Microbial synthesis of ZnO Nanoparticles against Antibiotic Resistant Pathogens

Fifth grade graduation project

By students

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2022-2023

Abstract

Nanoparticles (NPs) are gaining special interest due to their recent applications as antimicrobial agents to defeat the massive threat of resistant pathogens. This study focused on the utilization of soil bacteria in the biological synthesis of zinc oxide (ZnO) NPs. The isolates were related to Gram-positive and Gram-negative bacteria, in addition to *Candida*. The cell-free supernatant has been used for the synthesis of ZnO NPs. The synthesized NPs were characterized using Zetasizer and UV spectroscopy. This study showed the ZnO NPs have highly antibacterial effect against *Staphylococcus* aureus and moderate antibacterial effect against *Escherichia coli* and *Pseudomonas aeruginosa. Candida* was resistant to ZnO NPs synthesized by all isolates except one.

1. Introduction

Antibiotic-resistant bacteria are emerging pathogens whose resistance profiles generate a serious health crisis by holding their impact on human health. Misuse of antibiotics has directed the emergence of microbes' immune to presently accessible drugs. Pathogenic bacteria become resistant by employing various mechanisms, such as; antibiotic modification which is-one of the foremost effective bacterial approaches to resist the actions of antibiotics. This strategy is by the production of certain enzymes that deactivate the antibiotics by adding specific chemical moieties to the compound, rendering the antibiotic unable to interact with its target [1].

Other mechanisms to resist antibiotics are target site alteration, and biofilm formation, increasing the time they spend in the intracellular environment where antibiotics are unable to succeed at therapeutic levels. Due to this, attempts are being made to develop new alternative nano antibiotics as a promising approach to treat multidrug resistance disease-causing bacteria. Accordingly, there is considerable contemporary attention to the use of nanoparticles (NPs) as antibacterial agents against different pathogens and as target drug delivery toward specific tissues therefore microbes are eliminated by the biocidal properties because the conventional antimicrobial delivery system causes microbes to develop different resistance mechanisms, and one of the foremost encouraging approaches to advance the efficacy of antibiotics is to compound them with nano delivery materials[2][3]. Such vectors can protect drugs from enzyme degradation and increase the satisfying efficacy of the drugs [4].

Nanoparticles are typically defined as tiny solids, whose dimensions do not exceed 100 nm in all the three directions [5]. This new approach can be applied to the production of new antibiotics is a promising approach, since the use of nano metric size materials can result in greater contact between the compound and the bacteria with improved bioavailability, increased absorption, and the faster passage of the drug into the cell.

There is different type of nanoparticle one of them is metal nanoparticle. Metal-based nanoparticles are known to have non-specific bacterial toxicity mechanisms (they do not bind to a specific receptor in the bacterial cell) which not only makes the development of resistance by bacteria difficult, but also broadens the spectrum of antibacterial activity. And the metal nanoparticle can be obtained from a chemical and biological source. The use of chemical methods is very popular in the synthesis of nanoparticles but the use of hazardous chemicals limits their use in medical and clinical fields [6].

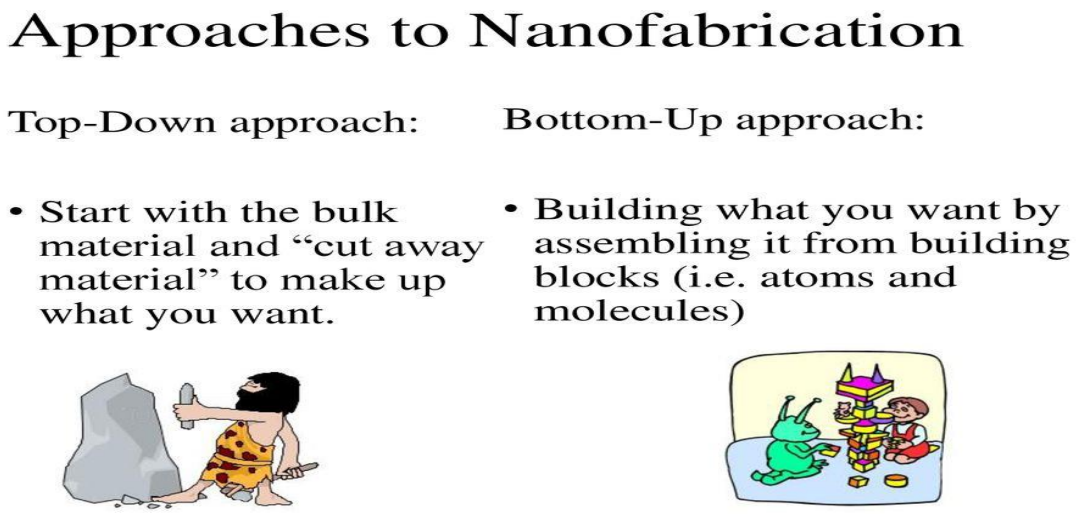


Figure (1): Approaches to nanoparticles

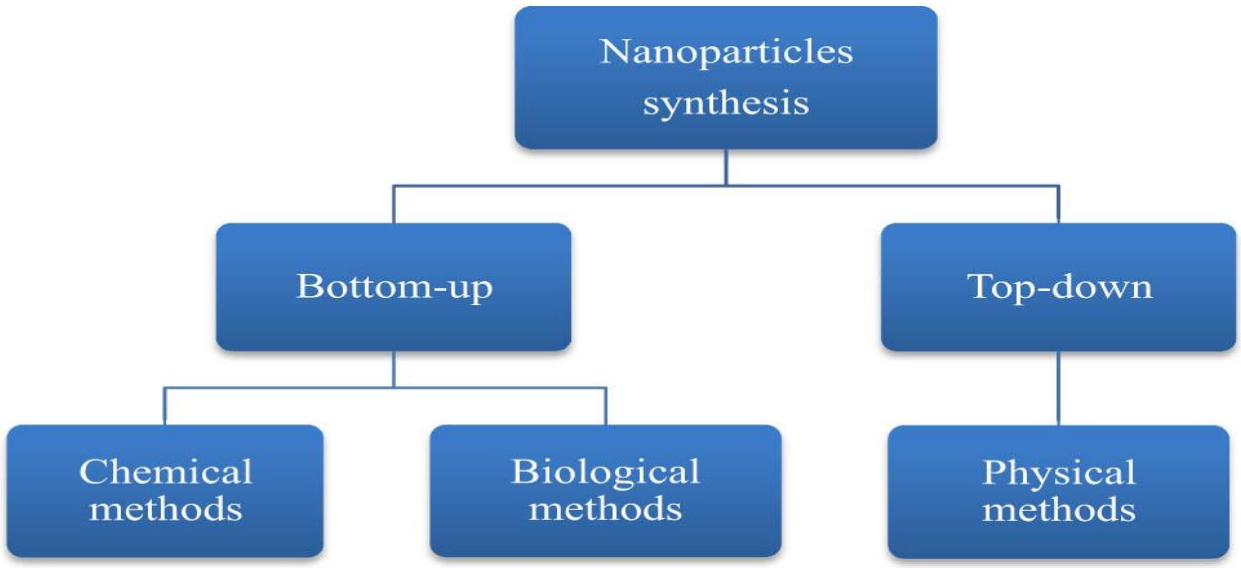


Figure (2): Nanoparticles synthesis types

The biological method is rapid, clean, simple, nontoxic, inexpensive and eco-friendly technology, their advantages coming from they are water soluble and biocompatible. Micro-organisms, enzymes and plant extracts has been suggested as possible biological methods to synthesize nanoparticles [7].

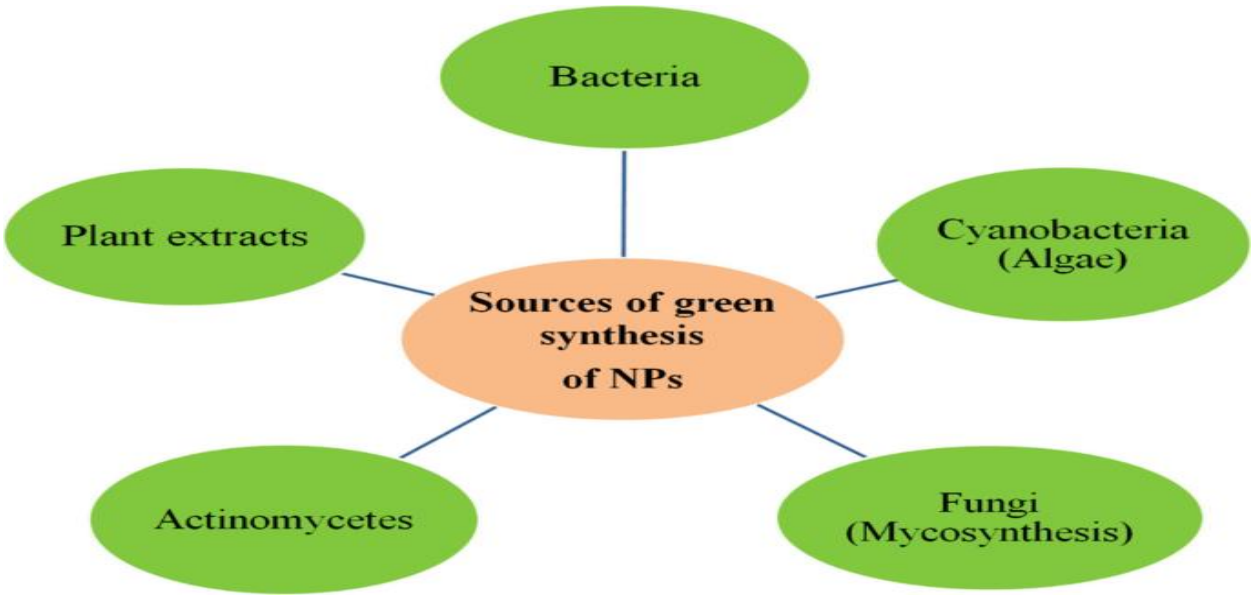


Figure (3): Sources of green synthesis of nanoparticles

Among biological system, microbes owing to their diversity emerge as a promising option for nanoparticle synthesis [8]. Bacteria are a good choice for study. They are also fast growing, inexpensive to cultivate and easy to manipulate. Growth conditions such as temperature, oxygenation and incubation time can be easily controlled. Bacteria possess remarkable ability to reduce heavy metal ions. For instance, some bacterial species have developed the ability to resort to specific defense mechanisms to quell stresses like toxicity of heavy metals. It was observed that some of them could survive and grow even at high metal ion concentrations [9]. Example on metal nanoparticle from biological sources is zinc oxide nanoparticle, which appears as a white powder, nearly insoluble in water. Zinc Oxide nanoparticles have been studied for their various applications in many fields including medicine.

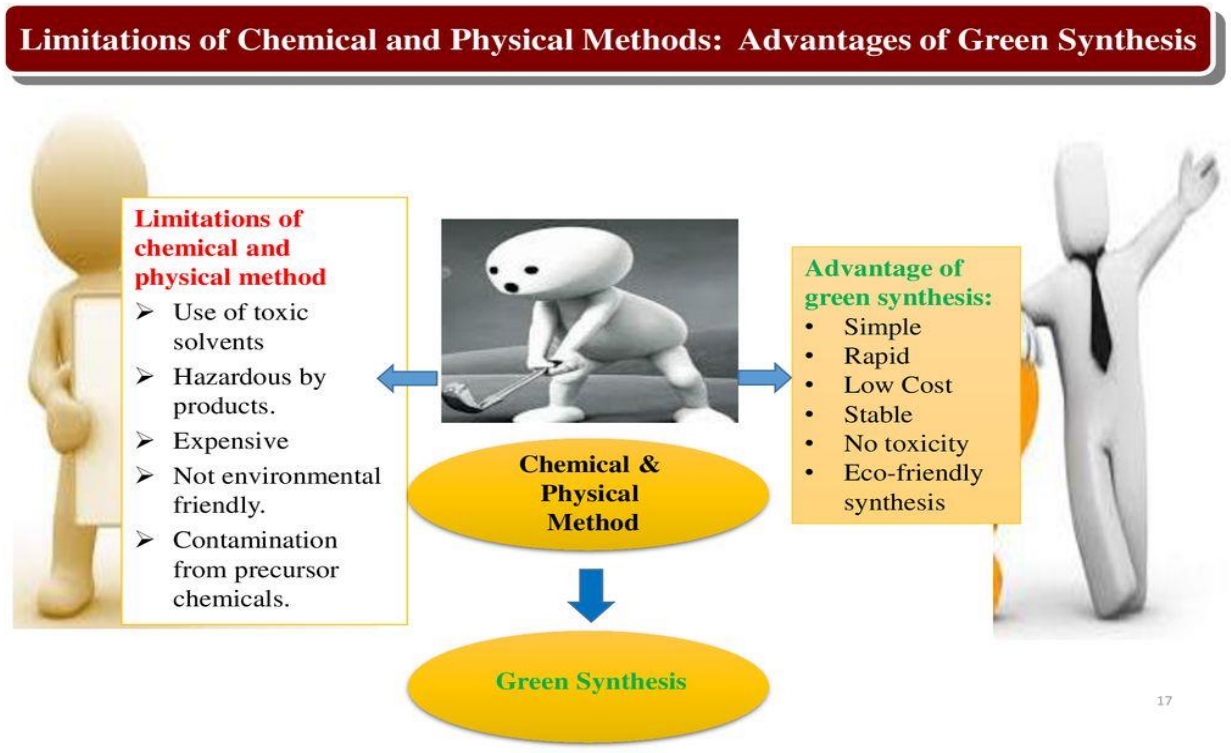


Figure (4): Properties of chemical physical and green synthesis of nanoparticles

Metal ions are initially trapped on the surface of the cell followed by reduction to NPs with the presence of bacterial enzymes [10].

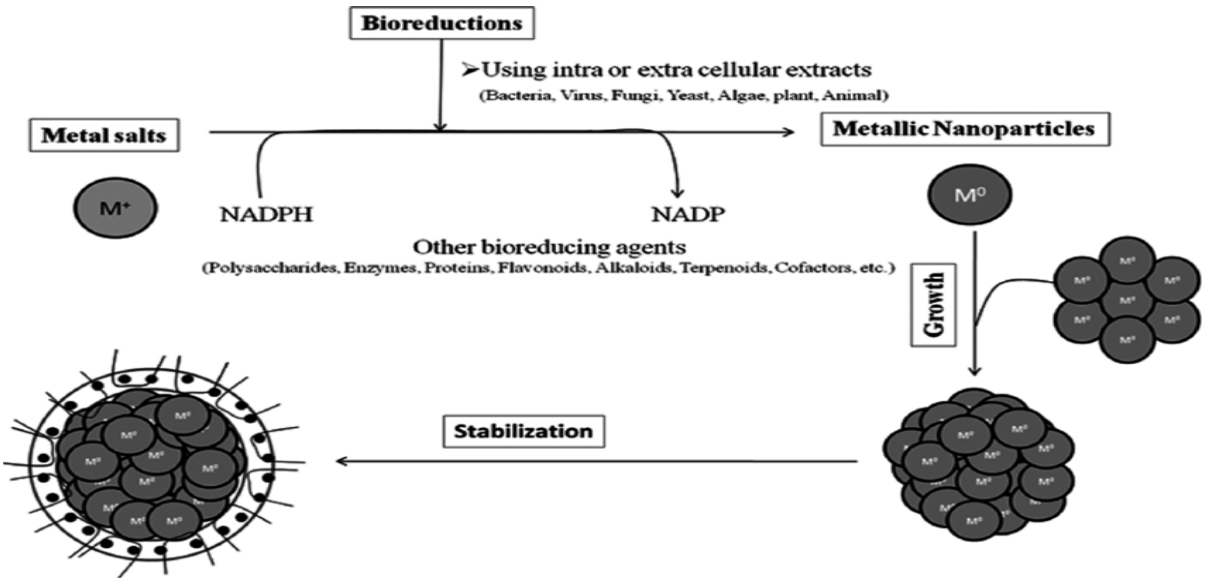


Figure (5): Bio reduction of metal salts for synthesis of nanoparticles

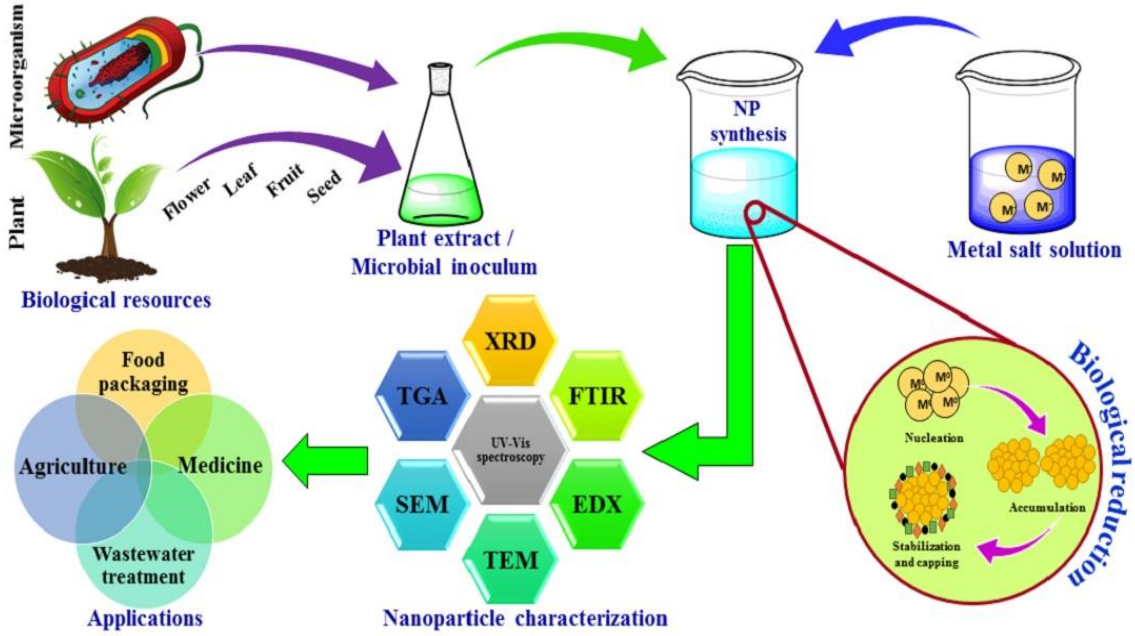


Figure (6): Nanoparticles synthesis steps

Zinc oxide exhibits antibacterial activity like many other metal oxide groups and that have been scaled down to the nano size and researched further. The advantage of using inorganic oxides such as zinc oxide as antimicrobial agents is that they contain mineral elements essential to humans and exhibit strong activity even when administered in small amounts. ZnO nanoparticles exhibit strong antibacterial activities on a broad spectrum of bacteria [11].

Zinc ions have been described to lead to bacterial cell death through direct membrane interaction leading to membrane destabilization and increased permeability [12], and through binding with nucleic acids and enzymes of the respiratory system, leading to disruption of enzyme function [13]. This has led some authors to consider Zn^{2+} ions as the true or major mediator of intracellular bacterial toxicity with internal cell disruption reportedly due to Zn^{2+} dissociation from ZnO NPs [13,14].

It has long been reported that the antibacterial activity of ZnO NPs is critically dependent on NP size, with higher antimicrobial activity commonly detected for smaller particles [15]. Some authors have suggested that a larger number of smaller particles can be accommodated at the surface of the bacteria [16] or that smaller, non-aggregated ZnO NPs are more likely to penetrate the cell membrane, leading to interior cellular damage [17]. The contact or accumulation of ZnO NPs at the cell surface can cause morphology changes and increased permeability within the immediate membrane contact area, lead to an increased dissolution of Zn^{2+} ions, and/or facilitate release ROS release directly at the bacterial surface [16,18,19].

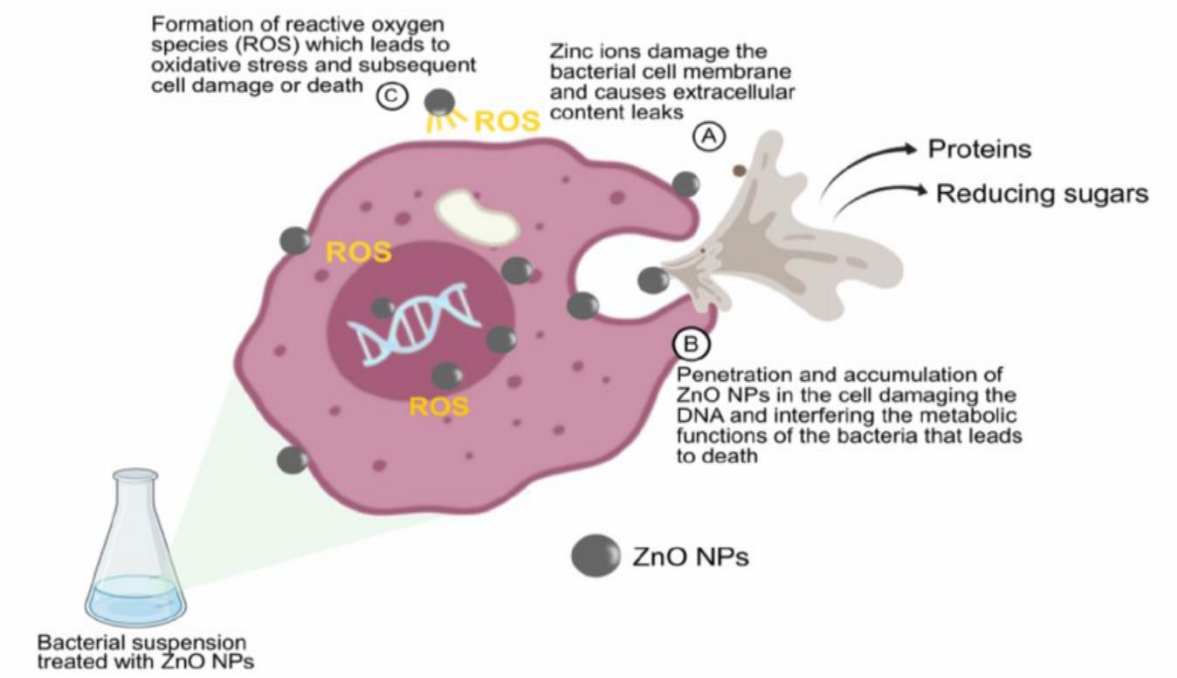


Figure (7): Mechanism of antibacterial action of green synthesized ZnO nanoparticles

2. Materials and Methods

2.1. Isolation technique

Soil samples in Basrah city were collected from gardens, the edges of the rivers and oily soil, at 11cm below the surface, in addition to compost samples. The samples were placed in plastic cases and were taken to the laboratory [20]. The soil samples were air dried and sifted. One gram of dried sifted soil sample was taken in 9ml of saline solution and agitated vigorously for 5 minutes, serial decimal dilutions of the supernatant were prepared. The soil dilutions were spreading on isolation media Sabouraud Dextrose Agar (SDA) contained 1 ml of Cycloheximide (100ùg/ml) as antifungal agents. The plates were incubated at 28-30 °C for 5 days. After that, the isolation bacteria were sub cultured by streaking on the same medium [21].

2.2. Microscopic visualization of soil bacterial isolates

The gram staining technique was performed to determine morphological characteristics (gram stain reaction, culture purity, and shape) of the isolates [22].

2.3. Screening for biosynthesis of ZnO NP

The isolated bacteria strains were examined to synthesize ZnO nanoparticles as follows: - each bacterial strain was seeded on a 250 ml conical flask containing nutrient broth medium for 24 hours at 35-37°C under 200 rpm shaking condition. After that, supernatant was used separately to synthesize ZnO nanoparticles according to [23, 24] with slight modification. 50 ml of 0.1 M zinc sulfate pentahydrate and 0.4 M sodium hydroxide were mixed with 50 ml of each culture filtrate of bacteria strains, followed by aggressive shaking and heating at 40°C for 15 minutes. The flasks were placed in the oven at 100C for 5-7 minutes before chilling for 1 hour to allow the nanoparticles to settle. The presence of white color deposition at the bottom of the flask confirmed the formation of nanoparticles.

2.4. Characterization of synthesized ZnO NP

The characterization of biosynthesized ZnO NPs was done by:

1.UV-Vis Spectrophotometry Analysis. The optical properties of the synthesized ZnO NPs were determined using a Perkin-Elmer lambda spectrophotometer. The reflectance spectra of the prepared samples were obtained at room temperature at a range of wavelengths between 320 and 380 nm [24].

2. Zeta sizer Nano Range. Zeta sizer gave information about the size and potential of the of ZnO NPs [25].

2.5. Antimicrobial activity of synthesized ZnO NP

Antimicrobial activity was measured using the agar well diffusion method against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. Briefly, microbial strains were cultured until the turbidity of 0.5 of McFarland standard was achieved. Using a sterilized cotton bud, the cultures were swabbed evenly on the nutrient agar plate. Next, the wells were made using a

sterile cork-borer with a diameter of 6 mm. About 100 µL of biosynthesized ZnO NPs were pipetted into each well and incubated at 37 °C for 24 h. Following incubation, the diameter (mm) of the inhibition zone was measured and recorded [26]. All isolates were subjected to Gram staining protocol and preserved on nutrient agar slants for further experiments [27].

3. Results and Discussion

3.1. Microbial isolation from soil

This study includes isolation of 9 bacterial isolates belonged to the Gram-negative bacteria and Gram-positive bacteria and 2 isolates of *Candida* from different soil samples and compost in Basrah city.

3.2. ZnO Nanoparticle Synthesis

The successful production of ZnO nanoparticles can easily be monitored by the presence of a white precipitate, which was an indication of the reaction mixture of the bacteria and the precursor salt $ZnSO_4.5H_2O$ As the 0.1 M solution of $ZnSO4.5H_2O$ was mixed drop by drop to the bacterial culture, the white precipitate was formed and the reaction mixture color changed from colorless to whitish as a consequence of the reduction of zinc ions; which indicated the materialization of ZnO nanoparticles (Figure 8 A and B).

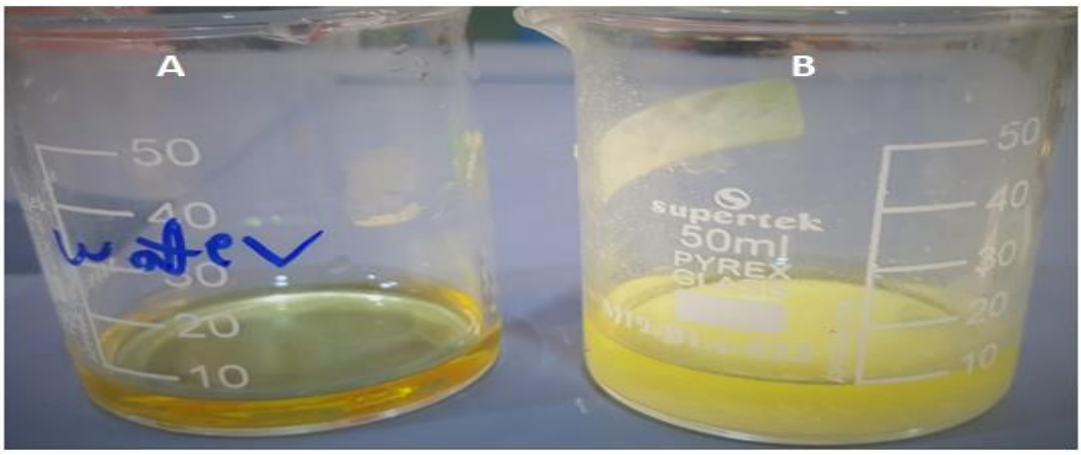


Figure (8-A): Synthesizing media (Nutrient broth) (A): before and (B) after Biosynthesis of ZnO nanoparticles

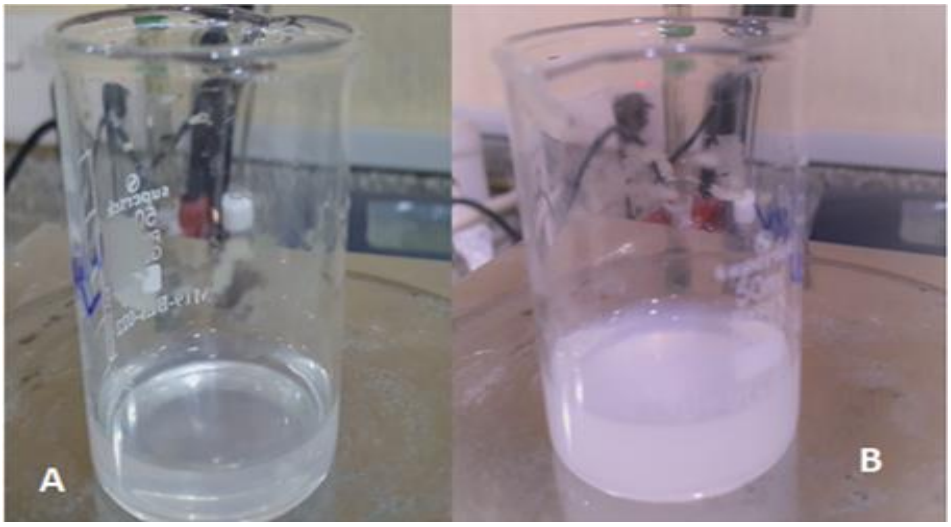


Figure (8-B) Synthesizing media(ISP4) (A): before and (B) after Biosynthesis of ZnO nanoparticles.

3.3. Screening of isolates for biosynthesis of ZnO NP

The formation of nanoparticles observed in 6 isolates belonging to Gram-negative bacteria, Gram- positive bacteria and *Candida*. Soil is an extensively explored ecological niche for sources of microorganisms that are involved in various interactions. Among these, Metal-microbe interactions have important roles with fascinating applications such as bioremediation, bio mineralization, bioleaching and microbial corrosion. However, recently that microorganisms have been explored as potential bio factory for synthesis of metallic nanoparticles such as cadmium, gold and silver [28]. Biosynthetic methods can be categorized into intracellular and extracellular synthesis according to the place where nanoparticles are formed [29].

3.4. Characterization of biosynthesized ZnO NP

1. Zeta sizer analysis of ZnO nanoparticles

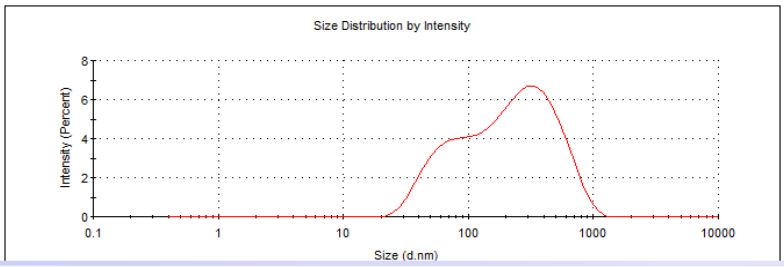
The synthesis of nanoparticle diameters was about obtained by Zeta sizer device from about 18-288 nm for all isolates, the poly disparity index (PDI) were about from 0.4-0.5 and that more acceptable range according to [30].

Records View | Intensity PSD (M) | Zeta Potential (M) | Molecular weight (M) | Autotitration (M) | Aggregation Point (M) | Chromatogram summary (M) | Expert Advice (M)

Sample Name:	grden1 1		
SOP Name:	mansettings.nano		
File Name:	dr rfef.dts	**Dispersant Name:**	Water
Record Number:	3	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, April 4, 2023 2:28:23 AM
Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	358.0	**Measurement Position (mm):**	3.00
Cell Description:	Disposable micro cuvette (40µl)	**Attenuator:**	8

		Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm): 136.1	**Peak 1:**	266.9	100.0	205.2
PdI: 0.493	**Peak 2:**	0.000	0.0	0.000
Intercept: 0.895	**Peak 3:**	0.000	0.0	0.000

Result quality : Good



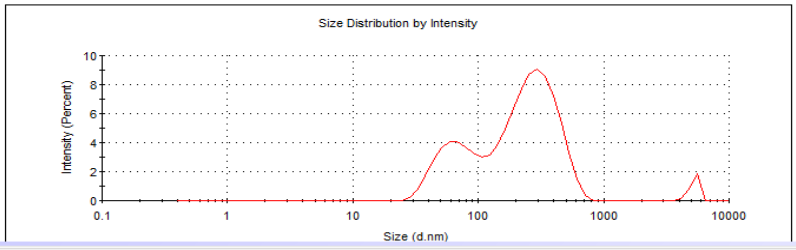
A (Gram-negative bacterium)

Records View | Intensity PSD (M) | Zeta Potential (M) | Molecular weight (M) | Autotitration (M) | Aggregation Point (M) | Chromatogram summary (M) | Expert Advice (M)

Sample Name:	1 2 زيتي		
SOP Name:	mansettings.nano		
File Name:	dr rfef.dts	**Dispersant Name:**	Water
Record Number:	4	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, April 4, 2023 2:33:16 AM
Temperature (°C):	25.1	**Duration Used (s):**	60
Count Rate (kcps):	359.9	**Measurement Position (mm):**	3.00
Cell Description:	Disposable micro cuvette (40µl)	**Attenuator:**	8

		Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm): 157.8	**Peak 1:**	288.0	70.8	124.7
PdI: 0.534	**Peak 2:**	67.17	26.5	21.32
Intercept: 0.895	**Peak 3:**	5276	2.7	422.1

Result quality : Good



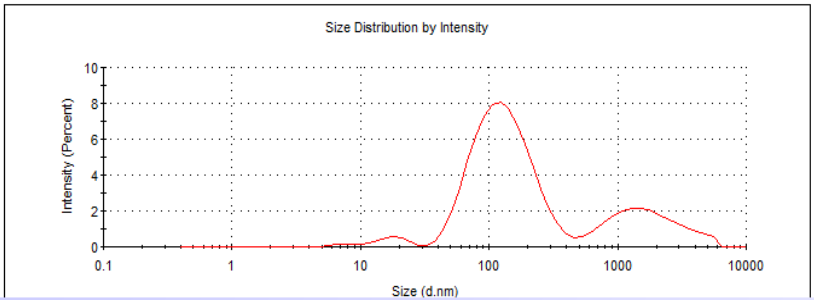
B (*Candida*)

Records View | Intensity PSD (M) | Zeta Potential (M) | Molecular weight (M) | Autotitration (M) | Aggregation Point (M) | Chromatogram summary (M) | Expert Advice (M)

Sample Name:	1 نهر داريه		
SOP Name:	mansettings.nano		
File Name:	dr rfef.dts	**Dispersant Name:**	Water
Record Number:	5	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, April 4, 2023 2:38:52 AM

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	244.6	**Measurement Position (mm):**	3.00
Cell Description:	Disposable micro cuvette (40µl)	**Attenuator:**	8

		Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm): 134.8	**Peak 1:**	141.0	72.8	75.68
PdI: 0.437	**Peak 2:**	1886	23.7	1203
Intercept: 0.942	**Peak 3:**	18.11	2.8	4.773
Result quality : Good				



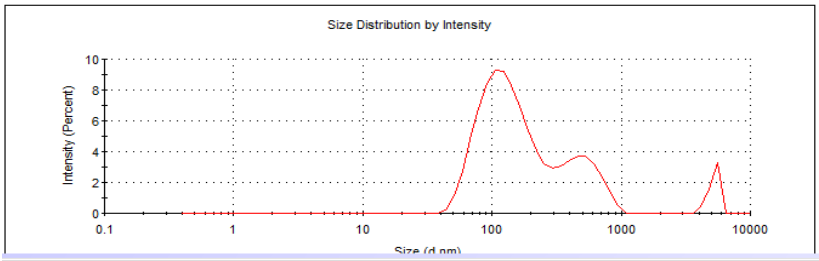
C (Gram-negative bacterium)

Records View | Intensity PSD (M) | Zeta Potential (M) | Molecular weight (M) | Autotitration (M) | Aggregation Point (M) | Chromatogram summary (M) | Expert Advice (M)

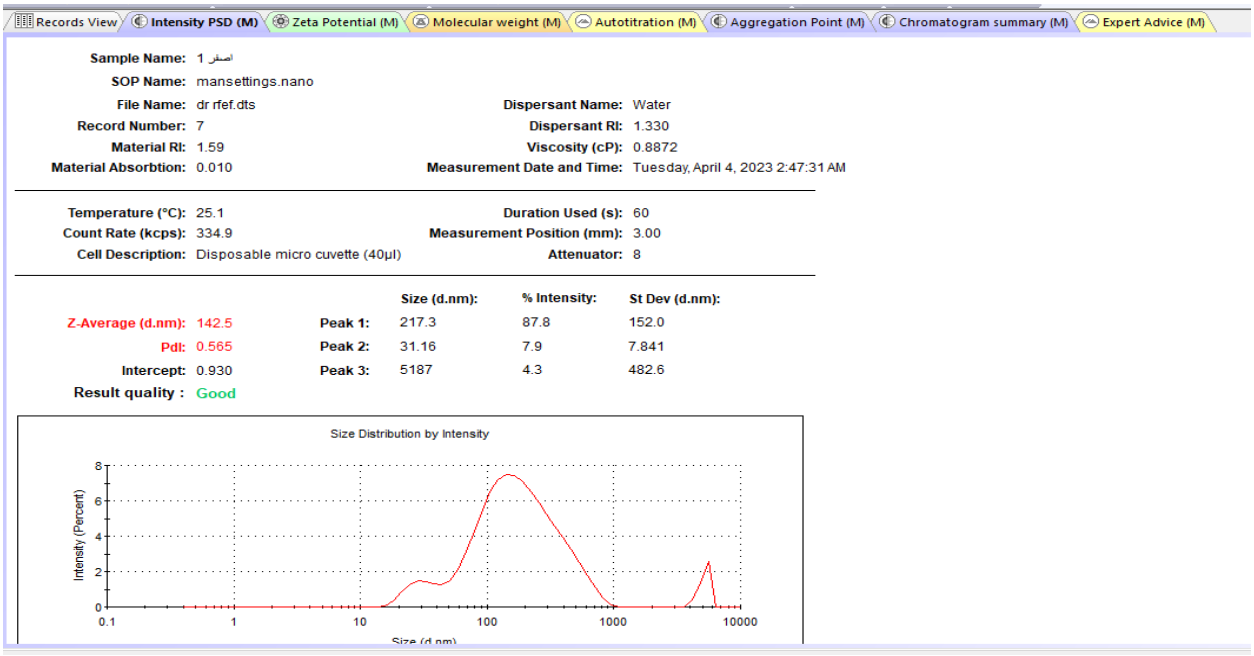
Sample Name:	1 سماد		
SOP Name:	mansettings.nano		
File Name:	dr rfef.dts	**Dispersant Name:**	Water
Record Number:	6	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.010	**Measurement Date and Time:**	Tuesday, April 4, 2023 2:43:05 AM

Temperature (°C):	24.9	**Duration Used (s):**	60
Count Rate (kcps):	278.7	**Measurement Position (mm):**	3.00
Cell Description:	Disposable micro cuvette (40µl)	**Attenuator:**	8

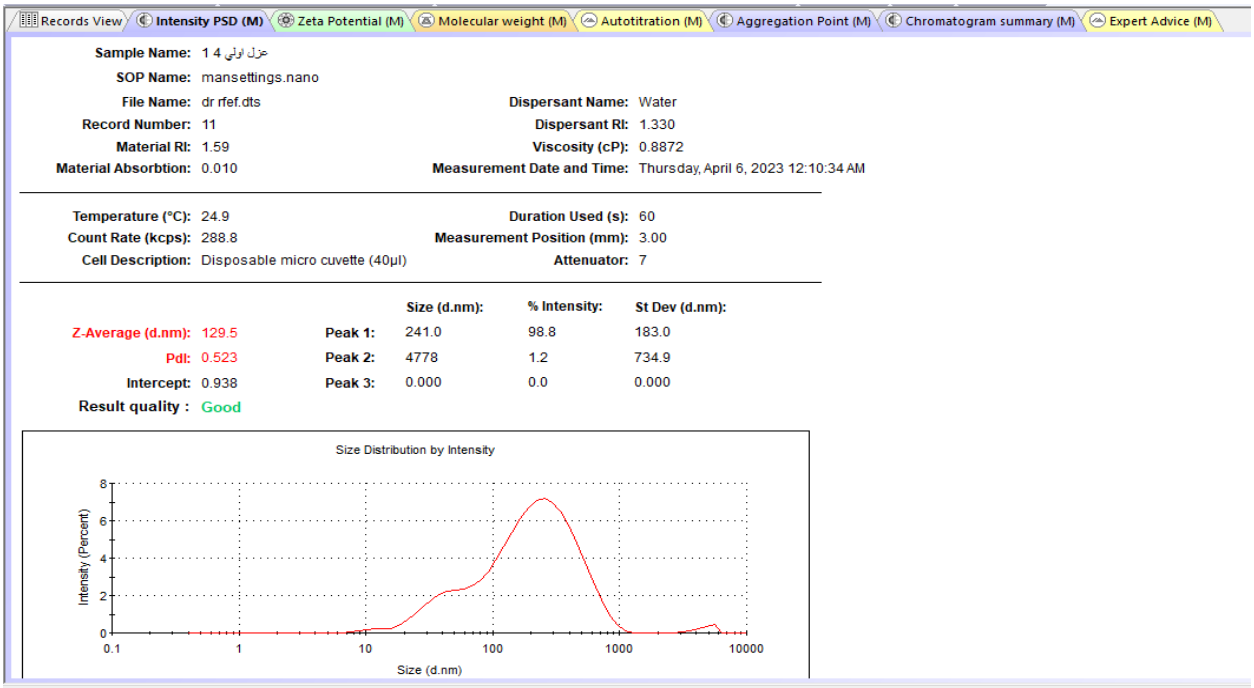
		Size (d.nm):	**% Intensity:**	**St Dev (d.nm):**
Z-Average (d.nm): 150.3	**Peak 1:**	134.6	71.2	60.32
PdI: 0.549	**Peak 2:**	503.7	23.7	163.8
Intercept: 0.912	**Peak 3:**	5222	5.1	462.0
Result quality : Good				



D (Gram-positive bacterium)



E (Gram-positive bacterium)



F (Gram-positive bacterium)

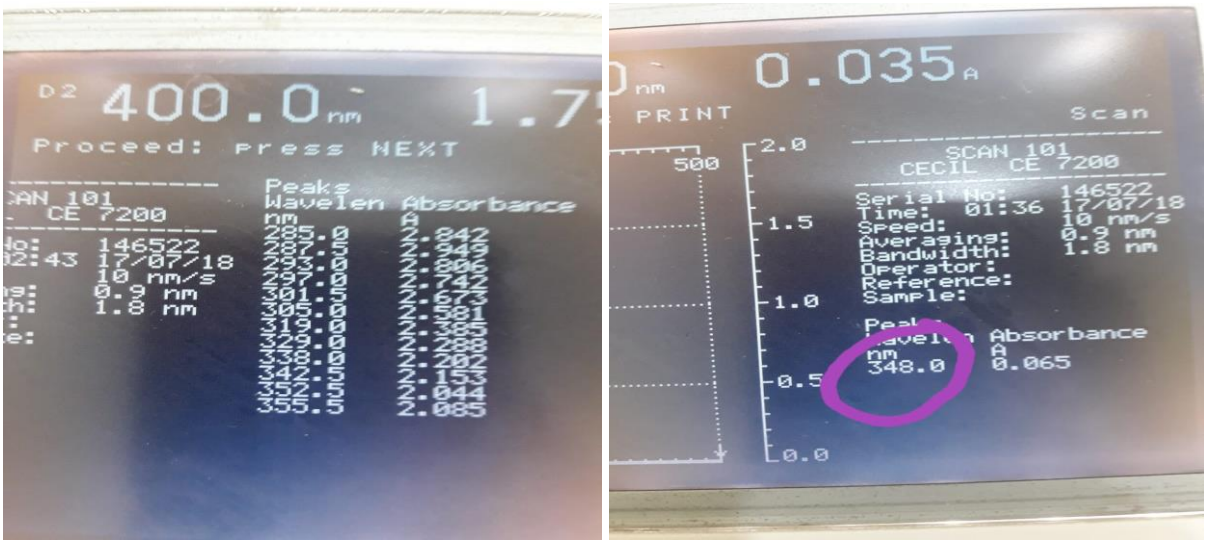
Figure (9): Zeta sizer analysis spectrum of supernatant samples of ZnO nanoparticles (A-F)

2. UV-Vis absorption analysis of ZnO nanoparticles

Figure (10) shows the UV-Vis absorption spectra of ZnO nanoparticles. The UV-Vis spectroscopic study shows the Plasmon resonance property, confirmed the reduction of metal ion and formation of nanoparticle (A-F) with peak at 355, 324, 331,323,355 and 348 nm respectively.

A (Gram-negative bacterium) **B (*Candida*)**

C (Gram-negative bacterium) **D (Gram-positive bacterium)**



E (Gram-positive bacterium) **F (Gram-posative bacterium)**

Figure (10): UV-Vis absorption analysis spectrum of supernatant samples of ZnO nanoparticles (A-F)

3.4. Evaluation of Antimicrobial Activity

A qualitative agar well diffusion test was performed to screen ZnO NPs for their antimicrobial properties against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. The growth-inhibiting effect of ZnO NPs was shown by the formation of clear zones on the Mueller–Hinton agar plates (figure 12). The larger the zone of inhibition, the more sensitive the test organism is to the nanoparticle, thus indicating the effectiveness of the nanoparticles.

Figure 11 shows the inhibition zones (mm) formed around the nanoparticles and positive control ($ZnSO_4.5H_2O$ solution). Nanoparticles were recorded the highest inhibition zones against *S. aureus*. Moderate inhibition zones were recorded against *E. coli* and *Ps. aeruginosa*. Result of this study showed that the antimicrobial effect of ZnO NP against Gram-positive bacteria was more than Gram-negative bacteria.

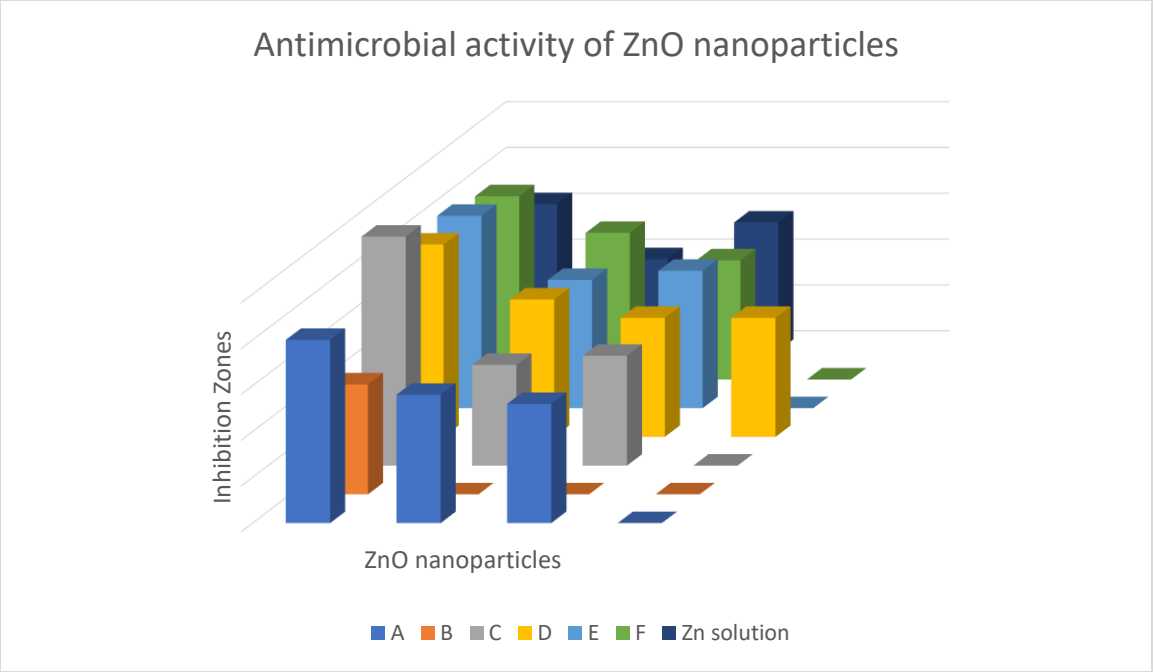
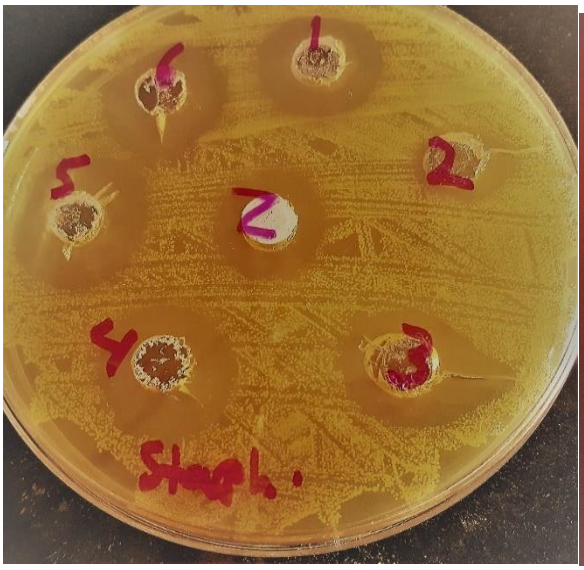


Figure (11): Inhibition zones (mm) of pathogens against ZnO nanoparticles by well diffusion method

This result is consistent with the result of [31] that was demonstrated the microbial activity of ZnO NPs to be higher against *S. aureus* than E. *coli*. The results of [32, 33] showed that ZnO nanoparticles have antibacterial effect against *E. coli* and *S. aureus*, respectively. Gram-negative bacteria seemed to be more resistant to ZnO nanoparticles than Gram-positive bacteria. However, in a study by [34], ZnO nanoparticles were found to be more effective against *E. coli* than *S. aureus*. The nanoparticles in this study were completely ineffective against *Candida* except one. This result doesn't agree with the study of [35] which concluded that ZnO as antifungal agent can well reduce the growth *Candida albicans*. ZnO Np exhibits inhibitory effects on biofilms of both isolates *Candida albicans*. These findings provide an important advantage of ZnO that may be useful in the treatment of catheter-related urinary tract infection [36].

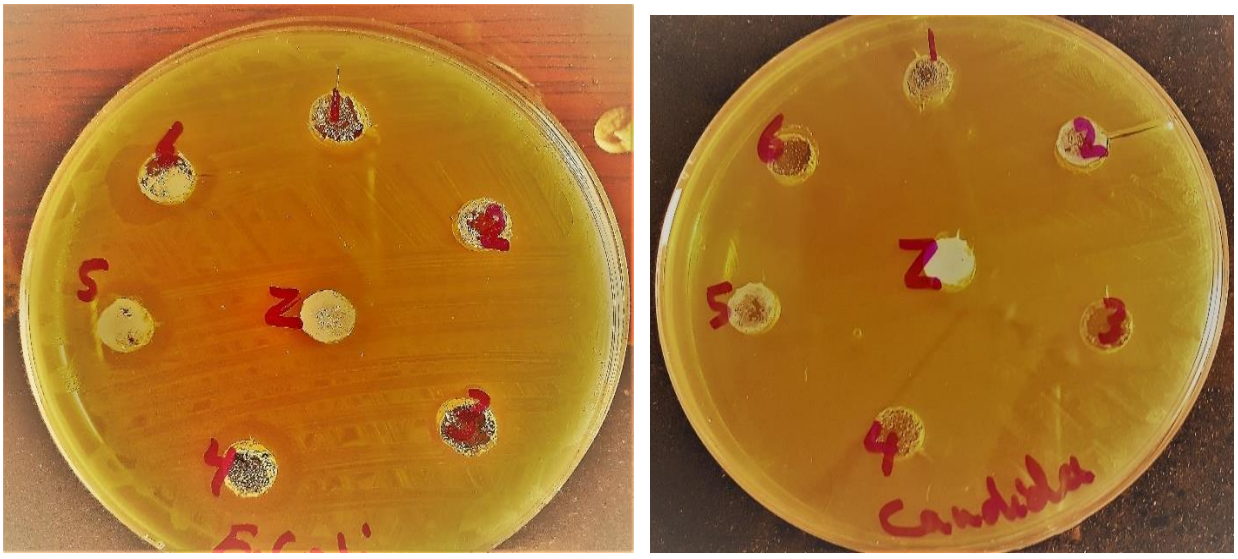
Several metal and metal oxide nanoparticles are successfully commercialized in the fields of electronics, textile, agriculture, environment, and health. ZnO NPs are one of the most used nanomaterials. They can be effectively used as antibiotic for pathogenic bacteria and viruses which show resistance towards the existing

commercially available antibiotics. They have unique physicochemical characteristics that can affect biological and toxicological responses in microorganisms. Due to its low toxicity, biocompatibility, and biodegradability, they are widely used in the industries of biomedicines and personal care products. They can be combined with antibiotics and anti-inflammatory drugs to enhance antimicrobial activity against pathogenic microorganisms without antibiotic resistance in non-clinical and clinical conditions. ZnO NPs interact electrostatically with the bacterial cell membranes and disrupt them leading to leakage of cell and death of bacteria. They are cost-effective and acts against a wide range of bacteria. The absorption of antibiotics by the bacterial cells can be increased by combining them with ZnO NPs. This is a novel substitute for the treatment of bacterial infections [37].



Staphylococcus aureus

Pseudomonas aeruginosa



Escherichia coli ***Candida albicans***

Figure (12): Inhibition zones (mm) of bacteria against ZnO nanoparticles by well diffusion method

Conclusion

Conclusively, bacterially synthesized ZnO nanoparticles from Gram-negative and positive bacteria and *Candida* have potential as antibacterial agents against resistant pathogens *S. aureus, E. coli* and *Ps. aeruginosa* but ineffective against *Candida albicans*

Recommendation

1. Making optimization study to determine the exact concentration of metal solution that can give highest amount of NP.

2. Some of the bacterial isolates have the ability to biosynthesis the metal NP inside the cell, therefore we recommended to detect the presence of NP inside the cell as well as in the outside " in the culture medium".

3. Trying to do all the characterization test for the NP like SEM, TEM, AFM and XRD.

4. Screening of soil bacterial isolates for secondary metabolites production.

6. Antimicrobial activity of secondary metabolites as antibiotics against pathogenic microorganisms and comparison with standard antibiotics.

5. Antimicrobial activity of combination between nanoparticles and secondary metabolites against pathogenic microorganisms and comparison with standard antibiotics.

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