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Effectivity of Iron Oxide Nanoparticles Synthesis by Intracellular *Lactobacillus* as Antibacterial Agent against *Pseudomonas aeruginosa*

Maha A. Qasim , Laith A. Yaaqoob

Department of Biotechnology, College of Science, University of Baghdad,

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Abstract: The aim of this study is to demonstrate the biosynthesis procedure of iron oxide nanoparticles (Fe_2O_3 NPs) by using intracellular components produced from environmental isolate *Lactobacillus Plantarum* as a reducing and stabilizing agent studied in the laboratories of the College of Science, University of Baghdad from November 2021 to March 2022. Take 1 g of ferric sulfate was added to 10 ml from intracellular for synthesis nanoparticles. The biosynthesized Fe_2O_3 nanoparticles have presented many applications such as catalysis, biosensing, anticancer, biomedical, etc. The study of optimum conditions for the synthesis of Fe_2O_3 was characterized by different techniques, such as UV-VIS, AFM, XRD, FTIR, and FE-SEM. The wavelength of biosynthesis of Fe_2O_3 from intracellular by using UV-VIS is (304 nm), Image FE-SEM displays Spherical Fe_2O_3 NPs in nano-cluster. The antibiotic susceptibility test of *P.aeruginosa* isolates was shown to be resistant to Tetracycline, Trimethoprim-Sulfamethoxazole, Ceftazidime, and Chloramphenicol, while sensitive to Amikacin, Norfloxacin, Meropenem, and Ciprofloxacin, and the effect of Fe_2O_3 NPs from intracellular on bacteria *Pseudomonas aeruginosa* on an inhibition zone 15 mm.

Keywords: Fe_2O_3 NPs, biosynthesis nanoparticles, Intracellular.

Corresponding author: (Email:maha.abd1206a@sc.uobaghdad.edu.iq).

Introduction

The category of *lactobacilli* known as facultatively heterofermentative includes *L. Plantarum*. It is a diverse and adaptable species that can be found in a wide range of environmental niches, such as dairy, meat, fish, and numerous plant or vegetable fermentations. Numerous types of cheese have also been reported to contain *L. Plantarum* strains. In addition, *L. Plantarum* strains have a history of successfully colonizing the intestines of mammals, including humans (1). *Lactobacillus Plantarum* can be distinguished from other bacteria because of its characteristic of Extracellular and Intracellular products, Extracellular and Intracellular are components composed of (Enzymes, proteins, polysaccharides, peptides, etc.) (2). These components are used as reducing agents and also used as biological defenses against various stresses such as phage attacks, toxic metal ions, and desiccation (3). Iron particles that are smaller than a micron is known as nanoscale iron particles. They have a large surface area, which makes them very reactive. They quickly oxidize to produce free iron ions when oxygen and water are present. They are extensively employed in medical and laboratory applications, and their

potential for cleaning up industrial areas contaminated with chlorinated organic chemicals has also been investigated (4,5). *Pseudomonas* is a widespread rod-shaped, gram-negative, encapsulated bacteria that can harm both humans and other animals and plants. It is positive for oxidase, catalase, and citrate (6,7). *P. aeruginosa*, a species of major medical significance, is an opportunistic bacterium resistant to several medicines and disinfectants that causes severe acute and chronic nosocomial infections in immunocompromised, catheterized, or burn patients (8). Biofilms are created when bacteria proliferate and attach to the surfaces of biomaterials. The bacterial cells are shielded by the biofilm growth phase from the host defense mechanism and antibiotic (9). Because of its multitude of scientific and technical uses, including biosensors, iron oxide has attracted particular interest (10). Antimicrobial activity (11). Additionally, due to its biocompatibility and magnetic characteristics, it has been extensively used in biomedical research (12).

Materials and methods

Species were by taking samples, of *Lactobacillus spp* isolated from a fermented food product. Isolates were cultured in MRS broth, By the manufacturer's instructions (13). All bacterial species isolate were identified via conventional biochemical assays and identifying procedures through a VITEK2 system.

Intracellular production

The bacterium was cultured on MRS broth media at 28 ˚C for 48 hrs, then centrifuged, the precipitate was taken, the filter was left, then twice washed with normal saline, and then an ultrasonic probe was used to break down the cell wall of the bacterium (14).

Synthesis of iron oxide nanoparticles from intracellular

Synthesis of iron oxide nanoparticles by biological method approach using Ferric Sulphate $Fe_2(SO_4)_3$ (Indian) with modification (15), used in the synthesis of Fe_2O_3 NPs from intracellular for *Lactobacillus SPP* In a typical procedure: 5gm of Ferric Sulphate $Fe_2(SO_4)_3$ was dissolved in 50 ml of the solution of intracellular produce from *Lactobacillus SPP* and dispersed by an ultrasonication bath for 10 min to more mix component, then put the flask on a shaker in the darkroom overnight. Then centrifugated for 20 min at 8000 rpm. The precipitate of a solution containing the iron oxide nanoparticles was washed twice with deionized distilled water to get rid of the remnant of the intracellular. the resulting nanoparticles precipitation was dried in the oven at 40 °C overnight. Finally, the brown powder was kept in a dark vial.

Pseudomonas aeruginosa isolate

The clinical specimens *of P. aeruginosa* collection are 121 clinical isolates and took place from November 2021 to February 2022. It's important to note that the samples contain burns and wounds. Under the specific condition, Cetrimide agar prepared according to the manufacturer was used to streak specimens taken from the hospital (16,17). Other identification tests included biochemical tests and morphological characteristics and the

Vitek-2 system was performed (18).

Antibacterial activity of Fe_2O_3 nanoparticles

Pseudomonas aeruginosa was cultured on Muller-Hinton agar and using the good method (MIC) of microbial inhibition by using Fe_2O_3 NPs synthesis from intracellular sources (19). Almost 25 ml of the Mueller Hinton agar sterilized medium was placed into sterile plates and enabled to solidify at room temperatures. The growth of the test species was transported and spread over. The agar medium by a sterile cotton swab separately and wells were made. Subsequently, Diverse ratios of Fe_2O_3 NPs from intracellular (500, 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625, 1.953125) mg/ml. Plates inoculated with Fe_2O_3 NPs were incubated at 37°C for 24 hours. The inhibition zone around the well was assessed after incubation (16, 20).

Detection of biofilm (quantitative method)

After incubating the *P. aeruginosa* isolates in BHI medium at 37°C for 24 hours, added 200µl of *P. aeurginosa* broth to each well in a microtiter plate, and then 200 µl of BHI medium was added to each well in a microtiter plate. After the incubation period for 24 hours, each well was washed three times with phosphate-buffered saline. Plates were then air-dried at room temperature for 15 minutes before adding 99% methanol 200 µL over 15 minutes. Plates were then air-dried at room temperature for 15 minutes and stained with 200 µl of 2% crystal violet for approximately 15 minutes. After the stain is completely removed, add 200 µL of 95% ethanol to each stained well and incubate at room temperature for 15 minutes. The optical density of the wells of the plate was measured by using a micro-ELISA auto reader at 550nm. Then they used Sterile BHI broth as a negative control of the test. To compensate for background absorbance, the mean of (OD) is optical density and the reading value of the control mean (C) was taken from the test (T) values (21). (Table 1) measures the intensity of biofilm:

Table (1): Intensity of biofilm.

Adherence of Biofilm Formation	Interpretation
ODs ≤ ODc	Non –adherent
ODc < ODs ≤ 2 * ODc	Weakly adherent
2 * ODc < ODs ≤ 4 * ODc	Moderately adherent
4 * ODc < ODs	Strongly adherent

Results and discussion

Bacterial isolation in culture media:

Lactobacillus samples were obtained from various sources such as dairy products including (cow milk, buffalo milk, cheese, and yogurt) (22). The samples were primarily cultured on MRS agar plates as selective media for isolation and incubated at 28°C for 48 hours with the presence of (3-5 %) CO2 by using Candle Jar. These bacteria can be examined using morphology, microscopy, and biochemistry testing to validate their identity (Figure 1).

Figure (1): *Lactobacillus Plantarum* cultured on MRS agar

Antibiotic susceptibility Test of *P.aeruginosa*

Of the 121 isolates, only 63 were tested for susceptibility to 12 different antibiotics using the disc diffusion method recommended by the Clinical and Laboratory Standards Institute (23). guidelines and the results are shown in Figure (2). Showed varying levels of resistance, In this study, *P.aeruginosa* showed the highest percent of resistance to Tetracycline followed by Trimethoprim-Sulfamethoxazole, Ceftazidime, and Chloramphenicol.

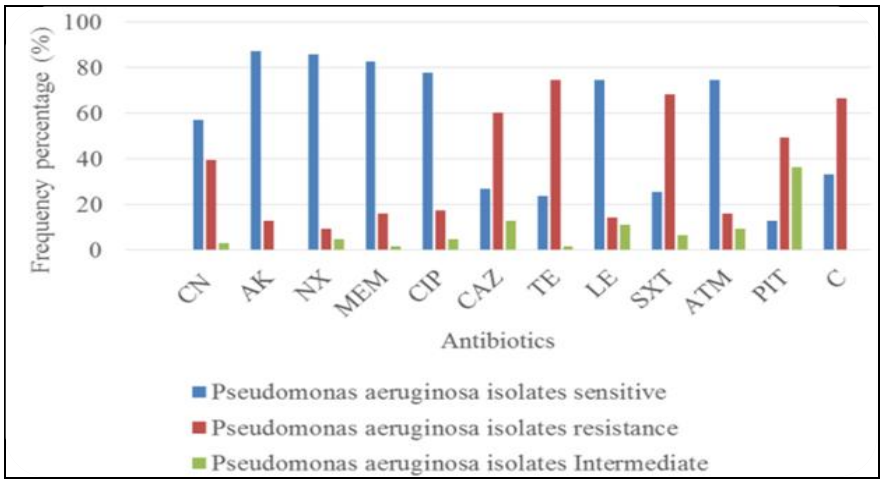


Figure (2): The results of Antibiotic Susceptibility Test of *P.aeruginosa*

Characterization of biological synthesis Fe_2O_3 NPs

UV–VIS spectral analysis Fe_2O_3 from intracellular

The optical properties of the nanoparticles were studied by exploiting ultraviolet – Visible spectrometer. (Figure 3) shows the absorbance of the sample in the nano range at room temperature. It has shown a peak at 304 nm wavelength was thus agreed with (24).

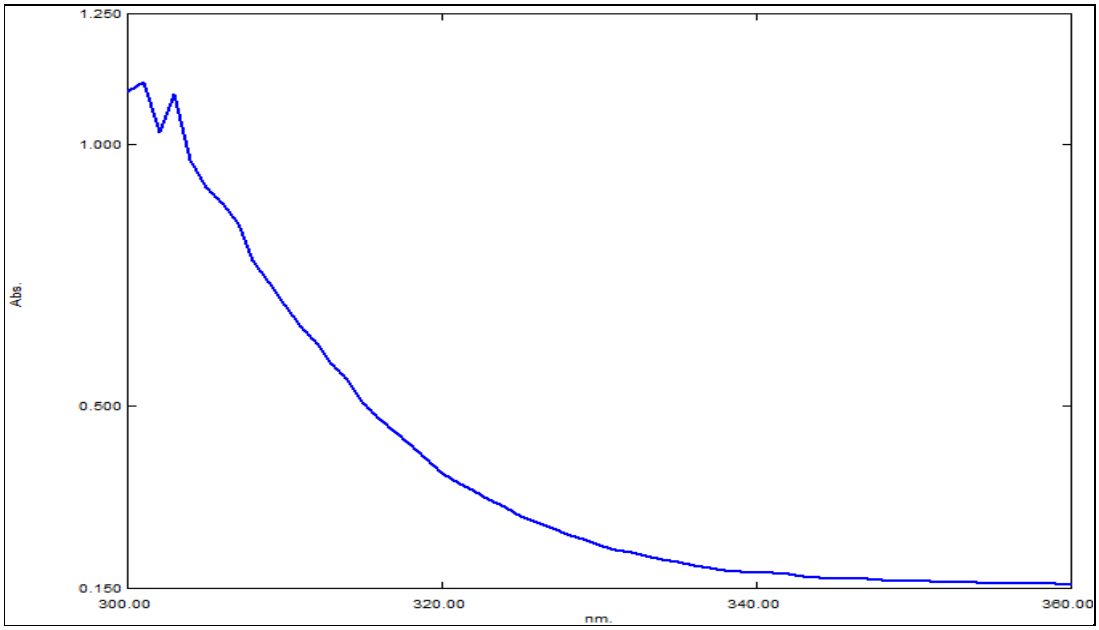


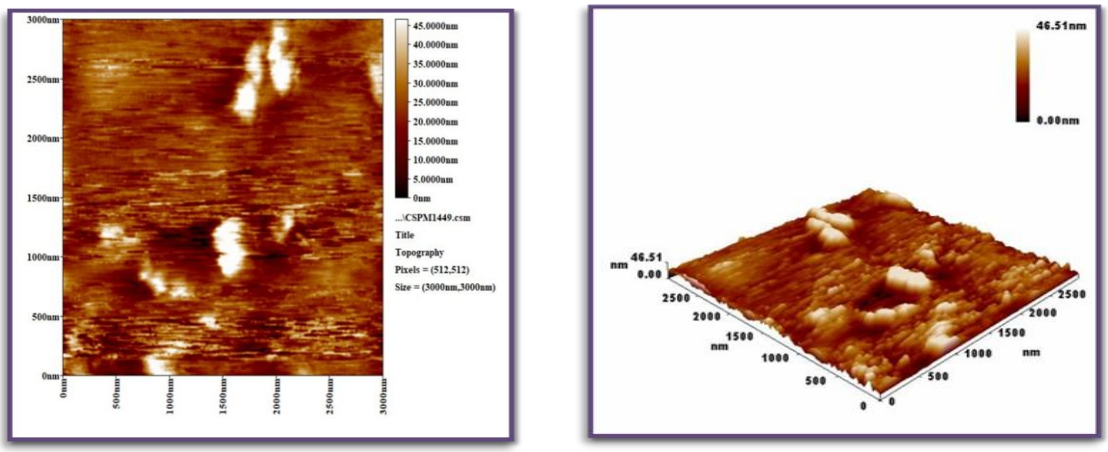
Figure (3): The UV-VIS of Fe_2O_3 nanoparticles synthesis using intracellular

Atomic force microscopy (AFM) analysis of Fe_2O_3 from intracellular

The surface shape formation of the Fe_2O_3 NPs was studied by atomic force microscopy to show that Fe_2O_3 NPs 2D and 3D (16). (Figure 4). AFM images show that the biosynthesized Fe_2O_3 NPs are spherical. The size of an average diameter of 57.23 nm, (Table 2) was also measured by AFM (Figure 5).

Table (2): The average diameter of Fe_2O_3 nanoparticles Synthesized using Intracellular.

Avg. Diameter: 57.23 nm	**<=10% Diameter: 25.00 nm**
<=50% Diameter: 50.00 nm	**<=90% Diameter: 90.00 nm**



(2D) **(3D)**

Figure (4): Atomic force microscopy (AFM) of Fe_2O_3 NPs synthesized using intracellular illustrate2D and 3D topological

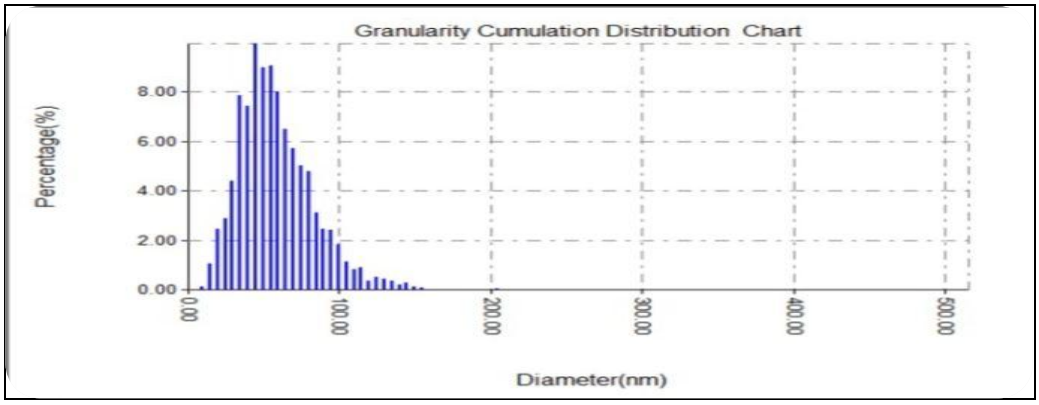
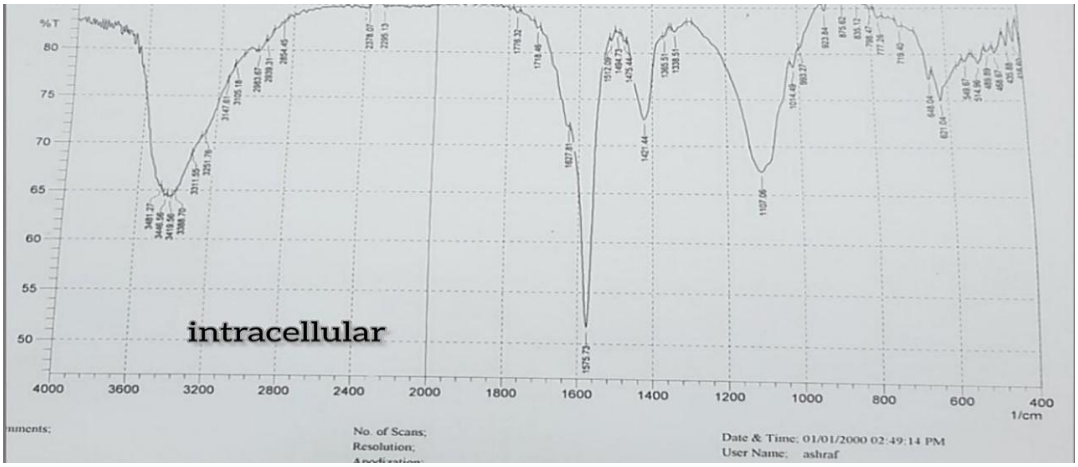
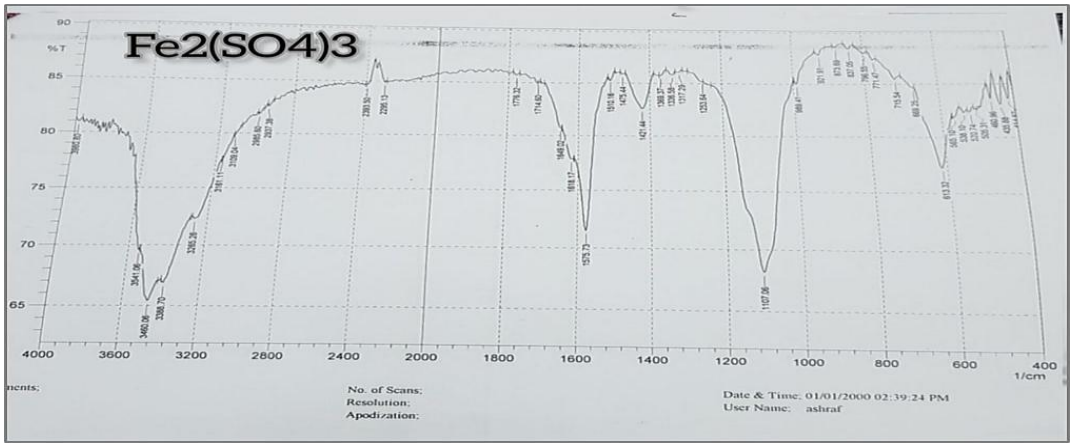


Figure (5): The average size of iron nanoparticles synthesized using intracellular

Fourier transform infrared (FTIR) analysis of Fe_2O_3 from intracellular

FTIR spectrum has determined the functional groups of nanoparticles. (Figure 6) Represents the absorption spectrum of Biologically synthesized nanoparticles in FTIR. An intense peak at 3398.34 cm^{-1} was visible due to OH stretching mode. The occurrence of the peak properties at 1629.74 cm^{-1} suggested the presence of crystallographic H_2O molecules, i.e. O–H bend. The wide peak at 455,17 cm-1 and 572,82 cm^{-1} respectively represented the Fe–O band and Fe–O–Fe skeletal frequency (16).





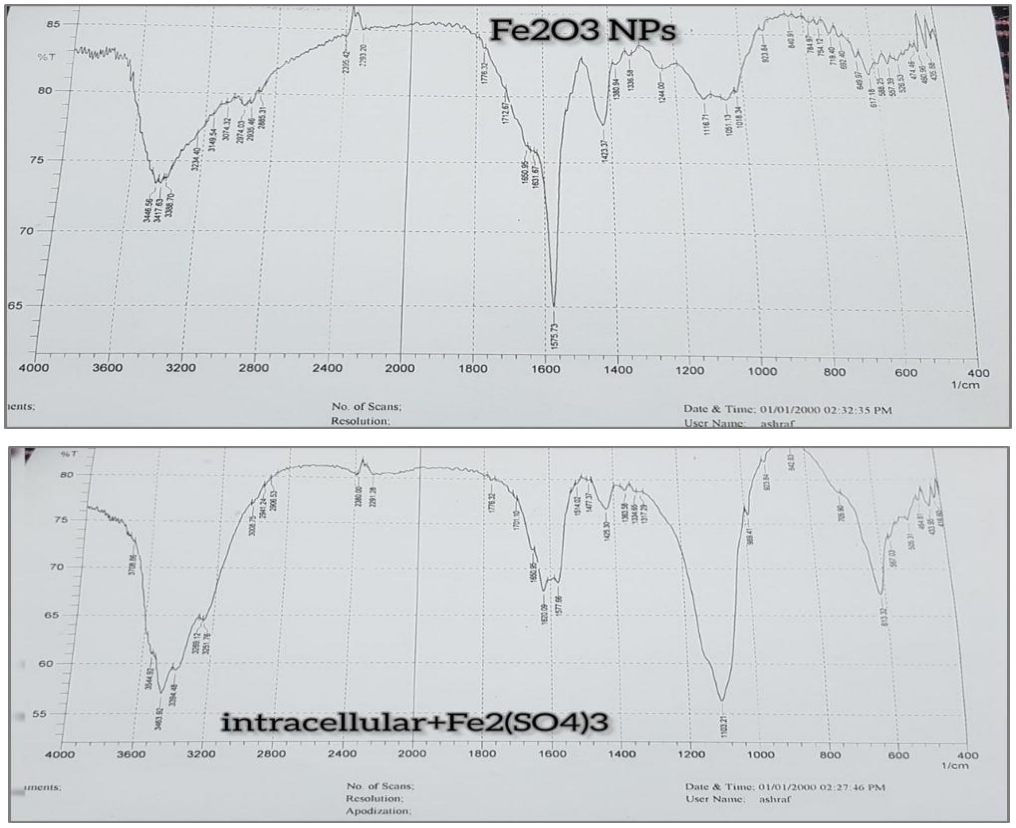


Figure 6: FTIR image of Fe_2O_3 NPs synthesized using intracellular, (A) Intracellular, (B) $Fe_2(SO_4)_3$, (C) Fe_2O_3 NPs, (D) $Fe_2(SO_4)_3$-Intracellular

Field emission scanning electron microscopy analysis of Fe_2O_3 from intracellular

Through applying FE-SEM, images were taken of the sample at a magnification of 50kx. Focused on (Figure 7). The whole sample has soft planes and a uniform shape in the form of Fe_2O_3 nanocluster centers (16).

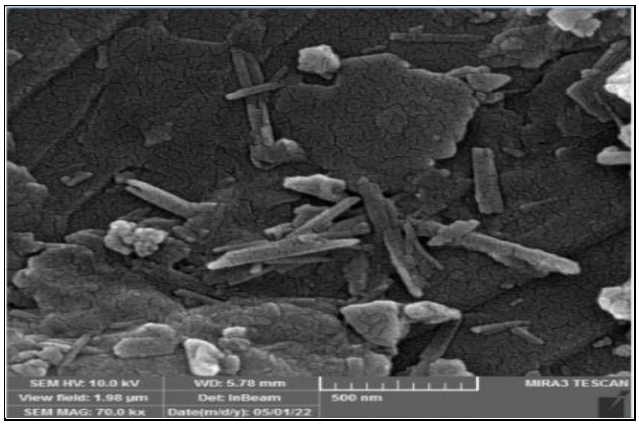


Figure (7): FE-SEM Images of Fe_2O_3 NPs synthesized using Intracellular.

Antibacterial susceptibility test

Iron Oxide NPs antibacterial activity was investigated using Gram-negative bacteria (*Pseudomonas aeruginosa*). The minimal inhibition concentration (MIC) of Fe_2O_3 NPs for microorganisms was calculated by the use of the agar well diffusion technique

(25, 26). Results of Fe_2O_3 NPs from intracellular antibacterial activity were shown in (Figure 8). The antibacterial activity was found to be directly dependent upon the Fe_2O_3 NPs concentration. Table (3) shows that the maximum inhibition zones of *Pseudomonas aeruginosa* were 15mm respectively at a concentration of 500 mg/ ml of Fe_2O_3 NPs from intracellular, Whereas the minimum inhibition zones were located at 62.5 mg/ml Fe_2O_3 NPs concentrations. The difference in inhibition diameter may be due to different interactions between Fe_2O_3 NPs and the microorganism, and due to the susceptibility of bacteria used in the current study. The main mechanism of toxicity of Fe_2O_3 NPs potentially associated with metal oxides carries the positive charge even though the microorganisms bear negative charges; this results in electromagnetic interaction between microorganisms and metal oxides leading to oxidation and finally death of microorganisms. The MIC was determined over a range from 500 to 1.953125 mg/ml for Fe_2O_3 NPs from extracellular and intracellular. by the serial dilution method, as described in CLSI (16). The bactericidal action of Fe_2O_3 nanoparticles on bacteria is of extreme importance due to the ability of pathogenic bacteria to join the food chain of the ecosystem (27). The antimicrobial effect of Fe_2O_3 against bacteria has been demonstrated (16, 27,28) and communicated in modern research.

Table (3): The inhibition zone of antibacterial effect of Fe_2O_3 from intracellular on *P.aeruginosa*

	Fe_2O_3 concentration (mg/ml)	**Inhibition zone (mm)**
A	500	15
B	250	11
C	125	9
D	62.5	6
E	31.25	Nill
F	15.625	Nill
G	7.8125	Nill
H	3.90625	Nill
I	1.953125	Nill

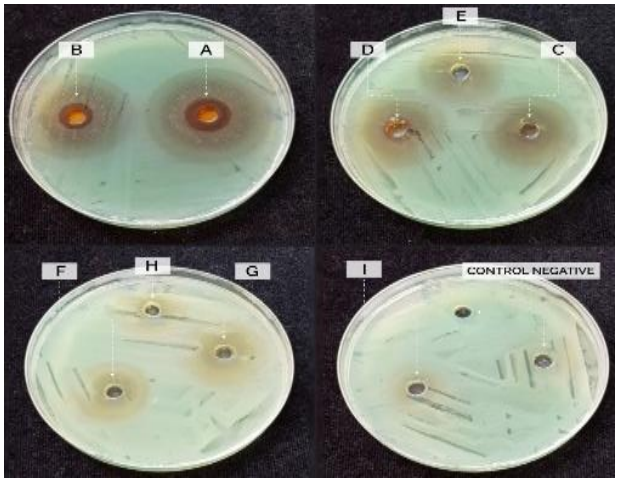


Figure (8): Antibacterial activity of Fe_2O_3 NPs from intracellular on *P.aeruginos*

Detection of biofilm production by microtiter plate assay

Under the same experimental conditions, all 20 *Pseudomonas*

aeruginosa isolates showed different potential biofilm-forming abilities (Figure 9). Separation results were limited to three groups. Six isolates were high biofilm producers (30%), 7 isolates were intermediate biofilm producers (35%) and 7 isolates were low biofilm producers (35%). These results were obtained after 24 hours of incubation *Pseudomonas aeruginosa* growth and biofilm formation. After 48 hours of incubation, 20 isolates were tested in microtiter plates. shows that 15 isolates (75%) are strong biofilm producers, 5 isolates (25%) are moderate biofilm producers, and 0 isolates (0%) are weak biofilm producers.

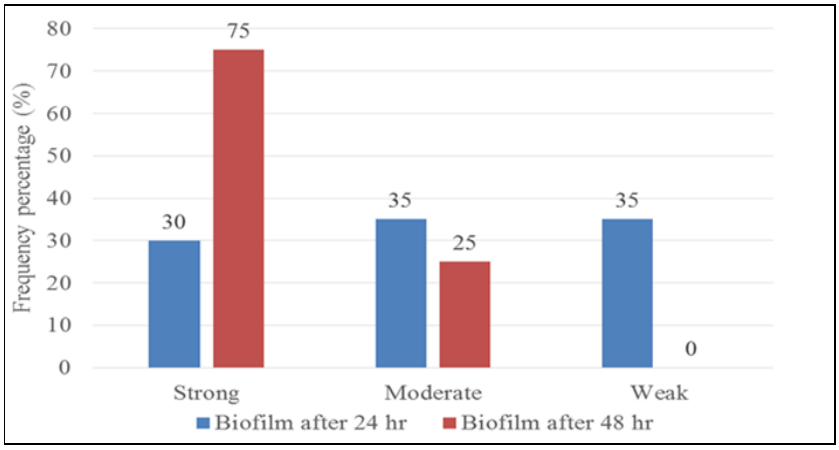


Figure (9): Biofilm produced of *Pseudomonas aeruginosa* after 24hr and 48 hr

Conclusion

In this study, the biosynthesis of iron oxide nanoparticles using intracellular as a reducing agent was demonstrated successfully. Additionally, the attained Fe_2O_3 NPs were characterized using UV-Vis, AFM, FT-IR, and FE-SEM. Techniques. In particular, the FE-SEM demonstrated that the prepared Fe_2O_3 NPs exhibited spherical particles as well as plate-like structures with an average diameter size ranging between 30-50 nm. While the AFM revealed an average diameter of 57.23 nm. In the antibacterial activity test, it was found that the bio-synthesized has a strong antibacterial activity against the introduced bacteria. The maximum inhibition zone was found to be 15 mm at a concentration of 500 mg/mL.

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