

Original Research Paper

Biosynthesis of Zinc Oxide Nanoparticles and Assay of Antibacterial Activity

¹Emad J. Ibrahim, ²Karkaz M. Thalij, ²Mahmood K. Saleh and ²Amin S. Badawy

¹Sulaimani Directorate of Health, Ministry of Health Kurdistan, Iraq

²Department Food Science, College of Agriculture, Tikrit University, Iraq

Article history

Received: 15-09-2015

Revised: 07-12-2016

Accepted: 29-3-2017

Corresponding Author:

Karkaz M. Thalij

Department Food Science,
College of Agriculture, Tikrit
University, Iraq

Email: kthalij@gmail.com

Abstract: In this study Out of 280 urinary tract infections samples, 212(75.7%) bacterial isolates were recovered. Based on, morphological, cultural and biochemical testes, further, confirmed the results by VITEK 2 System there were 54 (30.2%) of gram positive whereas the 158 (69.8%) of gram negative. The bacterial isolates were distributed as *Escherichia coli* followed by *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* which represented 96(45.2%), 48 (22.6%), 43(20.3%), 14 (6.6%) and 11 (5.2%) respectively. The synthesis of Zinc Oxide nanoparticles (ZnO NPs) using biological methods as eco-friendly from the use of *Aspergillus niger* filtrate in the extracellular synthesis, then identified and characterized by UV-Vis Spectrophotometer and Scanning Electron Microscopy (SEM). Furthermore, determine the antibacterial efficacy of biological synthesized ZnO NPs against isolated pathogens microbes *Staphylococcus aureus* and *Escherichia coli*. The results found the used of 100 mL of ZnCL₂ in cultivation of *Aspergillus niger* was obtained and biosynthesis of ZnO NPs at 46 mg. SEM was illustrate the morphology and practices of ZnO NPs sizes and appear as spherical in shape besides, the size range at 41-75 nm. The assurance by the UV-Vis spectrum appears the absorption bands of ZnO NPs at 380 nm. The results of 1.0 and 1.5 mg mL⁻¹ from ZnO NPs synthesized from *A. niger* were effect of inhibition against *S. aureus* and *E. coli* bacterial isolated from urine tracts infection sources. The Inhibition Zone Diameters (IZD) against *S. aureus* was appear at 24 and 26 mm respectively compared with the IZD from Ciprofloxacin alone at 20 mm. while the same concentration from ZnO NPs against *E. coli* causing in IZD at 25 and 28 mm respectively compared with Ciprofloxacin inhibition alone at 23 mm.

Keywords: Zinc Oxide NPs, *Aspergillus niger*, SEM, Antibacterial Activity

Introduction

Nano biotechnology has now emerged as integration between nanotechnology and biotechnology for developing biosynthetic and eco-friendly technology for synthesis of nanomaterials (Rai *et al.*, 2009). Applying nanotechnology has occurred rapidly and the use of nanoparticle is being used in all sciences in area of chemistry, physics, medicine and biology (Ozin *et al.*, 2009). Nanoparticles usually ranging in dimension from 1-100 nm the decrease in the dimensions of the materials to the atomic level, was caused to change in their properties (Gutierrez *et al.*, 2010).

Nanoparticles can be synthesized by physical, chemical and biological methods (Kathiresan *et al.*,

2009). 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). 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 their 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). The inorganic materials kill bacteria through various mechanisms, such as by binding to intracellular proteins and inactivating them, generation of reactive oxygen species and via direct damage to cell walls (Gao and Cranston, 2008). Lakshmi *et al.*, (2012) studied the antibacterial activity of ZnO NPs, namely, clinical isolates: *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Staphylococcus aureus*.

In this study, ZnO NPs were synthesized using biological synthesis mediated from fungi, characterization and also evaluation of their antimicrobial activity against *Staph. aureus* and *E. coli* isolated from UTI samples using disk diffusion and well diffusion assay.

Materials and Methods

Samples Collections

Urine samples of patients were collected from Microbial Laboratory in Emergency Hospitals, Sulimaini, Iraq. They were treatments to isolation and identification of pathogenic bacterial isolates according to the standard methods which recommended by (McFaddin, 2000; Steve *et al.*, 2004). Confirmed by VITEK 2 System (Version 5.01 BioMerieux).

Synthesis of ZnO Nanoparticles

The ZnO nanoparticles were prepared by using some modification in the method of Jacob *et al.* (2014). The *Aspergillus niger* ATCC 16404 was cultivation in Potato Dextrose broth (Difco, USA) used 250 mL Erlenmeyer flask with agitating by orbital shaker (Mimmert, UK) at 150 rpm, 28°C for 72 h. Fungal supernatant was obtained by passing through Whatman No.1. then 10 mL of supernatant was mixed with 100 mL of 0.25 mM solution of ZnCl₂ (Sigma Aldrich, Germany), in 250 mL Erlenmeyer flask and cultivation at 28°C with agitation at 150 rpm for 96 h. ZnO NPs was obtained through the fungal mycelium centrifuge at 3000 rpm for 15 min., then the participation was drying in oven at 60°C and harvested the ZnO NPs and weighted.

Characterization of ZnO NPs was describing using scanning electron microscope (Cam Scan 3200 LV; England) and record the micrograph images of ZnO NPs synthesized. Sample was fixated by glutaraldehyde, dehydrated by alcohol by SEM analysis (Vigneshwaran *et al.*, 2007; Talebia *et al.*, 2010). For assurance the ZnO NPs characterization also used the UV-Vis Spectrophotometer spectrum technique to measure the structural characterization of nanoparticles by determine the absorbance measurement (Haiss *et al.*, 2007).

Preparation of Microbial Suspensions

Antimicrobial activities of ZnO NPs were conducted against pathogenic bacteria isolates. Gram positive bacteria *S. aureus* and Gram negative *E. coli*. Agar well diffusion were used in this study. A suspension of bacterial isolates was freshly prepared by inoculating stock culture from each isolates into 3 mL of Mueller Hinton Broth (Difco, USA) separate broth tubes. The inoculated tubes were incubated at 37°C for 24 h. Bacterial accounts assay used 0.5 concentrations tube from McFarland technique for screening of antimicrobial activities.

Antimicrobial Activity

The technique was performed using agar well diffusion (Magaldi *et al.*, 2004) the suspension bacterial cultivation for 24 h from each isolates of *E. coli* and *S. aureus* adjusted to 0.5 McFarland turbidity standard described by Padmavathy and Vijayaraghavan (2008). 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 and 0.5 mg of Ciprofloxacin (Bioanalyse/Turkey) were injected in each wells. Then the plates were incubated at 37°C for 24 h, the diameters of inhibition were recorded in millimeter using metric ruler.

Statistical Analysis

Data were analyzed by the ANOVA analysis, using the general linear model of the Statically Analysis System (SAS Institute, 2001). Significant treatment differences were evaluated using Duncan's multiple-range test (Duncan, 1955). All statements of significance are based on the 0.5 level of probability.

Results and Discussion

Isolation and Identification of Bacterial Isolates

Table 1, shows the isolated microorganisms from urine source, which were identified according to the microscopic, macroscopic and biochemical tests and the results confirmed by VITEK-2 SYSTEM. The most frequently isolated bacteria significantly ($p < 0.05$) were *Escherichia coli* 96 (45.2%) followed by, *Klebsiella pneumoniae* 48 (22.6%), *Staphylococcus aureus* 43(20.3%), *Pseudomonas aeruginosa* 14 (6.6%) and *Staphylococcus epidermidis* 11 (5.2%). This result was in agreement with Inbaneson *et al.* (2011).

The isolates were cultured on Blood, MacConkey and Mannitol Salt agar plates and incubated at 37°C for 24 h. They were identified according to colony characteristics and microscopic examination of stained smear that

demonstrate microbial shape, structure, agreement, gram stain reaction and biochemical tests like indol (I), Methyl Red (M.R), Vogas Proskauer (V.P), citrate utilization, oxidase (C), motility test, catalase, coagulase and urease production; according to Brown (2007) as in Table 2. Then the identification was confirmed by using VITEK 2 compact system as recommended by Biomerieux. Our results were compared with the resource reported by (Collee *et al.*, 1996; McFadden, 2000).

Biosynthesis of ZnO NPs

The biosynthesis of ZnO NPs was conducted by cultivation of *Aspergillus niger* on potato dextrose broth media enrichments with ZnCL₂ at 28°C with agitation at 150 rpm for 96 h, (Fig. 1a and b). the range of ZnO NPs weight was at 46 mg the ability of *Aspergillus niger* on synthesis of nanoparticles from ZnCL₂ and reduced the particles from micro 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. The Nano-sized ZnO suspension clearly has a much higher activity than the micron-sized ZnO suspension (Zhang *et al.*, 2010). However, development of simple and eco-friendly route would help in promoting further interest in the synthesis and

application of Zn, Mg and Ti nanoparticles. Previous studies have indicated that NADH and NADH dependent enzymes are important factors in the biosynthesis of metal nanoparticles (Ahmad *et al.*, 2003a; 2003b).

Characterization of ZnO NPs

Scanning Electron Microscopy (SEM) performed has provided further insight into the morphology and size details of the synthesized nanoparticles. It is able to detect of their purity and particle size revealed on the formation of mono and poly dispersed nanoparticles and the ZnO NPs was appeared in the size range of 41 to 75 nm (Fig. 2). The morphology of the nanoparticles is variable with majority of them being spherical. SEM studies clearly establish that the reduction of Zn ions occurs extracellular, it would be important to identify the reducing agents responsible for this reaction.

The UV-Vis Spectrophotometer technique were used to measure the structural characterization of nanoparticles by determine the absorbance measurement and the UV-Vis spectrum of ZnO NPs was appear at 380 nm absorption bands, (Fig. 3). The absorption peak was appearing as similar the absorption band for the results obtained by Mashrai *et al.* (2013).



Fig. 1. Biosynthesis of ZnO NPs (a) *Aspergillus niger* extracts (b) ZnCL₂ with *Aspergillus niger* extract after 72 h of incubation

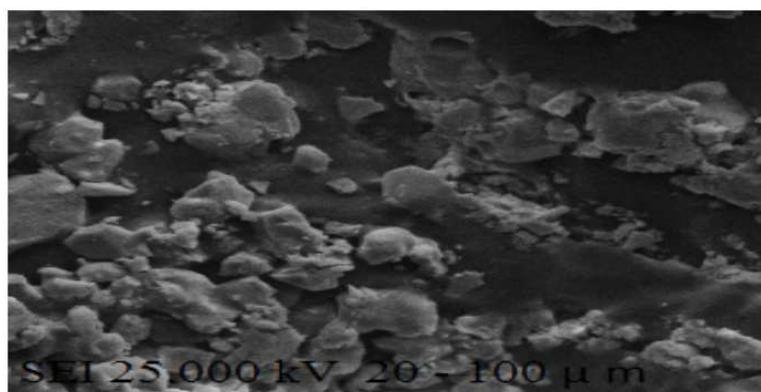


Fig. 2. SEM image of biosynthesized zinc oxide nanoparticles by *A. niger*

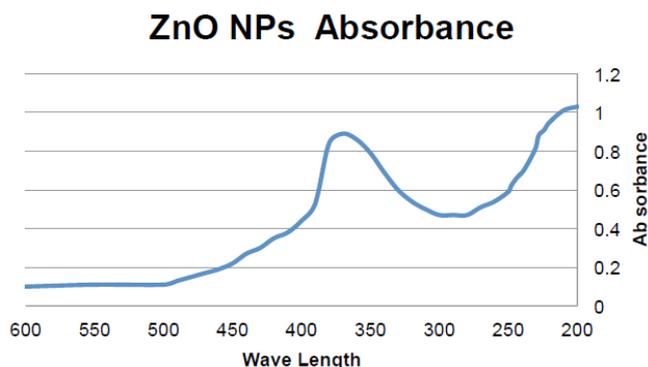


Fig. 3. The UV-Vis spectrum of ZnO nanoparticles synthesized by *A. niger*

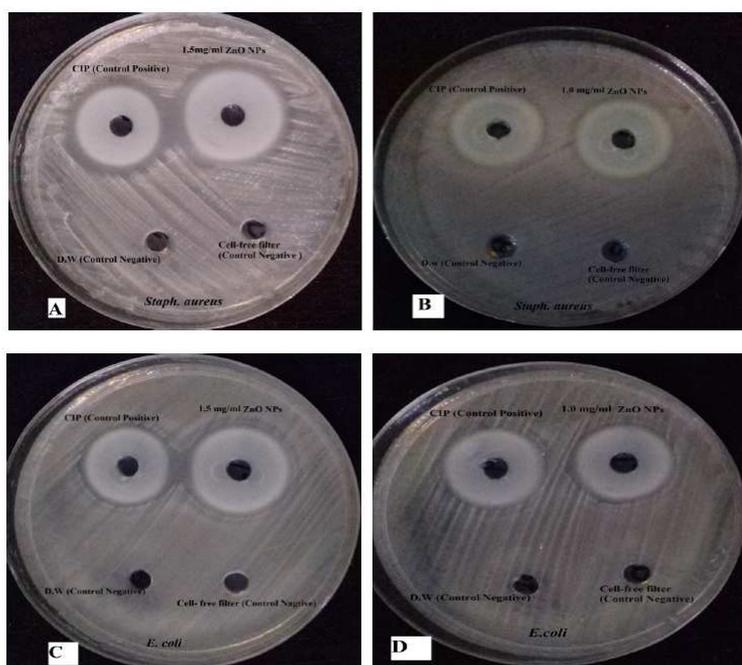


Fig. 4. Antibacterial activity of ZnO NPs used well diffusion test (A) at 1.5 mg mL⁻¹ against on *S. aureus*; (B) at 1.0 mg mL⁻¹ against on *S. aureus*; (C) at 1.5 mg mL⁻¹ against *E. coli*; (D) 1.0 mg mL⁻¹ against *E. coli*

Bacterial strain	Accounts of bacteria isolates	%
<i>E.coli</i>	96a±5.48	45.2 a±4.22
<i>K.pneumoniae</i>	48 b±4.05	22.6 b±2.57
<i>P. aeruginosa</i>	14 d±1.19	6.6 c±1.02
<i>S. Aureus</i>	43 c±2.65	20.3 b±3.38
<i>S. epidermidis</i>	11 e±1.37	5.2 c±0.70
Total	212	100

a-e: Values within columns followed by different letters differ significantly at 0.05

Antibacterial Activities of ZnO NPs

Antimicrobial activity of biological synthesized ZnO NPs of concentrations at 1.0 and 1.5 mg mL⁻¹ against gram positive *S. aureus* and gram negative bacteria *E. coli* were

tested by the well diffusion agar methods are represented in the Fig. 4 (A, B, C and D). Table 3 Illustrated the results of 1.0 and 1.5 mg mL⁻¹ ZnO NPs synthesized from *A. niger* effective antibacterial activity against *S. aureus* and the Inhibition Zone Diameters (IZD) were appear at 24 and 26 mm respectively compared with the IZD from Ciprofloxacin alone at 20 mm. while the same concentration from ZnO NPs against *E. coli* causing in IZD at 25 and 28 mm compared with Ciprofloxacin inhibition alone at 23 mm. The results were appeared the effects of ZnO NPs against gram positive bacteria was significantly more than effects on gram negative bacteria. Also, the results appeared that the inhibitory effect of ZnO was significantly increased with the increase in concentration. The results for the biologically synthesized ZnO were comparable to the results obtained for ciprofloxacin (0.5 mg mL⁻¹).

Table 2. Some biochemical tests used for identification of bacteria

Isolated Bacteria	Biochemical tests						
	Catalase	Coagulase	Oxidase	Indole	M.R	V.P	Citrate
<i>E. coli</i>	N	N	+	+	+	-	-
<i>K. pneumonia</i>	N	N	+	-	-	+	+
<i>P. aeruginosa</i>	N	N	+	-	-	-	+
<i>S. aureus</i>	+	+	-	N	N	N	N
<i>S. epidermidis</i>	+	-	-	N	N	N	N

+, positive; -, negative; N, not done

Table 3. Antibacterial activity of ZnO NPs synthesized by *A. niger*

Microorganism	Zone of inhibition (mm) well diffusion			
	Conc.1.0 mg mL ⁻¹	Conc. 1.5 mg mL ⁻¹	Ciprofloxacin 0.5 mg mL ⁻¹	D.W
<i>E. coli</i>	24 a±2.47	26 b±3.36	20 b±1.28	4 a±0.61
<i>Staph. aureus</i>	25 a±1.81	28 a±2.75	23 a±1.60	4 a±0.55

a-b: Values within columns followed by different letters differ significantly at 0.05

The presence of an inhibition zone clearly by ZnO NPs in this study were agreement with the previous study by (Rizwan *et al.*, 2010; Emami-Karvani and Chehrazi, 2011) whom have been seen that the increasing of ZnO NPs concentration causing in increased the inhibition zone diameters for gram negative and positive bacteria. This may be due to the destructive effect of ZnO NPs with the cells and increased production of active oxygen such as H₂O₂, leads to the cell death (Zhang *et al.*, 2010). Furthermore the ZnO NPs, was caused when adherence to the surface of the cell membrane, in disturbance of respiration mechanical through interact with enzymes of the respiration chains of bacteria (Jones *et al.*, 2008). Also from the results were appear that the inhibition zone was affected with the different in the bacterial types and shown the gram positive bacteria were more sensitive for ZnO NPs compared to the gram negative bacteria (Tam *et al.*, 2008; Tayel *et al.*, 2011) and these were related to differences in cell wall structure, cell physiology and metabolism pathway (Emami-Karvani and Chehrazi, 2011).

Conclusions

Concluded the ZnO NPs has broad-spectrum activity against bacteria and the inhibition against gram positive were more activity compared with the gram negative bacteria. Also the antibacterial activity was increased with the increased of ZnO NPs concentration.

Acknowledgement

Our thanks and appreciation to the Department of Biology, College of Education for pure Sciences, Tikrit University for funding supported.as well as to the laboratory staff in the Faculty of science, Sulaimani University, IRAQ in helping them to provide most the requirements for a successful search.

Author's Contributions

Karkaz M. Thalij, designed of the experiments and supervision for all the research work, statistical analyses and prepared the manuscript. Emad J. Ibrahim, participated in running all the experiments works besides, the preparing of draft manuscript. Mahmood K. Saleh and Amin S. Badowy, performed the experiments and helped to assisted in certain aspects of the experiments. All authors read and approved the final manuscript.

Ethics

The authors declare their responsibility for any of the ethical issues that may arise after the publication of this manuscript.

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