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Biological control by using bio- zno nanoparticles from *Aspergillus niger* and studying their inhibitor performance as antimicrobial agent

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ZnONPs showed significance antibacterial effects using MIC methods; the highest antibacterial activity was proved against E.coli and the same effect in *Staphylococcus aureus and Pseudomonas aeruginosa while the more resistance in Staphylococcus aureus* MRSA and *Escherichia coli* ATCC 25922.*Aspergillus brasiliensis (niger)*ATCC16404 produced ZnO nanoparticles (ZnO NPs), against pathogenic bacterial strains i.e *Staphylococcus aureus* MRSA(ATCC 43300), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* (ATCC 27853).ZnONPs application as immersion solution, for maintenance chicken eggshells, resulted in significant decrease in microbial load on the shells of 1.9 $\log^{10}$ CFU/eggshell. Consequences it could present an environmental advance in support of prospective antimicrobial to be used in food maintenance and supplementary physical condition security researches.

Keywords: ZnO nanoparticles, *Aspergillus niger*, eggshells and pathogenic bacteria

INTRODUCTION

Microbial infections have develop into a global health burden appropriate to promising and resistant strains of pathogenic fungi viruses, bacteria and protozoa defying clinical management. so, this has culminated into prolonged treatment, advanced health expenditure, humanity risk, and low life expectation (Inbaneson et al.,2011)

Nanotechnology is rising in Varity field of research interspersing objects science, bio nano-science, and tools (**Gutierrez et al., 2010)**

Nanoparticles used current cell membranes functions such as respiration and permeability. In addition, penetrating into bacterial cell, nanoparticles can also disturb sulfur-containing proteins function and phosphorus-containing compounds (Sangeetha et al., 2012)

Different pathogenic bacteria caused by infectious diseases and antibiotic resistance increase for the pharmaceutical companies. The researchers are discovering for new antibacterial agents. In the present situation, nanoscale materials have emerged up as original antimicrobial agents outstanding to their high surface area to volume ratio and the sole chemical and physical properties (Gao et al., 2008)

ZnO NPs creation with a wide range of sizes, compositions and shapes has been established by a variety of physical, biological and chemical methods. They create nanoparticles, that are intra

and/or extracellular in nature (Lakshmi et al., 2012)

MATERIALS AND METHODS

Soil samples were collected from farmer university of Sadat city in Sadat city, Egypt. They were treatments of pathogenic bacteria as *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* MRSA(ATCC 43300) and *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922,

Samples Collections

One gram of soil samples were serially diluted to 10^{-4} and dispensed on to petri-plates component potato dextrose medium that is especially used for cultivation of fungi and incubated at 28^{o} C for 72 h in incubator. Cultures were recognized on the origin of morphological and Biochemical test using BIOLOG kit. They were identified according to colony characteristics and microscopic examination.

ZnO Nanoparticles Synthesis from fungi

ZnO nanoparticles were organized by using various modification in the method of Jacob et al.**,** **(**Jacob et al., 2014). Fungus was cultivation in Potato Dextrose broth (Difco, USA) used 250 mL Erlenmeyer flask with agitating by orbital shaker at 150 rpm, 28°C for 72 h. fungal supernatant was obtained by passing through Whatman No.1. then 10 mL of supernatant was diverse with 100 mL of 0.25 mM solution of $ZnSO_4$ (Sigma Aldrich), in 250 mL Erlenmeyer flask and cultivation at 32°C with agitation at 150 rpm for 96 h. ZnO NPs was obtained throw the fungal mycelium centrifuge at 3000 rpm for 15 mint., then the participation was drying in oven at 60°C and harvested the ZnO NPs and weighted (Jones et al., 2008)

Characterization of ZnO NPs

ZnO NPs characterization used the UV-Vis Spectrophotometer spectrum method to determine nanoparticles structural characterization by absorbance measurement (Gunalan et al.,2012)

Antimicrobial activities of ZnO NPs

ZnO NPs were conducted against pathogenic bacteria. Agar well diffusion was used in this study. Suspensions of pathogenic bacteria were freshly prepared by inoculating stock culture. The inoculated tubes were incubated at 37°C for 24 h. (Mashrai, etal.,2013)

Antimicrobial Activity

The technique was performed by agar well diffusion (Magaldi et al .,2004)the suspension bacterial cultivation for 24 h from each bacterial of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* ATCC 29213 and, *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus* MRSA(ATCC 43300). About 100 µL of each bacterial suspension was spreading by swabs onto surface of Mueller-Hinton agar plates and were left (10-15 min) until the surface of agar has dried. Then the wells were made into agar at 4 mm diameter using cork borer with the distance between well and another more than 22 mm. About 50 µL from 1.0 and 1.5 of ZnO NPs were injected in each wells. Then plates were incubated at 37°C for 24 h, inhibition diameters were recorded in millimeter using metric ruler.

Determination of minimum inhibitory concentration

MIC and MBC were considered by dilution tests. The target bacteria growth on nutrient broth with different ZnO nanoparticles concentrations (1000, 500, 250, 200, 150, 100, 50 and 20 µg/ml), bacteria growth were determined by spectrometer in each tube. 0.1 ml of sterile distilled water was additional to these tubes to fresh medium that had not any ZnO nanoparticles. The minimum concentration from which the bacteria do not cultivate while transferred to new medium is MBC and MIC is The minimum concentration from which the growth on tubes (Priyanka et al.,2009)

Biological control for eggshells

Eggshells decontamination was using ZnO NPs as disinfecting solutions (ZnO NPs DS) at different period. ZnO nanoparticles (170 µg/ml), was practical for eggshells disinfecting solution (ZnO NPs DS). Chicken eggs “A” were gated clean from the laying farm, USC, Egypt. (ZnO NPs DS) treatment was subjected to treat groups (5 eggs each) was immersed in ZnO NPs DS for a range of immersion times, that are 1, 3, and 5 min, air dried, after that each each egg was immersed in 50 ml from sterilized buffered peptone water (BPW), well stirred, plated onto nutrient agar (NA) plates and incubated at 37 °C for 24–28 h to factor viable microbial load on eggshells subsequent to each experience period. Distilled water was used for the control egg group immersion **(**Abeer mohammed , 2015)

RESULTS AND DISCUSSION

1 Isolation and Identification of Bacterial Isolates

Table 1, shows the isolated fungus from soil source, which were identified according to the microscopic and biochemical tests using BIOLOG kit. The most isolated fungi significantly was *Aspergillus niger,* the end result was in conformity with 1.They were identified according to colony characteristics and microscopic examination of fungi that demonstrate fungus shape, structure, agreement according to Castro-longoria et al.,2010 .as in Table 2.Then the identification was confirmed by BIOLOG kit Our results were compared with the resource reported by Haiss et al.,2007.

3.2 ZnO NPs produced by fungi

ZnO NPs Biosynthesis was produced by cultivation of *Aspergillus niger* on potato dextrose broth media. The range of ZnO NPs weight was at 46 mg the ability of *Aspergillus niger* on synthesis of nanoparticles from $ZnSO_4$ and reduced the particles to Nano size were depended on the enzymes and the metabolic pathways that needed from fungal isolate. The metabolic reactions were obtained with catalytic activity. ZnO suspension clearly has a much higher activity (Talebia et al .,2010)Though, increase of easy and ecological way would assist in promoting advance significance in the synthesis and relevance of Mg, Ti and ZnO nanoparticles. subsequent studies have indicated that NADH and NADH reliant enzymes are important factors in the metal nanoparticles biosynthesis. (Ahmad et al.,2003a and Ahmad et al.,2003b)

3.2.1 Characterization of ZnO NPs

Transmission Electron Microscopy (TEM) performed has provided more interested in the morphology and nanoparticles size details. It is able to detect of their purity and particle size revealed on the formation of mono and poly dispersed nanoparticles and ZnO NPs was appeared in different range of size 20 nm Figure. 2. Nanoparticles morphology is changeable with bulk material. TEM studies obviously establish which Zn ions reduction into extracellular, it would be significant to determine the reducing agents dependable for this reaction. (Rizwan et al.,2010)

The UV–Vis Spectrophotometer way were used to determine nanoparticles structures by absorbance size of ZnO NPs was appear at 380 nm absorption bands, Figure. 3. The absorption band was appearing as like the absorption peak for the results obtained by Mashrai et al., 2013.

3.3Antibacterial Activities of ZnO NPs

ZnO nanoparticles effect on pathogenic bacteria strain. The values of zone of inhibition obtained from the assay are presented in Figure 1. Among gram positive bacteria, the diameter of inhibition zone produced by zinc oxide nanoparticles at different concentration 0.5,1,1.5 and 2 µg/ml against *Staphylococcus aureus* MRSA(ATCC 43300) showed significant increase as compared to *Bacillus subtilis* (ATCC 6051).Among Gram negative bacteria, the diameter of inhibition zone produced by zinc oxide nanoparticles against *Staphylococcus aureus* ATCC 29213 showed significant increase as compared to *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* ATCC 25922. Zinc oxide nanoparticles exhibit strong antibacterial activity on both Gram positive and Gram negative bacteria (Maleki et al.,2015)

The antagonism activity of ZnO nanoparticles was tested by MIC method (Tables 4 A,B,C,E). Appear of an inhibition growth obviously determined ZnO nanoparticles antibacterial effect. (Rizwan et al., 2010). it has been observed while increasing ZnO nanoparticles concentration in tubes, the growth of pathogenic bacteria has been decreased. In additional, inhibition zone was different according to bacterial type and ZnO nanoparticles concentrations such as *Staphylococcus aureus* MRSA(ATCC 43300), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 subsequent to incubation overnight at different concentrations of ZnO nanoparticles was shown in Figure (4 A,B,C,D,E). The lowest concentration of ZnO nanoparticles that inhibited the growth of bacteria was 1000 µg/ml for *Staphylococcus aureus* MRSA , 150 µg/ml for *Escherichia coli,* 1000 µg/ml for *Bacillus subtilis,* 250 µg/ml for *Staphylococcus aureus* and 250 µg/ml for *Pseudomonas aeruginosa*. This is conformity with before study on the antibacterial effort of ZnO nanoparticles with lowest concentration according to MIC, MBC and disc agar diffusion methods. it signification that in comparison with Gram-positive bacteria is lower than is the gram-negative bacteria at the highest concentrations of ZnO nanoparticles (Figure 4 A,B,C,D,E). Tayel et al., 2011 and abeer Mohammed et al.,2017 have determined the same results, it has been anticipated that the higher

susceptibility of Gram-positive bacteria could be associated to differences in cell wall structure, cell physiology and metabolism or level of contact.

Table 1 TEM character of biosynthesized zinc oxide nanoparticles by *A. niger*

Properties:	
Appearance (Color)	**Whitish color.**
Appearance (Form)	Powder.
Solubility	Stable colloid in mixture of Methanol and chloroform
Optical Prop. (Abs.)	λmax = 301 nm, and 380 nm
Avg. Size (TEM)	20 ± 5 nm
Shape (TEM)	Spherical shape

A1 Water	A2 Tween 80	A3 N-Acetyl-D-Galactosamine	A4 N-Acetyl-D-Glucosamine	A5 N-Acetyl-D-Mannosamine	A6 Adonitol	A7 Amygdalin	A8 D-Arabinose	A9 L-Arabinose	A10 D-Arabitol	A11 Arbutin	A12 D-Cellobiose
B1 α-Cyclodextrin	B2 β-Cyclodextrin	B3 Dextrin	B4 I-Erythritol	B5 D-Fructose	B6 L-Fucose	B7 D-Galactose	B8 D-Galacturonic Acid	B9 Gentiobiose	B10 D-Gluconic Acid	B11 D-Glucosamine	B12 α-D-Glucose
C1 Glucose-1-Phosphate	C2 Glucuronamide	C3 D-Glucuronic Acid	C4 Glycerol	C5 Glycogen	C6 m-Inositol	C7 2-Keto-D-Gluconic Acid	C8 α-D-Lactose	C9 Lactulose	C10 Maltitol	C11 Maltose	C12 Maltotriose
D1 D-Mannitol	D2 D-Mannose	D3 D-Melezitose	D4 D-Melibiose	D5 α-Methyl-D-Galactoside	D6 β-Methyl-D-Galactoside	D7 α-Methyl-D-Glucoside	D8 β-Methyl-D-Glucoside	D9 Palatinose	D10 D-Psicose	D11 D-Raffinose	D12 L-Rhamnose
E1 D-Ribose	E2 Salicin	E3 Sedoheptulosan	E4 D-Sorbitol	E5 L-Sorbose	E6 Stachyose	E7 Sucrose	E8 D-Tagatose	E9 D-Trehalose	E10 Turanose	E11 Xylitol	E12 D-Xylose
F1 γ-Amino-butyric Acid	F2 Bromosuccinic Acid	F3 Fumaric Acid	F4 β-Hydroxy-butyric Acid	F5 γ-Hydroxy-butyric Acid	F6 p-Hydroxyphenyl-acetic Acid	F7 α-Keto-glutaric Acid	F8 D-Lactic Acid Methyl Ester	F9 L-Lactic Acid	F10 D-Malic Acid	F11 L-Malic Acid	F12 Quinic Acid
G1 D-Saccharic Acid	G2 Sebacic Acid	G3 Succinamic Acid	G4 Succinic Acid	G5 Succinic Acid Mono-Methyl Ester	G6 N-Acetyl-L-Glutamic Acid	G7 Alaninamide	G8 L-Alanine	G9 L-Alanyl-Glycine	G10 L-Asparagine	G11 L-Aspartic Acid	G12 L-Glutamic Acid
H1 Glycyl-L-Glutamic Acid	H2 L-Ornithine	H3 L-Phenylalanine	H4 L-Proline	H5 L-Pyroglutamic Acid	H6 L-Serine	H7 L-Threonine	H8 2-Amino Ethanol	H9 Putrescine	H10 Adenosine	H11 Uridine	H12 Adenosine-5'-Monophosphate

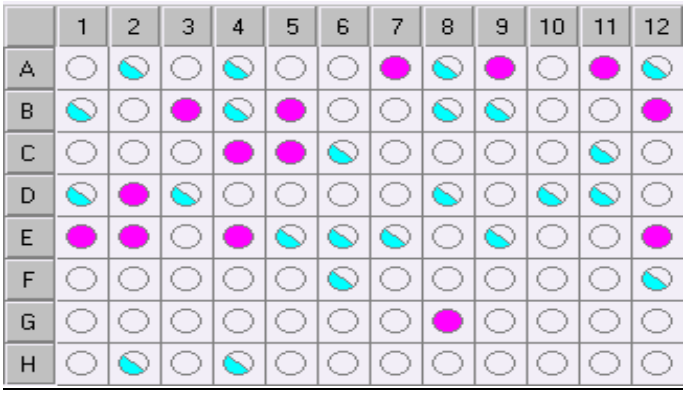

Figure1 BIOLOG result

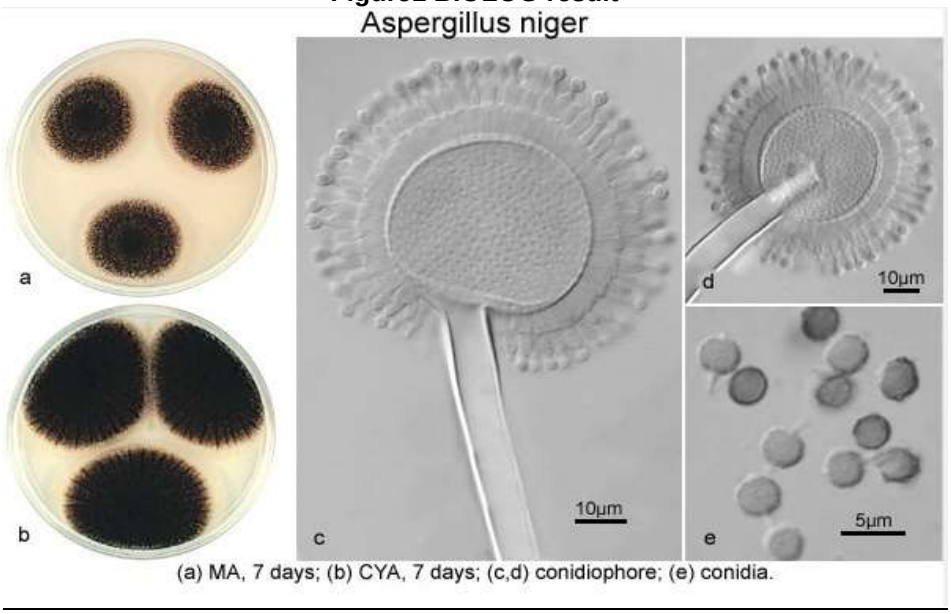

Figure.2. *Aspergillus niger* on the basis of morphological and Biochemical test using BIOLOG kit.

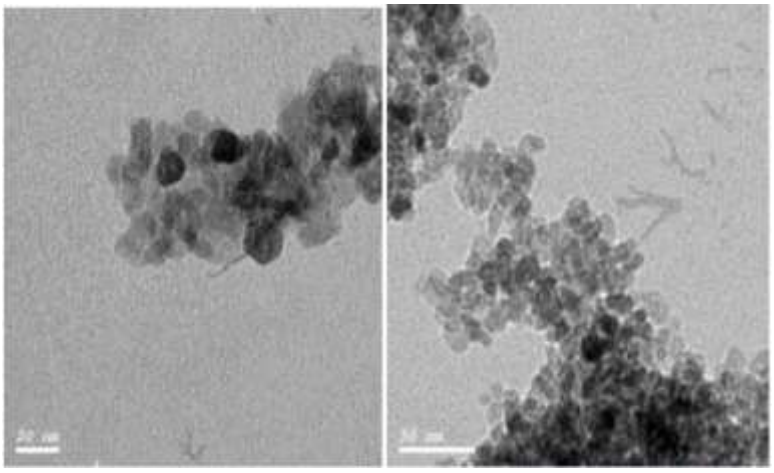

Figure. 3; TEM image of biosynthesized zinc oxide nanoparticles by A. niger

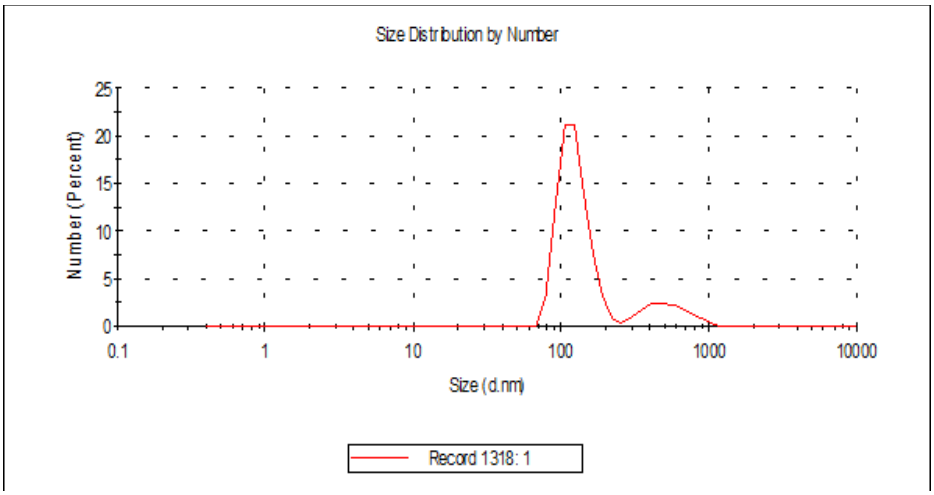

Figure. 4; DLS (dynamic light scattering) curve for average particle size, distribution ZnO nanoparticles synthesized by A. niger

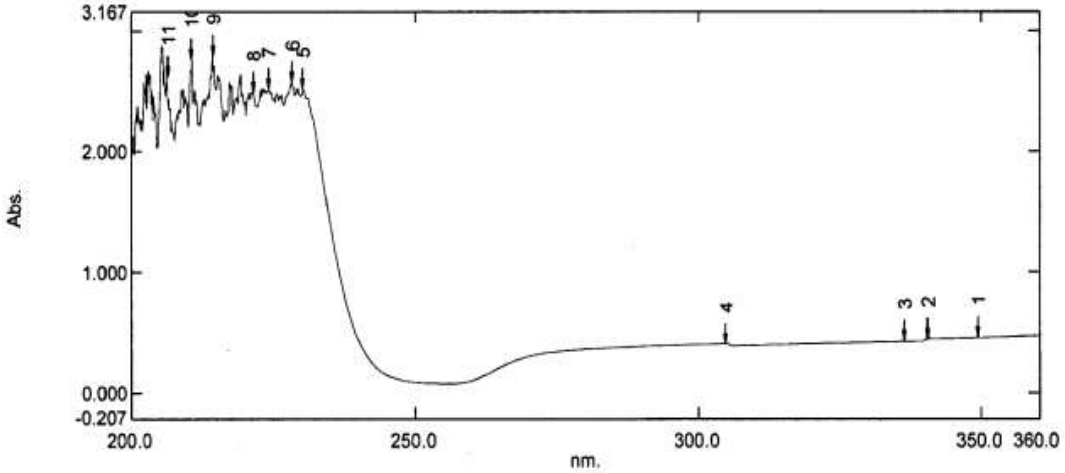

Figure. 5; The UV-Vis spectrum of ZnO nanoparticles synthesized by A. niger

Table 2. Antibacterial activity of ZnO NPs used well diffusion test at 0.5,1,1.5 and 2 µg mL^{-1} against on pathogenic bacteria

	Concentration of ZnO Nps (µg mL^{-1})	0.5 µg mL^{-1}	1 µg mL^{-1}	1.5 µg mL^{-1}	2 µg mL^{-1}
A	***Staphylococcus aureus MRSA ATCC 43300***	15	14	19	20
B	***Pseudomonas aeruginosa ATCC 27853***	18	19	20	21
C	***Escherichia coli ATCC 25922***	15	16	17	18
D	***Staphylococcus aureus ATCC 29213***	14	17	19	22
E	***Bacillus subtilis ATCC 6051***	15	17	18	23

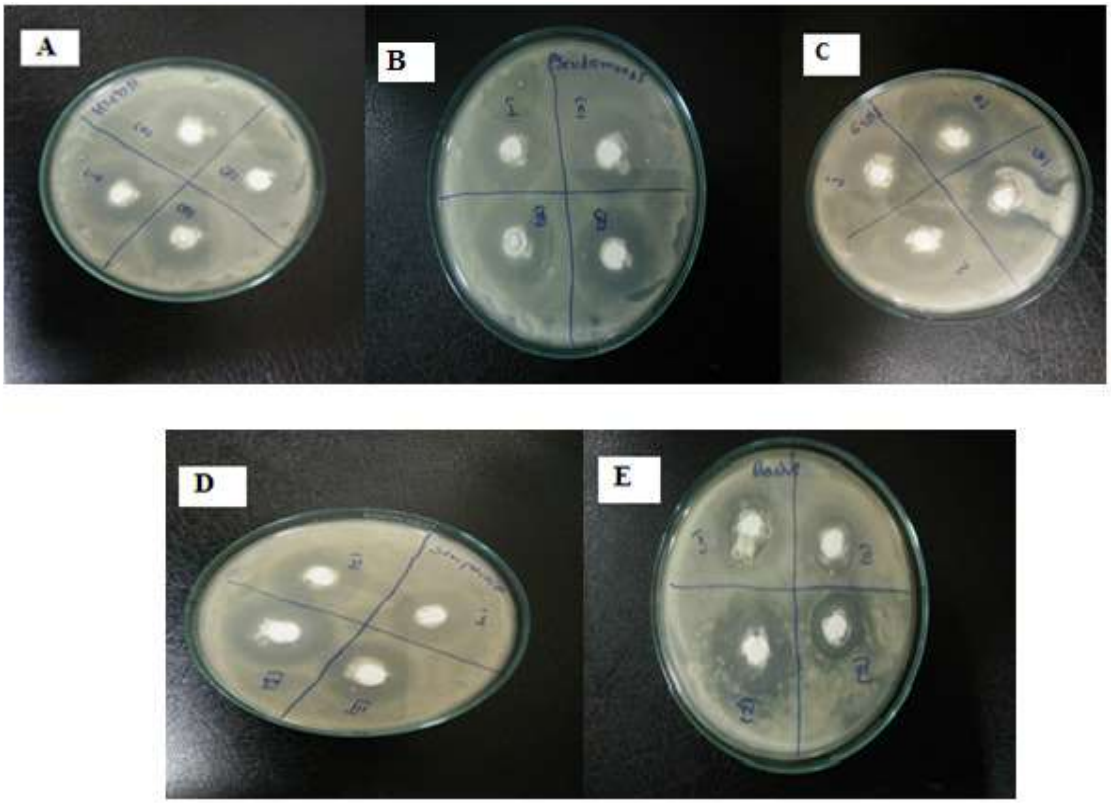

Figure.6; Antibacterial activity of ZnO NPs used well diffusion test (A) at 0.5,1,1.5 and 2 µg mL^{-1} against on (A) *Staphylococcus aureus* MRSA(ATCC 43300),(B) *Pseudomonas aeruginosa* (ATCC 27853), (C) *Escherichia coli* ATCC 25922, (D) *Staphylococcus aureus* ATCC 29213,(E) *Bacillus subtilis* (ATCC 6051).

Table 4. Antibacterial activity of ZnO NPs used MIC assay at different concentration µg mL^{-1} against pathogenic bacteria.

A. *Pseudomonas aeruginosa* ATCC 27853 MIC result

Conc. µg/ml	Pseudomonas aeruginosa			Mean
1000	0.040	0.045	0.043	0.043
500	0.039	0.047	0.039	0.042
250	**0.059**	**0.060**	**0.055**	**0.058**
200	0.111	0.100	0.096	0.102
150	0.105	0.120	0.110	0.112
100	0.129	0.136	0.126	0.130
50	0.243	0.247	0.244	0.245
20	0.365	0.363	0.365	0.364
Control	**0.312**	**0.318**	**0.315**	**0.315**

Pseudomonas aeruginosa MIC is 250µg/ml

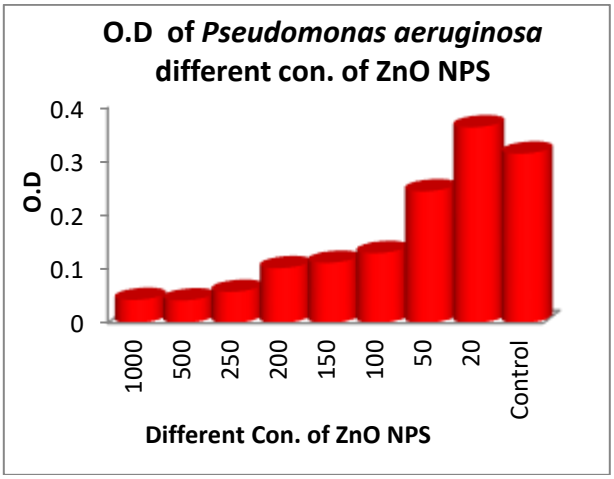

Fig. 6. **Pseudomonas aeruginosa MIC is 250µg/ml**

B. *Staphylococcus aureus* ATCC 29213

Conc. µg/ml	Staphylococcus aureus			Mean
1000	0.050	0.045	0.049	0.048
500	0.046	0.037	0.048	0.044
250	0.050	0.055	0.054	0.053
200	0.154	0.144	0.150	0.149
150	0.262	0.272	0.264	0.266
100	0.321	0.318	0.315	0.318
50	0.288	0.282	0.282	0.284
20	0.447	0.443	0.439	0.443
Control	0.512	0.515	0.520	0.516

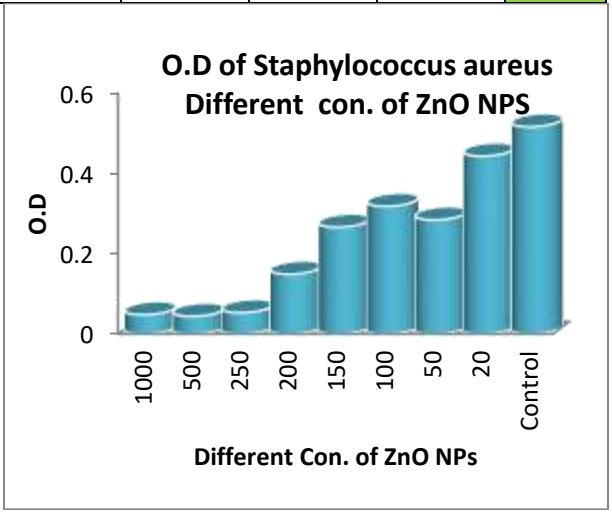

Figure. 7. Staphylococcus aureus MIC is **250µg/ml**

C. *Bacillus subtilis* ATCC 6051

Conc. µg/ml	Bacillus subtilis			Mean
1000	0.057	0.054	0.052	0.054
500	0.111	0.114	0.110	0.112
250	0.136	0.122	0.127	0.128
200	0.230	0.225	0.226	0.227
150	0.332	0.316	0.330	0.326
100	0.331	0.336	0.341	0.336
50	0.429	0.427	0.434	0.430
20	0.547	0.540	0.539	0.542
Control	0.545	0.548	0.547	0.547

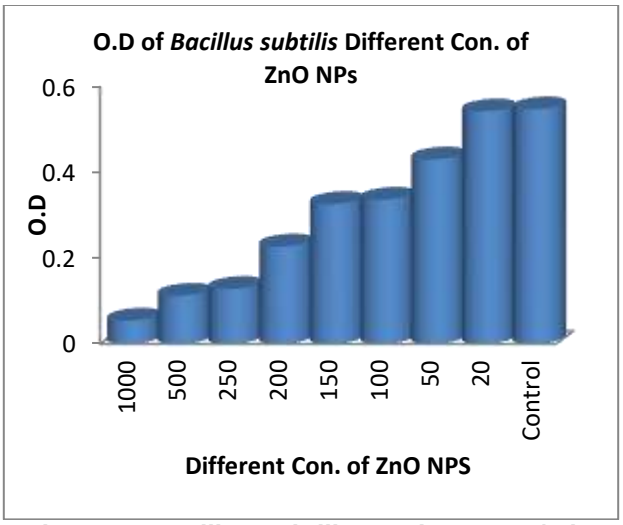

Figure. 8. Bacillus subtilis MIC is 1000µg/ml

D. *Escherichia coli* ATCC 25922

Conc. µg/ml	E coli			Mean
1000	0.040	0.035	0.038	0.038
500	0.058	0.056	0.055	0.057
250	0.063	0.055	0.062	0.060
200	0.062	0.069	0.069	0.067
150	0.081	0.066	0.070	0.072
100	0.250	0.254	0.259	0.254
50	0.398	0.398	0.400	0.399
20	0.462	0.464	0.468	0.465
Control	0.580	0.578	0.573	0.577

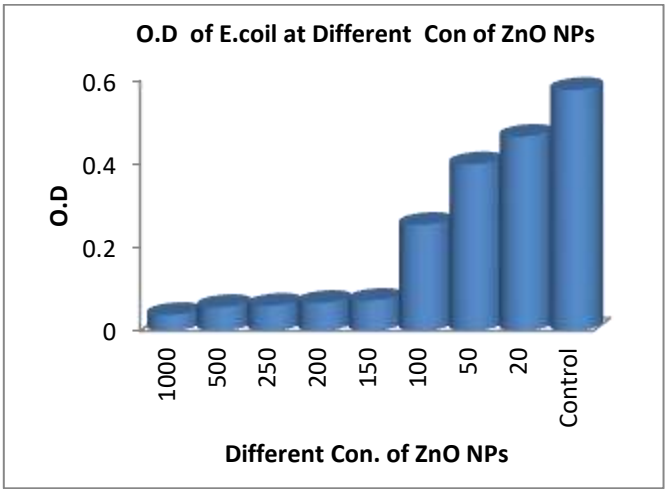

Figure. 9. *E.coli* MIC is 150µg/ml

E. *Staphylococcus aureus* MRSA ATCC 43300

Conc. µg/ml	MRSA			Mean
1000	0.054	0.058	0.057	0.056
500	0.110	0.111	0.106	0.109
250	0.117	0.116	0.120	0.118
200	0.278	0.270	0.273	0.274
150	0.276	0.280	0.270	0.275
100	0.348	0.353	0.346	0.349
50	0.460	0.453	0.457	0.457
20	0.475	0.474	0.469	0.473
Control	0.582	0.577	0.578	0.579

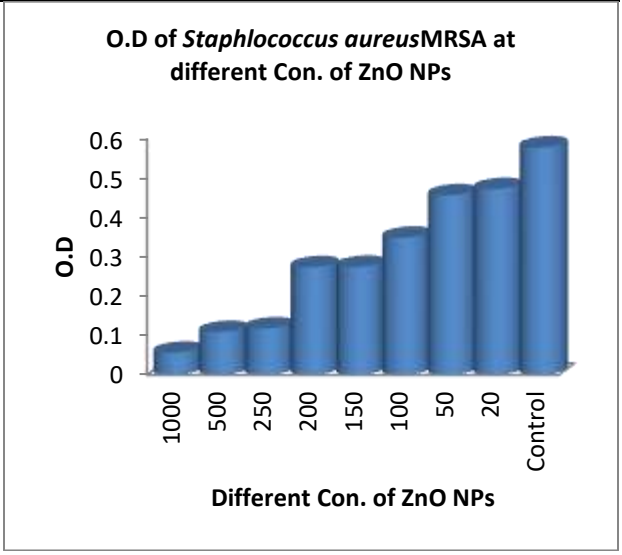

Figure.10. MRSA MIC is 1000µg/ml and it resistant to

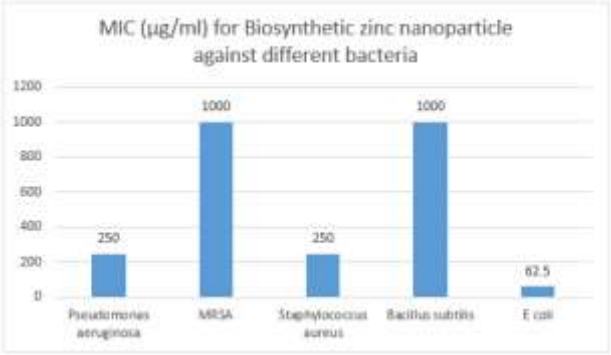

Figure. 11; MIC of ZnO against different microorganism If the number is large so it will be resistant

Eggshells Decontamination by using ZnO NPs solution

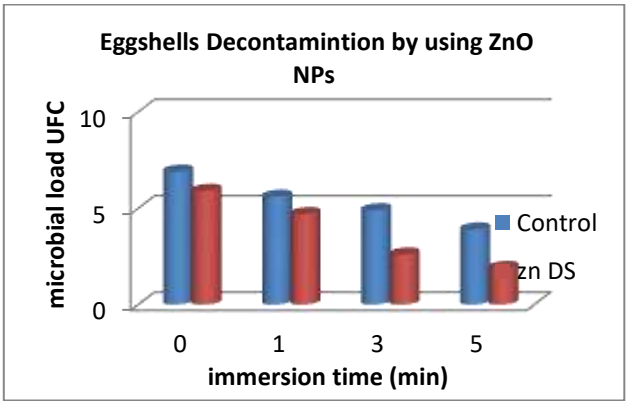

Figure . 12; Egg shells Decontamination by using ZnO NPs solution

3.4 Eggshells Decontamination by using (ZnO NPs DS)

The impact of eggshells disinfection, using ZnO NPs disinfectant solution (ZnO NPs DS) for eggshells decontamination, is observed in Figure. 4. the control group, which bacterial load decreased with washing with distilled water, for instance, from 6.9 to 3.9 $\log^{10}$ CFU/eggshell, while bacterial load decreased in treated eggshells with (ZnONPs DS) was supplementary and significant Figure 4. The total viable bacterial in disinfected eggs with ZnO NPs were 5.9, 4.7, 2.6 and 1.9 $\log^{10}$ CFU/eggshell after immersion time of 0, 1, 3, and 5 min, respectively.

CONCLUSION

Aspergullis niger supernatant is perfect for production of ZnO nanoparticles. ZnO its prefect for inhabtion the growth of E.coli and Pseudomonas *aeruginosa.* ZnO is not sufficient with inhabtion growth of Bacillus subtilis and MRSA. ZnO can be used in future more than antibiotics.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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