Evaluation of some Food Poisoning Bacterial Inhibition from ZnO and Ag Nanoparticles that Synthesized by Aspergillus niger

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ABSTRACT: The biosynthesis of ZnO and Ag nanoparticles (NPs) was conducted by using Aspergillus niger and determine the ability on the inhibition of E. coli and S. aureus. The morphology and particles sizes were found that ZnO and AgNPs at a rod to globular shapes, and the dimensions which were between 30 to 60 and 12 to 40 nm respectively. Also, determine the NPs synthesis absorbance of ZnO and AgNPs were to be at 368 and 420 nm respectively. The minimum inhibition concentration (MIC) assay was at 0.5, 1.0, 2.0, 5.0 10 and 20 % from each ZnO and AgNPs against S. aureus and E. coli have appeared that MIC act on inhibition for each bacterial growth at 1.2 %, and the Minimum Bactericidal Concentration (MBC) appeared at 20 mg/ml. The tested of 10, 15, 20 and 25 % from each ZnO and AgNPs on S. aureus and E. coli inhibition used wells method assay appear that inhibition diameter zone (IDZ) against S. aureus at 12, 13, 16 and 18 mm and 10, 14, 18 and 27mm respectively, While the IDZ against E. coli at 8, 10, 15 and 17 mm and 14, 16, 22 and 30 mm respectively. Thus, the ability of A. niger to synthesis of ZnO and AgNPs and these nanoparticles have the activity against bacterial species of S.aureus and E.coli.

1. Introduction

Nanotechnology have been considering one of the most modern technologies. It is a connection between the various sciences while applications are interfering in many aspects into human life. The role of this technology have been involved in many fields including chemistry, physics, materials science, molecular biology and biomedical sciences [1].

As Nanoparticles are distinguished in the techniques involved in that their molecules or the constituent atoms in them are in sizes between 1-100 nanometers. Therefore, the properties of their constituent materials are unique in contrast to the constituent substances that have molecules greater than 100 nanometers [2], as they are distinguished in high surface-to-volume ratio [3] and other new physical and chemical properties such as color, solubility, strength, diffusion, toxicity, magnetic, optical, thermodynamic, and many other characteristics [4].

There are many methods that have been involving in obtaining nanoparticles, including chemical, physical, and biological [5]. The chemical and physical methods are characterized by being economically costly and having negative effects if they are used for cases related to health and environment, in addition to their use requires providing special conditions of chemicals, energy sources, pressure and high temperature, so attention is focusing on preparing these nanoparticles through the use of biological methods that have been demonstrated to be easy to implement and safe to use in terms of health and environment outcomes as well as cheap and environmentally friendly [6].

Using the biological methods depends on the range of exposure of those organisms to the metabolic compounds which are susceptible to be used by the living organisms to synthesize nanoparticles from their primary compounds such as salts or chlorides of metallic elements which including gold, silver, zinc, aluminum, and others that have a specific dimensions and characteristics for each living organism that have been involved in the synthesis, and are the types of fungi as one of the most important organisms used in the production of nanoparticles [7]. The fungus Aspergillus niger is distinguished by their ability to form large numbers of conidia, and in its ability to produce many extracellular enzymes that have the primary role in analyzing the base material and converting it into its smaller components and facilitating its absorption by the fungus [8].

Numerous studies have been demonstrated that oxides of nanoparticles are having the ability to inhibit and to kill the microbes that are resistant to antibiotics through different numerous mechanisms of influence [9]. Including the nature of the charges carried by nanoparticles that are affected in the form.
of type and physical properties, as well as the size of the surface area that increases the possibility of contact with the walls of cells of microorganisms and their influence and penetration as these molecules are susceptible to the release of metal ions with a damping effect [10]. Thus, the aim of the study was to try to produce the nanoparticles of both Ag and ZnO of their two solutions of silver nitrate AgNO3 and zinc chloride ZnCl2 and to determine their nanoparticles characteristics, further to assay the inhibition ability against microorganisms causing the cheese spoilage after used with biofilm as gelatin after 21 days of storing.

2. Materials and Methods

2.1. Isolation and diagnosis of Aspergillus niger:

The isolation of A. niger was conducted after obtained the soil samples from different areas in Tikrit University gardens-Iraq. The obtained samples were treated by adding them in equal sizes to CaCO3 and then incubation at 37 °C for 4 days. Then the soil suspension was prepared by adding 25 g of each samples to 225 ml of sterile physiological solution in a 500 ml conical flask. Subsequently, the appropriate series of dilutions for obtaining colonies from mold were completed with a preparation that allows them to be diagnosed after their incubation at 30 °C for 5 days on the Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Rose Bengal Agar (RBA) medium (Oxoid, UK). Each isolate was subcultured and refined on MEA and PDA media. The isolates that appeared in black color on the plates were identified by using the classification key mentioned in [11].

2.2. Preparation of biomass and nanoparticles formation:

Prepare the fungal mass was conducted by taking a disc from the growing of pure fungal colony diagnosed with A. niger using a sterile 6 mm diameter cork bore, which was transferred to 100 ml of Potato Glucose Broth (PGB) medium in a 250 mL volumetric flask, and leave the fungal disc for 20 minutes to settle on the surface of the liquid medium, then incubate in the incubator at 29 °C for 5 to 7 days to obtain a thick fungal mass [12].

To prepare the nanoparticles, the biomass from the mold was filtered using sterile filter papers No.1. The filtrate obtained by drawing from each volume of 100 ml and added to it in a flask with a volume of 250 ml with a volume of 100 ml at a concentration of 1 mM for each of the silver nitrate solution AgNO3 and zinc chloride ZnCl2 and incubated in the incubator under completely dark conditions for 96 hours according to [13]. After changing the color of each of the solutions, which are preliminary evidence of the formation of nanoparticles, the two solutions underwent determination of their properties of volume using Transmission electron microscopy (TEM) (Shimadzu-Japan) that was done in the laboratories of the Department of Chemistry - College of Science - the University of Al-Nahrain and its forms using the X-Ray Diffraction technology (Shimadzu-Japan) and knowledge of the diffraction of the radiation at specific angles, which was accomplished in the Ministry of Science and Technology-Department of Materials. The absorption capacity of UV light was also determined using the Spectrophotometer, the size of the nanoparticles was also measured and distributed in the solution using a device nanoparticles Size Analyzer (Brookhaven-USA) by relying on the refractive index that was done in the packaging center laboratories-Ministry of Industry, Iraq.

2.3. Bacterial isolation and diagnosis:

The isolation of E. coli was conducted according to the method mentioned in [14], by mixing 10 ml of milk samples in 90 ml of normal saline. 100µl of the optimal final serial dilution was inoculated on the surface of MacConkey agar and Eosin methylene blue and spreading then incubated at 37°C for 24 hours. The bacterial colonies were taken and sub-cultured on the EMB medium to obtain pure isolates. Slants were kept in the refrigerator until the diagnosis was made to the species through morphologic, microscopic and biochemical characterizes according to [15].

The isolation of Staphylococcus aureus was done after the isolates were obtained in the same way, from cake samples that prepared by left it’s at room temperature for 4 days. Take 100µl of dilute cake sample and inoculated to the Mannitol salt agar medium then incubated at 37°C for 48 hours. Individual colonies were sub-culturing on the same medium used streaking to obtain pure bacterial isolates, then diagnosed by conducting morphological, microscopic and biochemical tests appropriate to reach the bacterial species of each as stated in [15].
2.4. Preparation of nanoparticle concentrations:
The preparation of nanoparticles was used as a solution after being filtered from the fungus, and they were prepared with the primary concentration of AgNPs and ZnNPs at 1 mM at 100% concentration. The concentrated levels were prepared by dilution with distilled water at 0.5, 1.0, 2.0, 5.0, 10, 15, 20 and 30%.

2.5. Determination of the minimum inhibitory concentration of AgNPs and ZnNPs. (MIC):
The Agar Dilution Method was used according to the [16]. Serial concentrations from each of AgNPs or ZnNPs were prepared at 0.5, 1.0, 2.0, 5, 10 and 20 mg/ml, and adding at 1ml to autoclaved Muller Hinton agar medium before solidification then, pour well into sterile dishes and keep at 4 °C until use. Prepare the bacterial suspension of each S. aureus and E. coli at 18-24 hrs., and compared with its turbidity with the 0.5 McFarland tube concentration, then inoculate 0.1 ml from each isolates on medium plates and left its until dried. Incubate the medium plates inverted at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was determined by assay the lowest concentration which inhibited the bacterial cell growth.

2.6. Determination of the antimicrobial activity of AgNPs and ZnNPs.
The effectiveness of both AgNPs and ZnNPs against the species of S. aureus and E. coli causing food poisoning isolated from food samples was evaluated according to [16] which included the following: Bacterial suspension from each isolates were treated with AgNPs and ZnNPs. The resulting solutions were compared with a standard MacFarland solution for the tube concentration of 0.5 to stabilize the bacterial account at 1.5 x 10^8 cells/ml. Draw 0.1 ml of each suspended solution to the surface of the Muller Hinton Agar culture medium and spread over the surface of the medium, leave for 15 minutes, then transfer 50 μl of nanoparticles of 10, 15, 20 and 25 mg/ml for each of the AgNPs and ZnNPs to the wells at 4 mm diameter, the inoculates medium then incubated at 37 °C for 24 hours. Then, the inhibition ability of each treatment against bacterial species were determined by measuring the diameter of the Inhibition zone in millimeters (mm).

3. Results and discussion
3.1. Isolation and diagnosis of Aspergillus niger:
A. niger mold isolation from soil models taken from different areas of Tikrit University gardens after culturing at 25 °C on the MEA, PDA and RBA media, and the fungal colonies have appeared after 3-5 days after incubation in the cultivation media in soft or slightly mystical shapes with a black color resulting from the color of the conidia. Picture (1). The phialides have appeared in black and the microscopic examination included the presence of the phialides at spherically radial or branched (cracked) divided into several irregular columns or a limited number of chains. While, the conidiophore had thick, smooth, translucent walls colored in brown, as the conidia appeared in spherical or elliptical shapes of Brown in color and many in accounts. After following the classification key mentioned in [11], it was found that the mold is of A. niger species.

3.2. Characterization of AgNPs and ZnNPs:
3.3. Color change
The results of this study showed the formation of AgNPs and ZnNPs by using the A. niger metabolites for biosynthesis and converting the primary compounds of AgNO₃ and ZnCl₂ to their nanoparticles (Fig. 1a). As the solution of silver nitrate AgNO₃ used with a concentration of 1 Mm added to the biomass filtrate has changed its color to brown (Fig. 1b), and the ZnCl₂ solution that was used with the same concentration above has changed its color to golden yellow (Fig. 1c). The color change is a preliminary indication of the ability of the metabolic products of the mold to dissolve the added metal compounds and the formation of nanoparticles from their decomposition resulting from the reductive action of both the AgNO₃ and ZnCl₂, and that color change is due to the excitation of the surface of the plasmon (the basis of this vibration is for electron connection totals).
3.4. Absorption UV-Visible light spectroscopy:

The results of the study showed that the ability of A. niger to synthesize silver and zinc nanoparticles through ultraviolet and visible absorption spectrum within the wavelength range of 200-800 nm for silver nitrate solution AgNO₃ and ZnCl₂ solution used in the preparation of nanoparticles (Figure 2). It was noted that the peak absorption of the silver nitrate solution was about the wavelength of 420 nanometers, which is the same wavelength that determined for the absorption of radiation for the nanoparticles of silver, the result in agree with that found in [17], while the highest peak of absorption of the zinc nanoparticles appeared at the wavelength of 368 nm which is the same wave length. That have been determined of those nanoparticles which was in agree with that found in [18].

3.5. X-Ray Diffraction

The results of the X-ray diffraction technique confirmed the susceptibility of a metabolite of A. niger to the synthesis of nanoparticles for both Ag and Zn (Fig. 3). The X-ray diffraction spectrum of AgNPs prepared using A. niger mold was shown. The diffraction peaks (103), (106) and (111) were at the angles of 77.44°, 44.31° and 77.45°, respectively (Fig. 3a). Figure (3) shows the X-ray diffraction spectrum of ZnNPs prepared using the same fungus and under the same conditions which appear had diffraction values (109), (118) and (152) at angles 34.59°, 31.97° and 70.79°, respectively (Fig. 3b). The results were identical to that of the International Center for Diffraction Data (JCPDS), these results in agree with that mentioned by [19] about silver nanoparticles, whereas the results [20] agreed with the results of X-ray diffraction of ZnNPs created using the same fungus.

Figure 2: X-ray diffraction spectrum for A: nanoscale silver B: zinc nanoparticles prepared in A.niger metabolites.
3.6. Nanoparticle Size

The nanoparticles size is the important tests to determine the shape and size of the nanoparticles using the Analyzer measuring device. Figure 3, illustrated the shape and size of the Ag and ZnNPs beside the nature of distribution in its solution used the Nanoparticles size analyzer system.

The results were showed that the size of AgNPs that distributed in the suspended solution was between 18-110 nm, whereas for ZnNPs was at 35-197 nm. The proportional of their density and distributed were over two separate curves, the first indicates the volumetric distribution of the smaller size nanoparticles, and the second curve was indicated for the larger size of nanoparticles.

![Figure 3: Volumetric distribution of nanoparticles in solution; A=AgNPs B=ZnNPs.](image)

3.7. Transmission Electron Microscopy

The dimension that determined for the Ag and the ZnNPs used the transmission electron microscope (TEM) were useful in knowing the shape and the size of nanoparticles of silver or zinc using the metabolites from A.niger and distributed with high accuracy and different magnifications. As shown in Figure 4 (A, B, C and D) obtained at different magnification capabilities. It observed from the figures that the nanoscale silver atoms are distributed in the form of spherical grains at a size rate between 12-40 nm, and these particles are homogeneous in the form of clusters. These results were agreement with the results that mentioned in [17], who found the same dimensions obtained. As for the distribution of nanoparticles of zinc, it was found from the shapes obtained from the microscope (EF, G and H) and that they were in the form of spherical granules with a volume rate between 33.5-69.1 nm, and these results were consistent with what both researchers obtained [19,20].
3.8. Determination of the MIC of nanoparticles

The determination of MIC use each of the AgNPs and ZnNPs with serial concentrations at 0.5, 1.0, 2.0, 5.0, 10 and 20% separately against S. aureus and E. coli bacteria were illustrated by the results in (Table 1). The results showed that the minimum inhibitory concentration (MIC) for both bacteria S. aureus and E. coli of the silver nanoparticles were at 2.0 and 1.0%, respectively. The inhibition ability was increased with increasing concentration of nanoparticles. The lowest inhibitory concentration of zinc nanoparticles against the two bacteria species was at the concentration of 5.0 and 2.0%, respectively.

Table 1. The minimum inhibitory concentration of AgNPs and ZnNPs against two tested bacteria.

| Bacterial species | Type of nanoparticles | Inhibitability of the test NPs depending on the concentration (%) |
|-------------------|-----------------------|---------------------------------------------------------------|
|                   |                       | 0.5  | 1    | 2    | 5    | 10   | 20   |
| Staph. aureus      | AgNPs                 | -    | -    | +    | ++   | +++  | ++++ |
|                   | ZnNPs                 | -    | -    | -    | +    | ++   | +++  |
| E. coli           | AgNPs                 | -    | +    | +    | ++   | +++  | ++++ |
|                   | ZnNPs                 | -    | -    | +    | ++   | +++  | ++++ |

(-) non sensitive, (+) low sensitivity, (+++) medium sensitivity, (++++) high sensitivity, (+++++) fully sensitive. AgNPs = silver nanoparticles. ZnNPs = zinc nanoparticles.

3.9. Inhibition of nanoparticles against bacteria

Results in Table (2). showed that nanoparticles at a concentration of 10, 15, 25 and 25% for both AgNPs and ZnNPs are susceptible to inhibition of S. aureus and E. coli. the addition of the AgNPs showed an inhibiting zone diameter within 10, 14, 18 and 27 mm, respectively and for 12, 13, 16 and 18mm respectively by ZnNPs against S. aureus. while the results of the use of AgNPs concentration against E. coli indicated that the inhibitory zone diameter at 14, 16, 22 and 30 mm respectively, and the ZnNPs was effects of inhibitory zone diameter at 8, 10, 15 and 17 mm respectively. These results were indicating that the sensitivity of the bacterial species to AgNPs were more than in the case of ZnNPs.
The nanoparticles showed inhibitory efficacy against the gram negative bacteria than that of the gram positive bacteria, and the results of the study were consistent with what was indicated by each of [21] who used the silver nanoparticles prepared from the fungus and observed increased inhibitory activity against the E. coli, P. aeruginosa is above its ability to inhibit S. aureus. The ability of nanoparticles comes from having positive charges that enable them to correlate with the negative charges of the surface of bacterial cell causing the accumulation of molecules on the surface of the cell membrane and to modulate some chemical and physical properties of the bacterial membrane, which lead to damage of bacterial cell membrane and caused to lose membrane functions such as permeability which lead to exit-out of electrons and nutrients from the cells [22].

That positively charged ions can be released by the nanoparticles inside the cell which can be bind to the bacterial ribosome and stop protein production or inhibiting the process of doubling the genetic material of bacteria by attaching those nanoparticles to the genetic material and destroying the DNA that leads to the death of bacteria, the more nanoparticles work inhibiting the respiratory chain enzymes and bacterial cell proteins by binding to the SH group of proteins, the more leading to the production of free radicals which causing the loss of their cellular components and thus in the bacterial cell death [23].

It is also known that the negative bacteria for gram stain possesses a layer that consists of the outer membrane LPS and proteins that have a role in the selectivity of the cell membrane of the bacteria and protect the bacteria from multiple harmful factors such as toxins, drugs, detergents and degradative enzymes and has a role in the delivery of nutrients to the cell and the outer surface. The bacteria have a negative charge by the carboxyl groups on them, and since the silver nanoparticles possess the positive charge, they will bind to the surface of the cell causing holes in the outer membrane layer and lead to a gradual release of the components from LPS and proteins. It is also believed that nanoparticles can be associated with proteins in the cell membrane by binding to functional groups of proteins causing damaging to them which leads to increased permeability of the cell membrane and the loss of the cell by its cellular components and thus in its death [21].

The inhibitory effect of ZnNPs against bacterial cells is achieved by producing active oxygen like H₂O₂ in the cells, causing cell death. The nanoparticles of Zn, after their attaching to the surface of the cell membrane which causing a disturbance in the metabolic processes of the cells, especially respiratory ones [22], thus causing bacterial cell death or inhibition and that depending on the concentration. These results were consistent with the results obtained by the researchers in [23].

The size of nanoparticle plays a major role in the antibacterial activity by the fact that the cell membranes in the bacteria have holes in the nanometer diameter, while the nanoparticles have a molecule sizes less than the holes so, they have distinctive properties to cross through the cell membrane. This, was explains the effectiveness of the AgNPs used in the study where the size of the molecules between 12-40 nanometers compared to the zinc molecules that showed the lowest efficacy where the size was about 30-60 nm [20] which clarifies the relationship of sizes of nanoparticles with its inhibitory action against bacterial species.

4. Conclusions
The conclusion of this study was investigating the ability of A.niger to synthesis the nanoparticles of ZnO and AgNPs which has been appeared that nanoparticles have broad-spectrum activity against bacterial species of S.aureus and E.coli.

Table 2. Inhibiting diameter zone (mm) for nanoparticles against bacterial species.

| Bacterial species | Type of nanoparticles | Diameter of inhibition zone (mm) against bacteria types depending on the concentration used (%) |
|-------------------|-----------------------|--------------------------------------------------------------------------------------------------|
|                   |                       | 10  | 15  | 20  | 25  |
| Staph.aureus      | AgNPs                 | 10  | 14  | 18  | 27  |
|                   | ZnNPs                 | 12  | 13  | 16  | 18  |
| E.coli            | AgNPs                 | 14  | 16  | 22  | 30  |
|                   | ZnNPs                 | 8   | 10  | 15  | 17  |

Ag-NPs = silver nanoparticles ... Zn-NPs = zinc nanoparticles.

The inhibitory diameter zone (mm) for nanoparticles against bacterial species.
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Author’s Contributions:

Karkaz M. Thalij, suggested of the experiments procedures and supervision for this research work, also the completion of the statistical analyses and prepared the manuscript setting. Kalaf N. Ahmad, participated in successively all the experiments procedures Also, the preparing of draft manuscript.

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References

[1] Sondi I and Salopek-Sondi B Journal of colloid and interface science, 275(1):177-182.
[2] Gupta A Eral H B, Hatton T A and Doyle P S 2016 Soft matter, 12(11):2826-2841.
[3] Rai M, Yadav A and Gade A 2009 Biotechnology advances. 27(1): 76-83.
[4] Ravindran A, Chandran P and Khan SS 2013 J. Colloids Surf B: Biointerfaces. 105:342–352.
[5] Zhang X F, Liu Z G, Shen W and Gurunathan S 2016 Int. J. Mol. Sci. 17(9):1534.
[6] Rai M, Gade A and Yadav A 2011 Biogenic nanoparticles: an introduction to what they are, how they are synthesized and their applications. In Metal nanoparticles in microbiology (pp. 1-14). Springer, Berlin, Heidelberg.
[7] Ahmed S, Ahmad M Swami B L and Ikram S 2016 Journal of Advanced Research. 7:17–28.
[8] Hoseinazadeh E Makhdoumi P, Taha P, Bossini H, Stelling J and Amjad Kamal M 2017 Current drug metabolism 18(2):120-128.
[9] Singh P 2018 Nanotechnology in food preservation. Food Science, 9(2). Hussein, A. (2013). Synthesis of zinc oxide and cobalt oxide nanoparticles in surfactant/antibiotics shell and investigating their anti-bacterial activities. Thesis of Master of Science in Biology, Faculty of Graduate Studies, An-Najah National University). 435-441.
[10] Brown A E and Smith H 2015 Benson’s microbiological applications: laboratory manual in general microbiology, Short Version, Thirteenth edition. McGraw-Hill Science, Engineering & Mathematics. pp:214.
[11] Samson R A, Houbraken J, Summerbell R C, Flannigan B and Miller J D 2001 Common and important species of fungi and actinomycetes in indoor environments. In: Microorganisms in Home and Indoor Work Environments. New York: Taylor & Francis. pp. 287-292.
[12] Al-Zubaidi S, Al-Ayafi A and Abdelkader H 2019 Journal of Nanotechnology Research 2:22-35.
[13] Ibrahem E J, Thalij K M, Saleh M K and Badawy A S 2017 Am J Biochem. Biotechnol. 13:63-69.
[14] Berkowitz F E and Jerris R C 2016 Practical Medical Microbiology for Clinicians. Wiley Blackwell:pp.125.
[15] Alfred E B 2005 Bensons Microbiological applications in laboratory manual in general microbiology 9th ed. McGraw– Hill Companies:pp.226.
[16] CLSI, 2011. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-First Informational Supplement. 30 (1): 145-157.
[17] Iyer R I, Selvaraju C and Santhiya S T 2016 Indian Journal of Science and Technology. 9:9.
[18] Gul S, Hashim Y Z H Y, Puad N I M and Samsudin N 2019 Fabrication and Characterization of Plant Mediated Green Zinc Nanoparticles for Antileishmanial Properties,pp:325.
[19] Gopinath V and Velusamy P 2013 Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy. 106:170-174.
[20] Supraja N, Prasad T N V K V, Krishna T G and David E *Applied Nanoscience*. 6(4):581-590.
[21] Roh J Y, Eom H J and Choi J 2012 *J. Toxicol. Res.* 28(1):19-24.
[22] Marambio-Jones C and Hoek EMV 2010 *A J. Nanopart. Res.* 12:1531-51.
[23] Karbasian M, Atyabi S M, Siyadat S D, Momen S B and Norouzian D 2008 *American Journal of Agricultural and Biological Science* 3(1):433-437.