	1600	2000	2500	3000	3200
<i>Aspergillus niger</i>	+++	+++	+++	+++	+++	+++	++	-
<i>Aspergillus tubulin</i>	+++	+++	+++	+++	+++	+++	++	-
<i>Aspergillus fumigatus</i>	+++	+++	+++	+++	+++	+++	++	-
<i>Fusarium oxysporum</i>	+++	+++	+++	+++	+++	++	-	-
<i>Penicillium citrinum</i>	+++	+++	+++	++	+	+	-	-

Extracellular Biosynthesis of Zinc Oxide Nanoparticles and Physical Characteristics

The ZnO nanoparticles were prepared in accordance with the methods described above. The extracellular synthesis of ZnO nanoparticles was carried out by exposure of a precursor salt solution (ZnSO₄, ZnO, Zn(NO₃)₂, ZnCl₂) (Zn⁺² ions; 3.0 mM) to fungal cell-free filtrate obtained by incubating the fungus in an aqueous solution. The viability experiment results showed that fungal cells were viable till the end of reaction (72 hr.). During the synthesis process of ZnO nanoparticles, the initially colorless solution will turn to the milky solution (Fig. 2). This has indicated that the ZnO nanoparticles were successfully produced by cell-free filtrate. **Jain et al. (2013)** found that the synthesis of ZnO spherical nanoparticles coated with protein molecules by *A. aeneus* which served as stabilizing agents. Also, they showed that the role of fungal extracellular proteins in the synthesis of nanoparticles indicated that the process is nonenzymatic but involves amino acids present in the protein chains. Several authors showed that *A. fumigatus* (**Rajan et al., 2016**), *A. niger* (**Kalpana et al., 2018**), *F. oxysporum* (**Ahmed et al., 2003**) and *P. citrinum* (**Honary et al., 2013**) have ability to synthesis nanoparticles from metal salts. The synthesized ZnO nanoparticles from tested fungi are presented in Fig. 1. All ZnO nanoparticles have shown a strong absorption peak at around 285 to 296 nm. **Rajendran and Sivalingam (2013)** found that ZnO nanoparticles have shown a strong absorption peak at around 360 nm. TEM results revealed that An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs exhibited a crystalline structure

with hexagonal wurtzite shape (30–100 nm size) (Fig. 2).

Evaluation of Antibacterial Activity

Inhibition Zone method

The results of inhibition zones of ZnO nanoparticles compared to three tested antibiotic compounds against *B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli*, *S. enterica* and *P. aerogenosa* are presented in Table 2. The larger zone was observed at *B. cereus* and *S. aureus* culture which is 14 to 26 mm compared to the *L. monocytogenes* culture with 17 to 25 mm of inhibition zone with all tested fungi except *Penicillium citrinum* which is 12 to 15 mm. While the inhibition zone by the antibiotics were observed at *B. cereus*, *S. aureus* and *L. monocytogenes* culture which is 20 to 30 mm compared to the *E. coli*, *S. enterica* and *P. aerogenosa* cultures with 7 to 27 mm. On the other hand, the inhibition zones of ZnO nanoparticles were observed at *E. coli*, *S. enterica* and *P. aerogenosa* cultures which is 11 to 18mm with all tested fungi. Based on the results obtained, it can be suggested that Gram-negative bacteria are more resistance to ZnO nanoparticles compared to Gram-positive bacteria. Also, ZnO nanoparticles was more effective against Gram negative compared to the antibiotics especially when compared to the antibiotic (AMP). This supports by **Premanathan et al. (2011)** who reports that ZnO nanoparticles showed a much stronger antibacterial effect on Gram-positive bacteria than on Gram-negative ones. **Stoimenov et al. (2002)** and **Fu et al. (2005)** elucidated by the possibilities of membrane damage caused by electrostatic interaction between ZnO and cell surface.

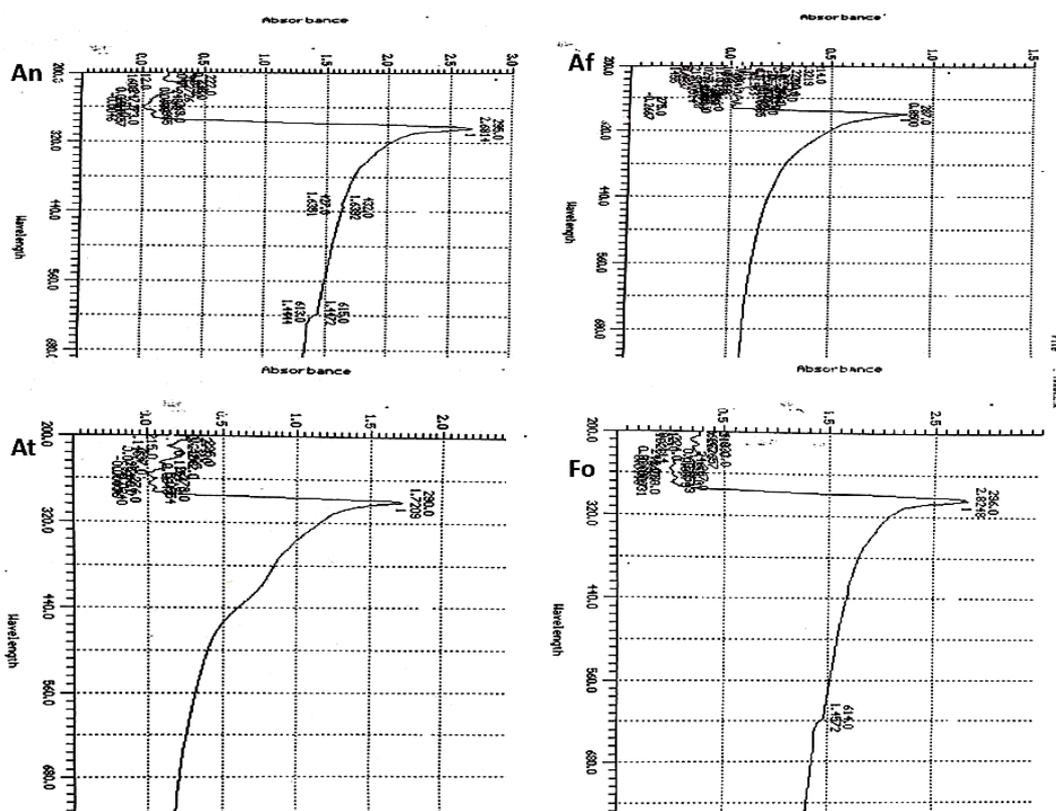


Fig. 1. UV-visible spectrum of ZnO nanoparticles suspension biosynthesized by *Aspergillus niger* (An), *Aspergillus tubulin* (At), *Aspergillus fumigatus* (Af) and *Fusarium oxysporum* (Fo).

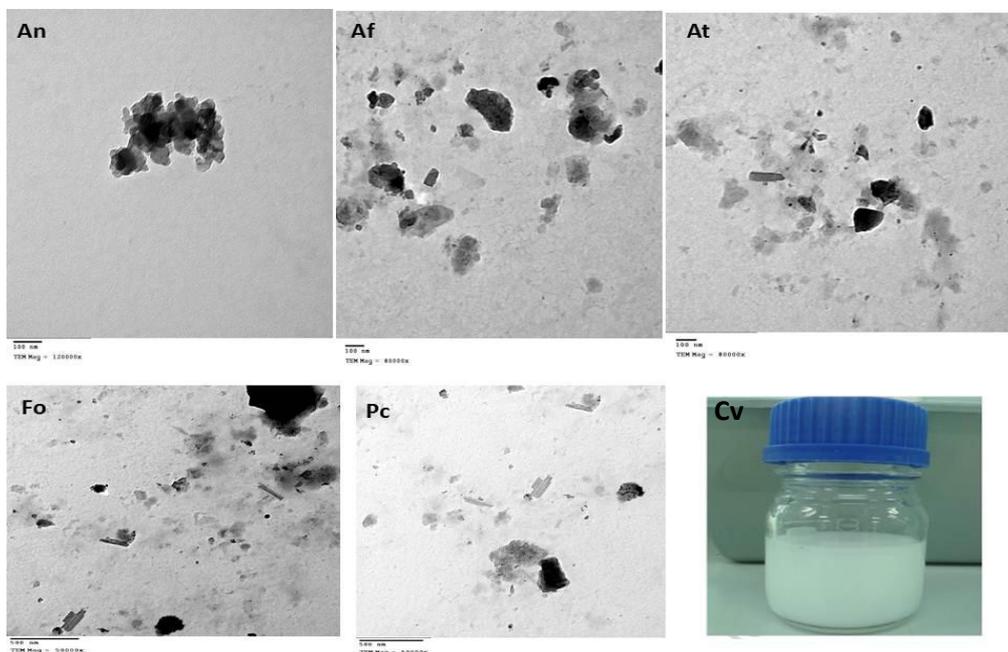


Fig. 2. Image of ZnO nanoparticles suspension biosynthesized by *Aspergillus niger* (An), *Aspergillus tubulin* (At), *Aspergillus fumigatus* (Af), *Fusarium oxysporum* (Fo) and *Penicillium citrinum* (Pc) under Transmission Electron Microscope (TEM), the view of the formed colour due to the formation of ZnONPs (Cv)

Table 2. Inhibition zone of ZnO (100g/ml) nanoparticles biosynthesized by tested fun against Gram positive and Gram negative bacterial strains compared to some tested antibiotics

Tested bacteria	Diameter of inhibition zone (mm)					
	<i>B. cereus</i>	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>S. enterica</i>	<i>E. coli</i>
Tested fungi						
Stadard fungi						
<i>A. tubingensis</i> Mossery Aumc No. 6915	25±0.23	17±0.14	19±0.11	16±0.10	13±0.11	17±0.25
<i>A. fumigatus</i> Fresenius No. 48	23±0.16	18±0.19	21±0.12	14±0.09	16±0.08	15±0.11
<i>A. terreus</i> Thom Aumc No. 75	24±0.11	23±0.17	22±0.15	11±0.15	13±0.16	13±0.15
Local isolates						
<i>A. niger</i>	26±0.21	23±0.11	24±0.15	17±0.18	15±0.26	18±0.26
<i>A. tubulin</i>	25±0.13	22±0.13	25±0.12	14±0.16	18±0.18	15±0.17
<i>A. fumigatus</i>	26±0.16	25±0.18	26±0.19	15±0.17	16±0.13	17±0.18
<i>P. citrinum</i>	14±0.13	12±0.19	15±0.10	12±0.13	10±0.23	12±0.19
<i>F. oxysporum</i>	25±0.15	24±0.17	26±0.14	13±0.22	12±0.27	14±0.16
Control (Antibiotics)						
Ofloxacin (5 µg)	27±0.11	29±0.15	30±0.23	25±0.11	10±0.25	24±0.13
Ampicillin 10µg)	20±0.23	21±0.25	23±0.34	7±0.23	11±0.34	14±0.18
Amikacin (30µg)	22±0.32	28±0.16	23±0.18	25±0.16	25±0.23	27±0.15

Time-kill study

In this study, the time kill measurement was determined by the actual reduction in bacteria growth curve at 24 hr., of the culturing period for *S. aureus* and *E. coli*. Results from the time-kill studies are shown in Figs. 3 and 4 which demonstrates the growth curve of Gram positive bacteria *S. aureus* and Gram negative *E. coli*. *S. aureus* and *E. coli* bacteria were in contact with 0.1 ml of ZnO nanoparticles. The results exhibited that ZnO nanoparticles have inhibitory effects on the growth as compared to bacterial culture without nanoparticles as a control. These results demonstrated that ZnO nanoparticles showed inactivation effect against both types of bacteria from Fig. 3, Gram-negative bacteria is more resistance to the ZnO nanoparticles

compared to the Gram-positive bacteria. This can be related to the cell wall-structure, as the Gram-positive bacteria have one cytoplasmic membrane with the multilayer of peptidoglycan polymer, and a thicker cell wall (Fu et al., 2005) Whereas Gram-negative bacteria wall is composed of two cell membranes, an outer membrane and a plasma membrane with a thin layer of peptidoglycan (Sirelkhatim et al., 2015). Besides according to Padmavathy et al. (2008), ZnO nanoparticles have an abrasive surface texture which influences the antibacterial mechanism, which in sequence destroys the bacterial membrane.

Antibiofilm formation

The results of antibiofilm formation by An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc-ZnO-

NPs and Fo- ZnO-NPs against Gram negative and Gram positive bacteria was assessed by

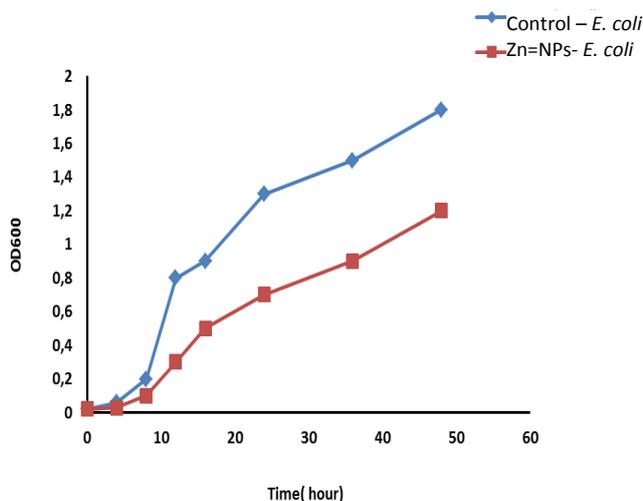


Fig. 3. Effect of ZnO nanoparticles on the growth of *E. coli* in TSB at 37°C

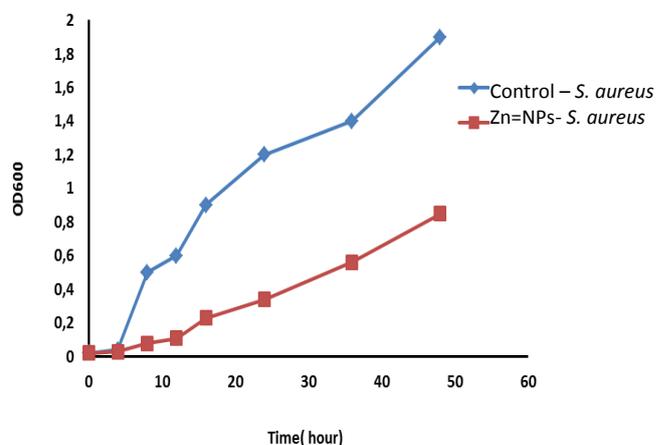


Fig. 4. Effect of ZnO nanoparticles on the growth of *S. aureus* in TSB at 37°C

microtiter plate-based assay, and shown in Table 3. The effectiveness of inhibition of the microbial biofilms of *S. aureus*, *L. monocytogenes* and *B. cereus* compared to *S. enterica*, *E. coli* and *P. aerogenosa* by An-ZnO-NPs, At-ZnO-NPs, Af-ZnO-NPs, Pc- ZnO-NPs and Fo- ZnO-NPs were shown at a concentration of 100 µg/ml. The An-ZnO-NPs were more effective to inhibit the formation of biofilm by all the tested bacterial strains compared to all the other treatment. *S. aureus* was more resistant than all the tested bacterial strains against all ZnO nanoparticles. Because, in the process of staphylococcal biofilm formation, the accumulation and development of a mature stage depend mainly on the polysaccharide intercellular adhesions (PIA) that promote bacterial accumulation, especially polysaccharide poly-N-succinyl-β-1-6 glucosamine (PNAG). PNAG biosynthesis is regulated by enzymes encoded by the *ica* ADABC operon (Maira-Litrán *et al.*, 2002). Further studies should be done on the efficiency of ZnO nanoparticles against pathogenic bacterium and fungi such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Trichophyton rubrum* (*T. rubrum*) including the changes of morphological of the strains after in contact with ZnO nanoparticles. This study will contribute to

the understanding of inactivation effect of ZnO nanoparticles against pathogenic bacteria.

Underline numbers, strong biofilm formation, normal numbers, moderate biofilm formation, bold numbers, weak biofilm formation. Optical density (OD) values. The mean OD value of negative controls (OD_{NC}) was 0.070. The treatment was considered biofilm producers when their OD values were three times greater than the standard deviation of the mean OD_{NC} . Additionally, the treatments are showing ability to produce biofilm were classified as weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$) or strong ($OD > 4 \times OD_{NC}$ biofilm producers).

Conclusion

ZnO nanoparticles were successfully been biosynthesized by five different isolates of fungi (*i.e.* *Aspergillus niger*, *Aspergillus tubulin*, *Aspergillus fumigatus*, *Penicillium citrinum* and *Fusarium oxysporum*) as compared to three standard strains of fungi. UV-vis absorption analysis showed that the ZnO nanoparticles exhibited a peak at around 286 to 296 nm. ZnO nanoparticles exhibited excellent antibacterial activity against Gram-positive and Gram-negative bacteria. ZnO nanoparticles showed

better antibacterial activity against *S.aureus*, *S. enterica*, *E. coli* and *P. aerogenosa*. *L. monocytogenes* and *B. cereus* compared to

Table 3. Antibiofilm formation by Zn-NPs against some pathogenic bacteria

ZnO nanoparticles	OD ₅₇₀				Biofilm formation=3×OD _{NC}
	<i>P. aeruginosa</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>S. aureus</i>	
OD _{NC}	0,061	0,069	0,076	0,071	0,280
Positive control	0,426	0,637	0,493	0,465	0,280
An-ZnO-NPs	0,074	0,053	0,058	0,071	0,280
Af-ZnO-NPs	0,076	0,066	0,056	0,304	0,280
At-ZnO-NPs	0,086	0,067	0,073	0,462	0,280
Fo-ZnO-NPs	0,096	0,092	0,103	0,124	0,280
Pc-ZnO-NPs	0,304	0,25	0,22	0,226	0,280

An-ZnO-NPs, *A. niger*; At-ZnO-NPs, *A. tubulin*; Af-ZnO-NPs, *A. fumigatus*; Fo- ZnO-NPs, *F. oxysporum* and Pc- ZnO-NPs, *P. citrinum*

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التخليق الحيوي لجسيمات الزنك النانوية باستخدام مرشحات مزارع فطرية لأنواع من *Aspergillus* و *Fusarium* و *Penicillium* وخصائصهم المضادة للبكتيريا الموجبة لجرام والسالبة لجرام

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توضح الدراسة الحالية وجود علاقة إيجابية بين قدرة تحمل معدن الزنك للفطريات المعزولة من التربة وقدرتها على تخليق جزيئات نانو أكسيد الزنك (ZnO) وتم عزل ٥ أنواع فطرية من ريزوسفير بعض النباتات و التي تنمو بشكل طبيعي في محافظة الشرقية في مصر وتم تعريفها بناءا علي الخصائص المورفولوجية وكانت هذه العزلات تنتمي إلى *Aspergillus niger* (An), *Aspergillus tubulin* (At), *Aspergillus fumigatus*(Af), *Penicillium citrinum* (Pc) and *Fusarium oxysporum* (Fo) وقد استخدمت هذه العزلات في تخليق جسيمات أكسيد الزنك النانوية، (An-ZnO-NPs و At-ZnO-NPs و Af-ZnO-NPs و Pc- ZnO-NPs و Fo- ZnO-NPs) باستخدام كبريتات الزنك مقارنة إلى سلالات المراجع من *A. tubingensis* Mosseray AUMC No.6915, *A. fumigatus* Fresenius AUMC No.48 and *A. terreus* Thom AUMC No.75 ZnO-NPs وقد تم اختبار وجود ال ZnO-NPs المتكون من خلال الكشف عن ذروة امتصاص ضوئي ملحوظ عند ٢٨٥ إلى ٢٩٦ نانومتر، والتي تظهر في أطياف-UV Vis بسبب ظاهرة صدق السطح البلازمي، وأظهرت نتائج الميكروسكوب الالكتروني النافذ أن جزيئات النانو زنك وهي An-ZnO-NPs و At-ZnO-NPs و Af-ZnO-NPs و Pc- ZnO-NPs و Fo- ZnO-NPs كانت ذو تركيب بلوري علي شكل سداسي الاضلاع (٣٠ إلى ١٠٠ نانومتر) ولقد اظهرت جسيمات الزنك النانوية نشاطاً ممتازاً مضاداً للبكتيريا المختبرة ضد كلا من البكتيريا الموجبة لجرام والسالبة لجرام. وقد أظهرت جزيئات النانو نشاطاً مضاداً ضد البكتيريا الموجبة لجرام *S. aureus* و *L. monocytogenes* وبشكل أفضل مقارنةً بالبكتيريا السالبة لجرام *S. aureus* و *E. coli* و *P. aerogenosa*. تم تحليل فعالية تثبيط الأغشية الحيوية الميكروبية لـ *S. aureus* و *L. monocytogenes* و *B. cereus* مقارنة بـ *S. enterica* و *E. coli* و *P. aerogenosa* بتركيز ١٠٠ ميكروجرام / مل.

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