Research Article **CODEN: IJRPJK** **ISSN: 2319 – 9563**

International Journal of Research in Pharmaceutical and Nano Sciences

Journal homepage: **www.ijrpns.com**

ECO-FRIENDLY APPROACH FOR BIOSYNTHESIS OF ZINC OXIDE NANOPARTICLES USING SOME SOIL FUNGI

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ABSTRACT

Biosynthesis of zinc oxide nanoparticles using fungi as an environmentally benign approach is of prime interest due to the vast rate of applications of these nanoparticles and due to the excellent fermentation characteristics of fungi. *Aspergillus carneus* was selected in a preliminary screening of twenty fungal species isolated from the Egyptian soil. The zinc oxide nano-colloidal solution of this fungus revealed a characteristic turbid yellow color with absorption intense peak at 310 nm. The fungus reached its highest yield of the nanoparticles when its mycelium formed on Sabouraud's medium was contacted with 1mM solution of zinc nitrate adjusted at pH 9 under submerged conditions after 24 h at 30^{o}C. The biosynthesized ZnO NPs showed quasi-spherical shape from the high resolution-transmission electron micrograph. The biosynthesized zinc oxide nanoparticles were confirmed to have an antibacterial activity against four different human pathogenic bacteria.

KEYWORDS

Zinc oxide, Nanoparticles, Fungi and Antibacterial activity.

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INTRODUCTION

Nanotechnology is a multidisciplinary promising area that influences human life and the environment. One active and pronounced area of research in nanotechnology is the production, characterization and manipulation of nanoparticles (NPs). The NPs is the stone corner of the different applications of nanotechnology. They have many applications as starting point in chemistry, electronics, medicine and biotechnology for preparing many nanostructured materials and

devices[1]. Great efforts of many researchers all over the world are devoted for the biosynthesis of metal NPs since the beginning of the new millennium. Most works were on either silver or gold NPs. The green synthesis of both types of NPs was achieved using different plant extracts, bacteria and fungi.

A relatively few numbers of workers are interested in the biosynthesis of other metal and metal oxide NPs. These metal and metal oxides have attracted increased attention over the last decade due to their ability to withstand harsh process conditions[2]. Zinc is well known as an important metal involved in different metabolic processes. It is an integral component of many enzyme structures and is the only metal present in structure of enzymes of all classes[3]. Metal oxides-including zinc oxide (ZnO)-are stable under harsh process conditions. They also regarded as safe material to human beings and animals[4]. Moreover, ZnO has attracted wide interest because of its good photo catalytic activity, high stability, antibacterial property and nontoxicity[5]. Of the oxide NPs, ZnO NPs received some attention in the last years. These ZnO NPs can be used in various fields of application due to their unique characteristics like good conductivity, chemical stability, catalytic property, photonics and optoelectronics in addition to antimicrobial and UV filtering properties[6-8].The main advantages of ZnO NPs are their excellent stability with organic antimicrobial agents[9]. They are used in next-generation biological applications as chemotherapeutic agents[10-12].

ZnO NPs can be synthesized using physical or chemical methods. The first methods are expensive[13] at the time that the second use toxic chemicals that may have adverse effects in medical application[14-16]. On the other hand, the biological methods are cost effective and safe technique avoiding disadvantages of the other methods. They can use plant extracts for the biosynthesis of ZnO NPs[17-19]. Role of microorganisms especially fungi in this biosynthetic process is so far limited. Although some fungi are utilized for the biosynthesis of ZnO NPs[20-22], yields and characteristics of the synthesized NPs are not sufficiently suitable to be used in different fields of applications particularly in the biological and the medical fields.

This work was performed to study the potentiality of some fungi isolated from the Egyptian soil aiming to obtain an organism with good potentiality for biosynthesis of ZnO NPs. The best favorable parameters controlling production of the NPs by the most promising fungus has been optimized. Exploitation of the biosynthesized ZnO NPs as an alternative antibacterial agent was also investigated.

MATERIAL AND METHODS

Organisms and cultivation

Twenty different fungal species and strains were isolated in this work from soil samples collected from certain localities of Egypt. These fungi were identified by Assuit University Mycological Center (AUMC) where they were deposited with their accession numbers. The isolated fungi were grown on Capek's- Dox agar medium at 30^{o}C and monthly sub-cultured. The fungi were screened using triplicate sets of 250 ml Erlenmeyer flasks each containing 50 ml, of Capek's-Dox broth of the following composition (g/100 ml) sucrose, 3; $NaNO_3$, 0.2; KH_2PO_4, 0.1; KCl, 0.05; $MgSO_4.7H_2O$, 0.05 and $FeSO_4.5H_2O$, 0.001. The flasks were sterilized, cooled, inoculated with one ml of spore suspension (~10^6 conidia) obtained from 7-day-old cultures and finally incubated in the dark on rotary shaker adjusted at 150 rpm for 72 h. At the end of the incubation period, the biomass of each fungus was separated by filtration using Whatman No.1 from the culture supernatant (CS) that then centrifuged at 3000 rpm for 10 min.

Biosynthesis of ZnO NPs

For each fungus, both biomass and CS were used for the biosynthesis of the investigated NPs by adding them separately to 1 mM zinc nitrate solution ($ZnNO_3$. $6H_2O$). Typically 10 g fresh biomass were brought in contact with 90 ml of 1 mM of zinc nitrate, kept on the rotary shaker at 30^{o}C and agitated at a velocity of 150 rpm for 24 h in the dark. Both positive (biomass in deionized water) and negative (1mM $Zn(NO_3)_2.6H_2O$)

controls were run along with the experimental flasks. Moreover, triplicate sets of 250 ml Erlenmeyer flasks each containing 90 ml of the CS and10 ml of 10 mM 1mM $Zn(NO_3)_2.6H_2O$in deionized water was added and mixed well so the final concentration of $Zn(NO_3)_2.6H_2O$would be 1mM. Simultaneously, a positive control (CS) and negative control (1mM $Zn(NO_3)_2.6H_2O$) was also checked for comparison. All sets were kept under agitation (150 rpm) at 30ºC in the dark.

Optimization of NPs biosynthesis

A series of experiments were conducted in a trial to increase the yield of the NPs biosynthesized by the most potent fungus. The first step was cultivating the selected fungus on four different fermentation media beside the Czapek-s-Dox medium that used in the screening. These media were (g/ 100ml): MGYP (malt extract, 0.3; glucose, 1; yeast extract, 0.3 and peptone, 0.5), Sabouraud's (dextrose, 4 and peptone 1), semi- definesd (KH_2PO_4, 0.7; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.01; yeast extract, 0.06 and glucose, 1) and potato- dextrose (potatoes infusion 20 and dextrose 2.).The second step was optimization of the reaction conditions. Influence of pH values of the reaction mixture (fungal biomass in 1mM $Zn(NO_3)_2.6\ H_2O$) on the biosynthesis process was studied by incubating different sets of flasks containing the reaction mixture as well as the two controls in the range of 6-11 and the work was completed as above. To study the effect of reaction temperature on formation of the ZnO NPs, the reaction mixtures adjusted at pH 9 along with the controls were separately incubated at different temperatures under the previously specified conditions. Effect of different concentrations of the salt was then ascertained where the pH was adjusted at 9 and incubation was lasted for 24 h at 30ºC. In the last step, the biosynthesis of the investigated NPs was followed at different periods of incubation under the best favorable conditions.

Characterization of the biosynthesized ZnO NPs

Any change in color from colorless to yellow or yellowish with slight turbidity was taken as a preliminary sign for the biosynthesis of these NPs. Spectrophotometric analysis was then used to confirm formation of these NPs. The biosynthesized NPs colloidal solution was firstly filtered through 0.22µm membrane filter (Millex-GS, Millipore, Madrid, Spain). Absorption measurements were carried out at wavelengths from 200 to 800 nm using a double beam spectrophotometer (Metash UV-Vis, model UV-8500) at a resolution of 1 nm. Morphology of ZnO NPs was performed in central lab of national research center (NRC), Dokki, Giza using high resolution-transmission electron microscope (HR-TEM). For this purpose, an aliquot of the aqueous suspension of ZnO NPs was transferred onto a carbon coated copper grid. Samples were dried and kept under vacuum in desiccators before loading them onto a specimen holder. The grid was then scanned using a Jeol JEM-2100 (Made in Japan Model Year 2000) operated at a voltage of 200 kV.

Antibacterial activity of the produced CuO NPs

Antibacterial assay

The ZnO NPs suspended in deionized water were examined for their antibacterial activity by a standardized single disk method[23]. Four bacterial strains, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Klebsiella pnemonia* ATCC 27736 and *Staphylococcus aureus* ATCC 8538 were used for this assay. Triplicate sets of Petri plates containing 20 ml of melted nutrient agar medium (5-6 mm in depth) were used. The bacterial strains were grown on nutrient broth medium for 24 h prior to the experiment. One ml of the bacterial suspension containing 10^7 colony forming units (cfu) was mixed well with the medium by gently swirling the plates on the table top. Sterile filter paper discs approximately 5 mm in diameter were impregnated with solutions of ZnO NPs and positive controls, dried, placed on the surface of the solidified seeded medium and gently pressed down to ensure contact. The plates were incubated at 37ºC for 24 h and diameters of the inhibition zones were measured and expressed as the mean values along with the standard deviation. Ciprofloxacin was used as standard for the antibacterial activity.

Determination of minimal inhibitory concentrations (MIC)

The micro dilution method in culture broth **w**as used for determination of MIC which is the lowest concentration of the NPs that did not permit any visible bacterial growth of after 24 h of incubation. Tested bacterial strains were refreshed on nutrient agar by sub-culturing under sterile incubated at 37ºC for 24 h statically. Different concentrations of ZnO NPs (10-100 µg/ml) prepared using deionized water were added to conical flasks of 50 ml capacity each contain 10 ml of sterilized Mueller Hinton broth. One flask devoid of NPs was used as negative control and other flask devoid of inoculum was used as a positive control. All flasks were inoculated with approximately 10^7 cfu/ml of actively dividing bacterial cells. All experimental flasks were incubated on rotary shaker adjusted at 150 rpm and 37ºC for 24 h. At the end of the incubation period, absorbency value of OD 600 nm was determined. All experiments were performed in triplicate and the averages were obtained.

RESULTS AND DISCUSSION

Screening

The work was started with screening of the isolated fungi to test their potentiality for biosynthesis of the extracelluar ZnO NPs. Twenty different fungal species belonging to ten genera were investigated for biosynthesis of CuO NPs. The results (Table No.1) demonstrate that some of isolated fungi had the ability for synthesis of ZnO NPs when using their CS or biomass. *Aspergillus flavus var.columnaris* represents the first case (using CS), *Corynoascus sepedonium* and *Penicillium aurantiogriseum* represent the second case (using biomass). Moreover, four different fungi were recorded to be capable of biosynthesis of ZnO NPs using both CS and biomass. They were *Alternaria alternata*, *A. aureoterreus, A. carneus* and *A. sydowii*. Analysis of the UV-Viz spectra for the NPs produced by the other studied fungi clearly shows that there is fluctuation in their maximum absorption ranged from 310 for biomass of the fungus *A. carn*eus, 315 nm for biomass of the fungus *P. brevicompactus*, 335 nm for *Alternaria alternata* CS, 345 nm for the fungus *A. aureoterreus* CS and 355 nm for CS of *A. flavus var. columnar is*. There are different shapes of the absorption peaks *i.e.* intense, good, broad and flattened. The most potential case was that of *A. carneus* biomass. The UV-Viz spectrophotometric analysis for NPs revealed an intense peak with maximal absorption at 310 nm.

Optimization of the biosynthesis of ZnO NPs using *A. carneus*

Optimizing the conditions required for the best synthesis of ZnO NPs using the preformed biomass from *A. carneus* was achieved in two steps. In the 1st, thebiosynthesis of ZnO NPs was achieved after growing the fungus on five different media.The data recorded (Table No.2) reveal that the potentiality of *A. carneus* biomass was greatly affected on different fermentation media. No synthesis of the investigated NPs was occuurred on MGYP medium. The best biosynthesis was achieved on Sabouraud's medium. The maximum absorption of the produced NPs was recorded at 310 nm with an intense absorption peak. The reaction conditions affecting the biosynthesis of ZnO NPs by *A. carneus* were investigated in the 2nd step. pH of the reaction fluid was adjusted at different values varied from 6 to 11 to test its effect on the biosynthesis ability of the biomass of the investigated fungus. The results (Figure No.1) demonstrate an increase in the formation of the NPs by increasing the pH towards alkalinity and reached their maximal at pH 9. Increasing of pH value towards alkalinity may cause more competition between protons and metal ions for negatively charged binding sites resulting in better synthesis of the NPs. This is in complete accord with a previous suggestion[24]. An intense absorption peak was formed at the optimum pH value with a maximum at 310 nm. A decrease in the yield formation was recorded on both sides of the optimum.

The effect of reaction temperature on the biosynthesis of ZnO NPs by *A. carneus* biomass was achieves in the temperature range from 28ºC to 40ºC. The results (Figure No.2) revealed that the

optimum formation of the NPs was at 30ºC. Further increase above the optimum temperature slowed down the biosynthesis process. This can be explained on the basis of the mesophilic characteristics of the experimented fungus. The absorption spectrum of the formed NPs and their type of peak was also affected. While the maximum absorption at 30ºC was 310 nm with an intense peak, it was blue shifted to 300 nm and 305 nm at 35ºCand 40ºC with different peaks, respectively.

Different concentrations of zinc nitrate were then used to obtain the most suitable concentration. The results (Figure No.3) demonstrate that 1mM solution was the best. It is of interest to note that the absorption spectrum of the formed NPs was greatly changed towards red shift reaching 350 nm with a hump-like peak when 0.5 mM solution was used. On the other hand, formation of NPs was prevented at 5 mM and this may be due to the intolerance of the fungus to the high salt concentration. A similar explanation was stated previously[21].

Finally, the biosynthesis of these NPs was followed at different periods of incubation (Figure No.4). A slow biosynthesis was observed in the first 12 h where it being initiated sluggishly but the biosynthesis process was strongly accelerated in the following hrs reaching its maximal after 24 h with intense absorption peak at 310 nm.

It is of interest to note that no other peaks were recorded in the absorption spectrum suggesting complete purity of the biosynthesized ZnO NPs. HR-TEM was used to determine the morphology details of the biosynthesized NPs. The micrograph (Figure No.5) reveals that the NPs are quasi-spherical in shape without any signs of aggregation.

Antibacterial activity of the biosynthesized ZnO NPs

The antibacterial activity of ZnO NPs biosynthesized using the preformed biomass of *A. carneus* was tested using disk diffusion qualitative method against four bacterial strains i.e. *B. subtilis* ATCC 6633, *E. coli* ATCC 8739, *K. pnemonia* ATCC 27736 and *S. aureus* ATCC 8538. Diameters of the inhibition zones measured in mm are shown in Table No.3. Photograph for this application was demonstrated in Figure No.6. The antibacterial activity of the biosynthesized ZnO NPs expressed a fluctuating effect against various test organisms but the effect was more pronounced against the Gram +ve bacteria. They showed the higher activity against *S. aureus* (20.6 ± 0.27) followed by *B. subtilis* (17.6 ± 0.41). The activity was moderate against *E. coli* (16.6 ± 0.25) and *K. pnemonia* (16.0 ± 0.40). Effect of the reference antibiotic ciprofloxacin accord completely with the previous trend but was more effective.

The antibacterial activity against the four bacterial strains was then studied quantitatively in term of MIC. The results (Figure No.7) revealed a fluctuation in the MIC of ZnO NPs towards them. It was varied from 40µg/ml for the *B. subtilis*, to 50µg/ml for *S. aureus* and *E.coli* and 60µg/ml for *K. pneumonia*. These results are in coincidence with previous findings[25,26] and can be attributed to the difference of cell wall composition[1]. The antibacterial activity of the NPs was rendered to some reasons but their precise mechanism is yet to be fully understood. Generation of excess free radicals and formation of oxidative stress was proposed by some workers[26-29]. The small particle size was proposed as an additional factor in the antibacterial activity of the NPs due to their large surface area to volume ratio[30].

Table No.1: potentiality of the Isolated Soil Fungi in Biosynthesis of ZnO NPs

Fungus	Source	Formation of NPs	Yield (Au)	SPR band (nm)	Type of peak
Alternaria alternata (Fries) Keissler AUMC 13015	Biomass	±	0.20	370	Good
	Cs	++	1.06	335	Good
Aspergillus aureoterreus Samson *et al.* AUMC 13006	Biomass	±	0.30	365	Broad
	CS	±	0.21	345	Feeble
Aspergillus carneus Blochwitz AUMC 13007	Biomass	++	1.44	310	Intense
	CS	+	0.45	350	Broad
Aspergillus flavus Link AUMC 8653	Biomass	-	-	-	-
	CS	-	-	-	-
Aspergillus flavus var. columnaris Raper and Fennell AUMC 13012	Biomass	-	-	-	-
	CS	+	0.72	355	Broad
Aspergillus fumigatus Fresenius AUMC 13024	Biomass	-	-	-	-
	CS	-	-	-	-
Aspergillus niger Van Tieghem AUMC 13022	Biomass	-	-	-	-
	CS	-	-	-	-
Aspergillus sydowii (Bainier and Sartory) Thom and Church	Biomass	+	0.81	335	Flattened
	CS	±	0.20	335	Broad
Aspergillus terreus Thom AUMC 13019	Biomass	-	-	-	-
	CS	-	-	-	-
Cladosporium cladosporioides (Fresenius) de Varies AUMC 13021	Biomass	-	-	-	-
	CS	-	-	-	-
Corynoascus sepedonium (Emmons) Von Arx AUMC 13016	Biomass	+	0.62	365	Good
	CS	-	0.31	390	Broad
Emericella nidulans (Eidam) Vuillemin AUMC 8623	Biomass	-	-	-	-
	CS	-	-	-	-
Eupenicillium hirayamae Sott and Stolk AUMC 13009	Biomass	-	-	-	-
	CS	-	-	-	-
Fusarium semitectum Berkeley AUMC 13018	Biomass	-	-	-	-
	CS	-	-	-	-
Fusarium subglutinans (wollenweber and Reinking) Nelson *et al.* AUMC 13008	Biomass	-	-	-	-
	CS	-	-	-	-
Humicola grisea Traaen AUMC 13020	Biomass	-	-	-	-
	CS	-	-	-	-
Penicillium aurantiogriseum Dierckx AUMC 13013	Biomass	±	0.10	350	Broad
	CS	-	-	-	-
Penicillium brevicompactum Dierckx AUMC 13014	Biomass	+	0.90	315	Intense
	CS	±	0.30	305	Broad
Penicillium pinophilum Hedgcock AUMC 13011	Biomass	-	-	-	-
	CS	+	-	-	-
Scedosporium apiospermum (Sacc.) Sacc. AUMC 13017	Biomass	-	-	-	-
	CS	-	-	-	-

Table No.2: Biosynthesis of ZnO NPs using biomass from *A. carneus* grown on five different media

S.No	Medium	Formation of NPs	Yield (Au)	SPR (band)	Type of peak
1	Czapek's	++	1.44	310	Intense
2	MGYP	-	-	-	-
3	Potato – dextrose	+	0.63	310	Broad
4	Sabouraud's	+++	1.61	310	Intense
5	Semi- defined	+	0.88	305	Broad

Table No.3: Size of inhibition zone for ZnO NPs from *A. carneus* biomass against some bacterial strains in comparison with ciprofloxacin

S.No	Test organisms	Inhibition Zone (mm)	
		NPs	AB
1	*B. subtilis* ATCC 6633	17.6 ± 0.41	21.3 ± 0.31
2	*E. coli* ATCC 8739	16.6 ± 0.25	18.3 ± 0.24
3	*K. pneumonia* ATCC 27736	16.0 ± 0.40	16.6 ± 0.37
4	*S. aureus* ATCC 8538	20.6 ± 0.27	22.0 ± 0.32

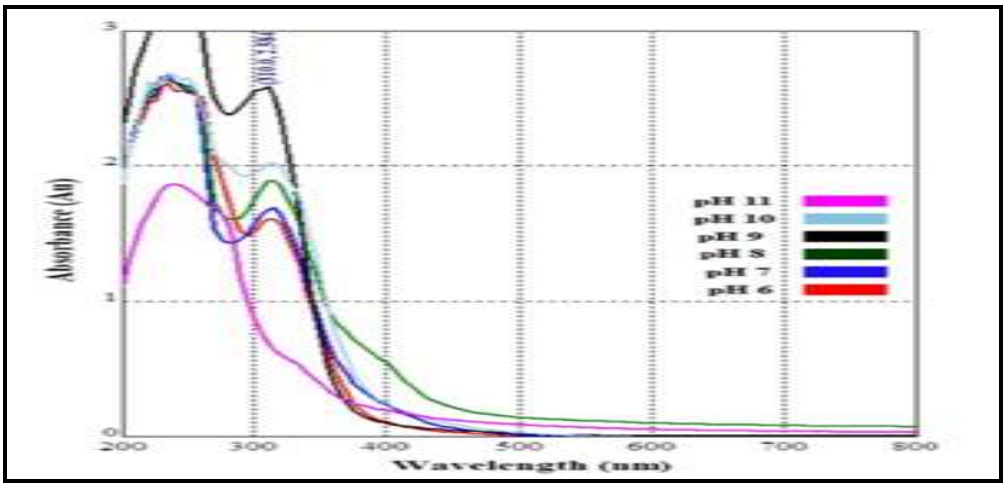

Figure No.1: Biosynthesis of ZnO NPs at different pH values using *A. carneus* biomass

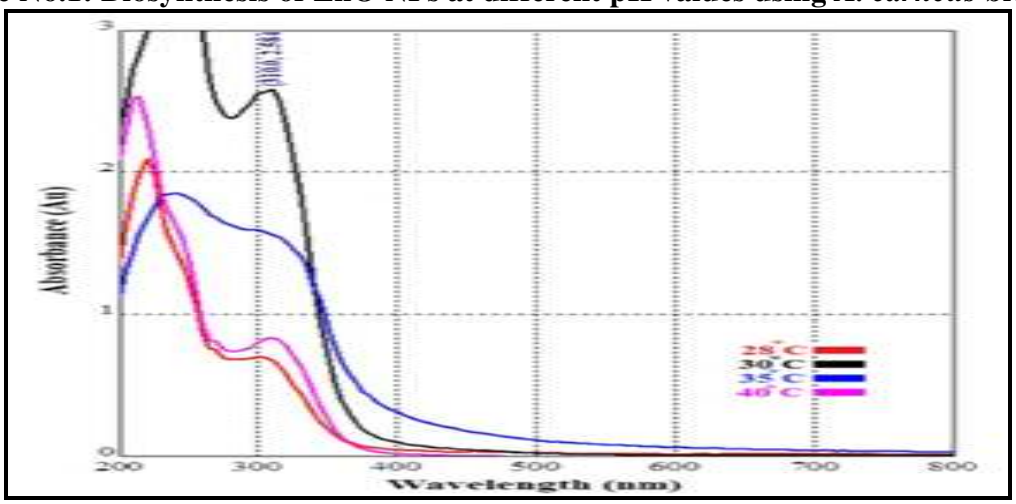

Figure No.2: Biosynthesis of ZnO NPs using *A. carneus* as influenced by different reaction temperatures

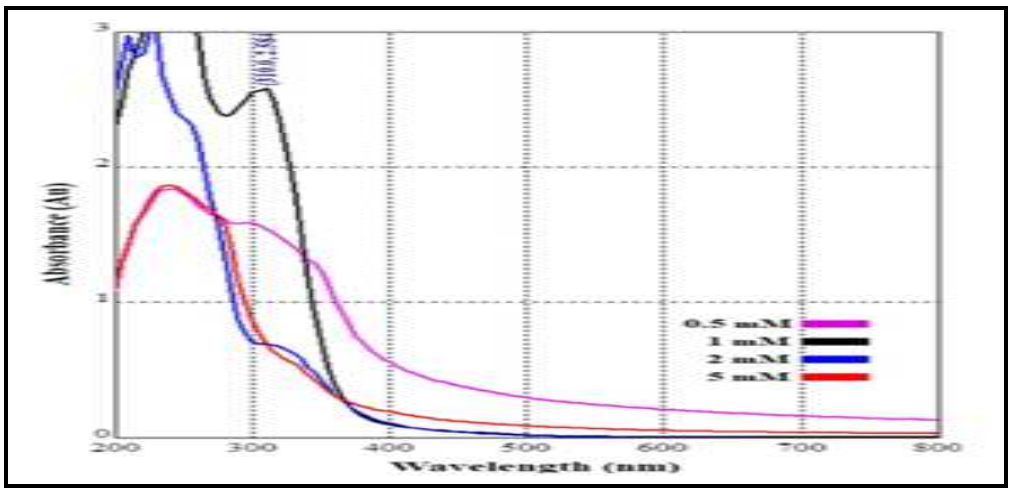

Figure No.3: Biosynthesis of ZnO NPs using *A. carneus* biomass incubated with different concentrations of zinc nitrate

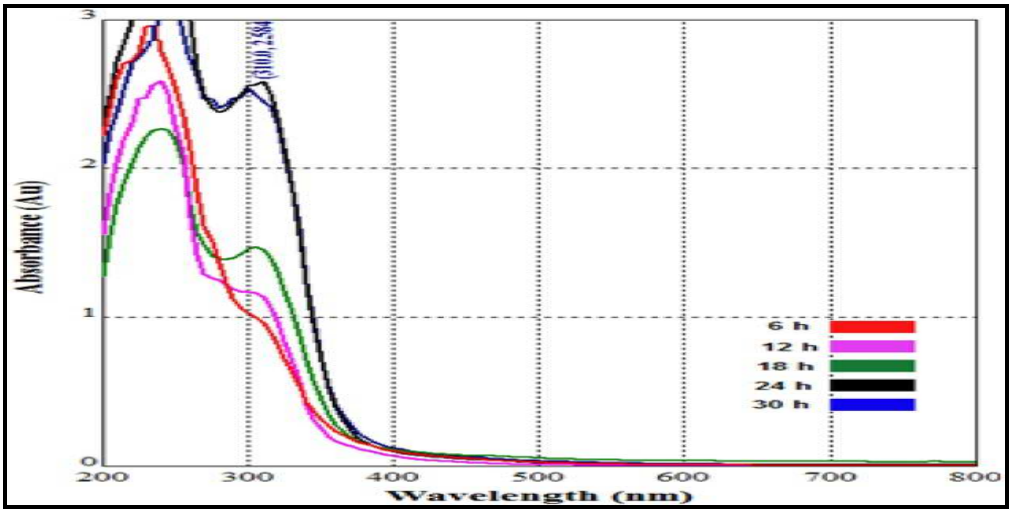

Figure No.4: Biosynthesis of ZnO NPs using *A. carneus* biomass after different periods of incubation

Figure No.5: High magnification HR-TEM micrograph of ZnO NPs biosynthesized by *A. carneus* biomass

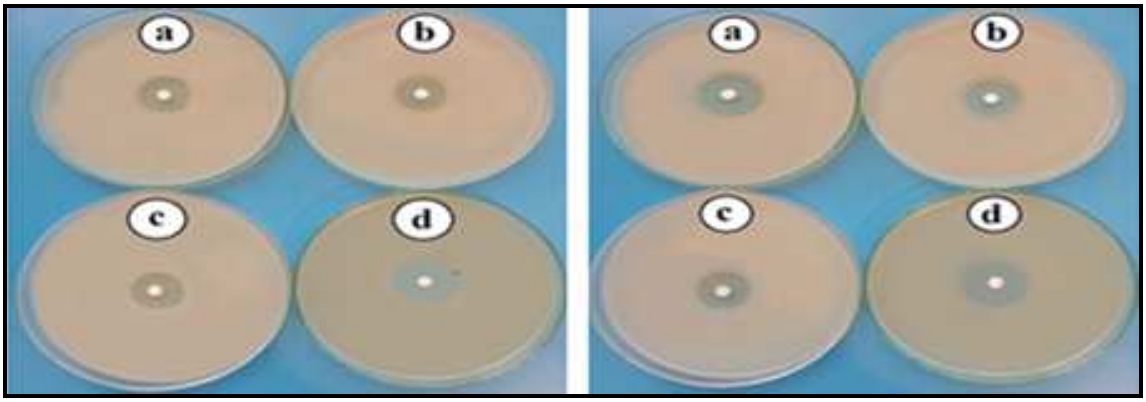

Figure No.6: Antibacterial activity of ZnO NPs biosynthesized by A. carneus biomass (Left) and the antibiotic ciprofloxacin (Right) against four different bacterial strains *B. subtilis* (a), *E. coli* (b), *K. pneumonia* (c) and *S. aureus* (d)

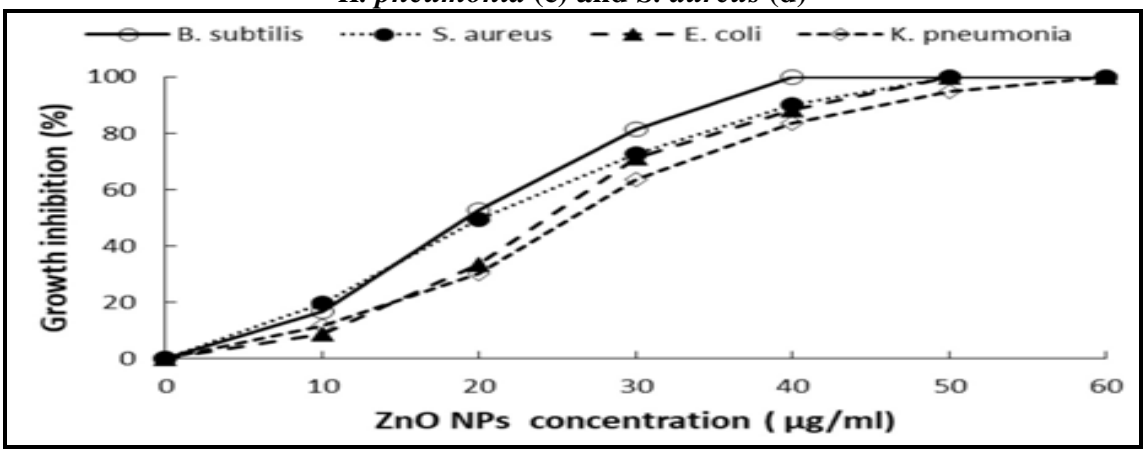

Figure No.7: Antibacterial activity of ZnO NPs biosynthesized by A. carneus biomass against four different bacterial strains

CONCLUSION

Aspergillus carneus was selected in screening program of twenty fungi isolated from the Egyptian soil as promising source for biosynthesis of zinc oxide nanoparticles. The synthesis process using this fungus was found to be affected by the reaction conditions. The produced nanoparticles were confirmed to have antibacterial activity against four different human bacterial pathogens.

ACKNOWLEDGEMENT

The authors wish to express their sincere gratitude to Department of Biological and Geological Sciences, Faculty of Education, Ain Shams University, Roxy 11757, Cairo, Egypt for providing necessary facilities to carry out this research work.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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Please cite this article in press as: Mohamed Ghareib *et al.* Eco-friendly approach for biosynthesis of zinc oxide nanoparticles using some soil fungi, *International Journal* of *Research in Pharmaceutical and Nano Sciences,* 7(3), 2018, 108-118.