Available online at http://bjas.bajas.edu.iq
https://doi.org/10.37077/25200860.2024.37.2.19
College of Agriculture, University of Basrah

Basrah Journal of Agricultural Sciences

ISSN 1814 – 5868 Basrah J. Agric. Sci., 37(2), 249-263, 2024 E-ISSN: 2520-0860

Biosynthesis of Iron Oxide Nanoparticles Using Food Origin Citrobacter freundii in Optimized Conditions

Marwa H. AlKhafaji[1*], Raghdan H. Mohsin[2] & Mokhtar Jawad Kadhim[3]

[1] Department of Biology, College of Science, University of Baghdad, Iraq
[2] Department of Animal Production, College of Agriculture, University of Basrah, Iraq
[3] College of Agricultural Engineering Sciences, University of Baghdad, Iraq

***Corresponding author email**: marwa.alkhafaji@sc.uobaghdad.edu.iq; **R.H.M.**: raghdan.mohsin@uobasrah.edu.iq; **M.J.A.**: mokhtarjawad91@gmail.com;

Received 2nd July 2024; Accepted 3rd December 2024; Available online 31st December 2024

Abstract: Sustainability, Ecofriendly, and green technology are key principles guiding the biosynthesis of nanoparticles in this research. This work aimed to utilize Iron oxide nanoparticles (IONPs) as antimicrobial agents, what offers a promising solution to combat antibiotic-resistant pathogens. In this study, 120 food samples were analyzed. Food origin *Citrobacter freundii* was isolated and identified accurately to be used then for the biosynthesis of Iron oxide nanoparticles. Iron Oxide Nanoparticles were synthesized and characterized using different assays. Atomic force microscope was the principle characterization technique. Their antimicrobial activity was tested against foodborne and clinical bacterial isolates. The results of this study revealed that the biosynthesized IONPs were in a diameter of 32.86 nm with magnetic properties. The biosynthesized IONPs inhibited the biofilm formation of both food and clinical isolates. The main conclusion of this work is that food origin *C. freundii* is an excellent reducing agent in the biosynthesis of these bioactive nano-scale materials. This research is the first to synthesize Ferric oxide NPs using *C. freundii* marking a new approach in the field. Clinical *C. freundii* required a higher IO-NPs dose more than foodborne isolates. This calls for stronger therapies, while foodborne *C. freundii* still poses contamination risks despite lower resistance. Addressing both could improve antimicrobial treatments and food safety.

Keywords: Food, Nanotechnology, Iron, Ecofriendly, Food safety.

Introduction

Antimicrobial resistance (AMR) is a critical public health concern that causes significant rates of death and morbidity (Tacconelli & Pezzani 2019). AMR of bacteria causes approximately 700,000 deaths annually, with an estimated increase to 10 million by 2050 (Tang *et al*., 2023). Furthermore, bacteria acquire antibiotic resistance genes and evolve a diverse set of AMR mechanisms to protect themselves from commercially available antimicrobial drugs (Aslam *et al*., 2018). The severity of antibiotic resistance and the scarcity of therapeutic options are being addressed. Stronger, more effective antibacterial medicines are being developed by scientists. Recently, nanoparticle-based materials have gained popularity in the treatment of microbial diseases (Jain *et al*.,

2021, Mussin *et al*., 2021). Nanotechnology is a promising solution for the antibiotic resistance issue (Gudkov *et al*., 2021). It is a multidisciplinary field that combines chemistry, biology, biochemistry, physics, and materials science (Ramsden, 2018). Nanotechnology was first introduced in 1960 by Richard Feynman who proposed manipulating matter at the atomic and molecular level (Feynman, 2018). Professor Norio Taniguchi from Tokyo University of Sciences popularized the term. His definition was "the treatment, separation, consolidation, and deformation of materials into atoms or molecules" (Taniguchi, 1974). Iron oxide is the most widely investigated substance for FDA-approved nanomedicines (Bobo *et al*., 2016). Magnetic iron oxide NPs (MNPs) consisting of magnetite (Fe_3O_4) or maghemite (Fe_2O_3) have been shown to be useful. May be as contrast agents, drug delivery vehicles, and thermal-based therapies at particular diameters (from 15 nm to 100 nm) (Arias *et al*., 2018). This material is highly efficient for biomedical applications, including diagnostics, imaging, and photothermal treatments. Biocompatibility and stability make them ideal for situations that cannot be achieved with organic materials. Low solubility and toxicity limit their use in certain clinical applications (Manshian *et al*., 2017). These nanoparticles have a unique sensitivity to external magnetic fields (Socoliuc *et al*., 2020). The field of iron oxide nanoparticles has garnered significant attention. This was because of its chemical and structural diversity, great availability, low cost, non-toxicity, and wide range of chemical methods that facilitate the synthesis of desired physiochemical properties (Chaudhari *et al*., 2024).

This study is carried out to use the nanotechnology as a promising control strategy to combat the drug-resistant bacterial isolates, isolated from Iraqi animal origin foods.

Materials & Methods

Collection of food samples

This work comprised a total of 120 food samples. The food samples were collected from markets in Baghdad city. These foods included: 40 leafy vegetables, 40 fish, and 40 chicken meat samples.

Isolation of foodborne bacteria

The collected food samples were labeled and one gram was weighed from each sample. It was added to nine mL of peptone water and left to stand for 15 min. The diluted samples were cultured on selective and differential culture media using the pour plate method .

One mL of each diluted sample was placed in the center of a sterile Petri dish. The Petri dish containing the inoculum was then filled with 20 mL of cooled, molten MacConkey agar that was thoroughly mixed. The agar plate was incubated at 37°C for 24 hours after it had solidified. The grown colonies were then inspected further to confirm their identification (Salfinger &Tortorello, 2015).

Clinical bacterial isolates

Clinical bacterial isolates of *C. freundii* were provided by the microbiology laboratory in the Department of Biology/ College of Science/ University of Baghdad. These isolates were cultured for activation. Thereafter they were subjected to confirmation of their purity and identification.

Identification of the bacterial isolates

All isolated bacteria were identified based on colony morphology on selective and differential culture media and biochemical

tests (Benson, 2001). Then VITEK2 system was used to verify the findings.

Detection of the ability of *C. freundii* to form biofilm

Biofilm formation by clinical and foodborne isolates of *C. freundii* was tested using two assays:

Congo red agar

Congo red agar was used to test the bacterial ability to produce a slime layer (Freeman *et al*., 1989). CRA was composed of 50 $g.L^{-1}$ of sucrose, 37 $g.L^{-1}$ of brain heart infusion broth, 15 $g.L^{-1}$ of agar-agar, and 0.8 $g.L^{-1}$ of congo red dye. The CRA constituents were mixed, heated, and autoclaved, then cooled to 55 to 60°C (except the dye). Thereafter the congo red dye solution was added. The prepared medium was immediately poured into sterile Petri plates. Test bacteria were streaked on the agar plates, and incubated aerobically at 37°C for 18-24 hours. Black colonies were considered as a positive result.

Tissue culture plate method

Tissue culture plate assay (TCP) was used to detect the ability of *C. freundii* to form biofilm. The protocol of Coffey & Anderson (2014) was dependent in this study. *C. freundii* isolates were cultured on tryptic soy broth for 18 hrs at 37°C in a shaker incubator. Then the activated isolates were transferred to a fresh broth in the wells of the TCP. After being inoculated, the TCP was incubated at 37°C for 24 hrs. Then the TCP was washed with phosphate buffer saline three times to get rid of the non-biofilm producers. On the polystyrene wells the adherent bacteria were stained with 0.1% w/v Crystal violet. Negative control wells were filled with the uninoculated medium to define the reference optical density (OD). The OD of stained adherent biofilm was measured by Micro ELISA auto reader at wavelength 630 nm. The experiment was repeated three times and the average values of OD were calculated for all the tested bacterial isolates and the negative controls. The Cutt-off OD c value was assessed and the bacterial isolates were categorized as in Table (1) (Hassan *et al*., 2011).

Table (1): The estimation of biofilm formation

O. D. at 630 nm	Biofilm
2*OD c <OD ≤ 4*OD c	strong biofilm-forming
OD c <OD ≤ 4*OD c	moderate biofilm producer
OD c <OD ≤ 2*OD c	weak biofilm-forming
OD ≤ OD c	non- biofilm producer

* OD c: The absorbance of negative control wells

Biosynthesis of Iron-oxide Nanoparticles using bacterial cell filtrate

Preparation of bacterial cell filtrate

A fresh foodborne *C. freundii* culture was grown in nutrient broth for 18 hours at 37 °C. The bacterial supernatant was then collected by centrifugation of the tubes at 10,000 rpm for 10 minutes.

Iron salt ($FeSO_4$) stock solution Preparation

To prepare 1M of stock solution, 27.8 gm of $FeSO_4$ were dissolved in 100 mL of D.D.W. to be used for biosynthesis.

Biosynthesis of Iron-oxide Nanoparticles

The IO-NPs biosynthesis protocol of Nahari *et al*. (2022) was dependent with some modifications. To biosynthesize the iron oxide nanoparticles, 1 mL of *C. freundii* filtrate and 3 mL of 1 M ferric sulfate solution were combined at 40 °C. After several hours, the colour finally changed from yellowish brown to brownish black. Colour change indicates the formation of iron nanoparticles (Fig. 1). The biosynthesized IO-NPs were separated by centrifugation at 10,000 rpm for 15 min. Centrifugation was used to remove the generated ferric oxide nanoparticles for 30 min. at 10,000 rpm (Gao *et al*., 2017). After centrifuging and three washes, the pelleted IO-NPs were removed. After drynig at 40°C, the resulting nanoparticles were pure and highly durable, then were kept in storage at -4°C.

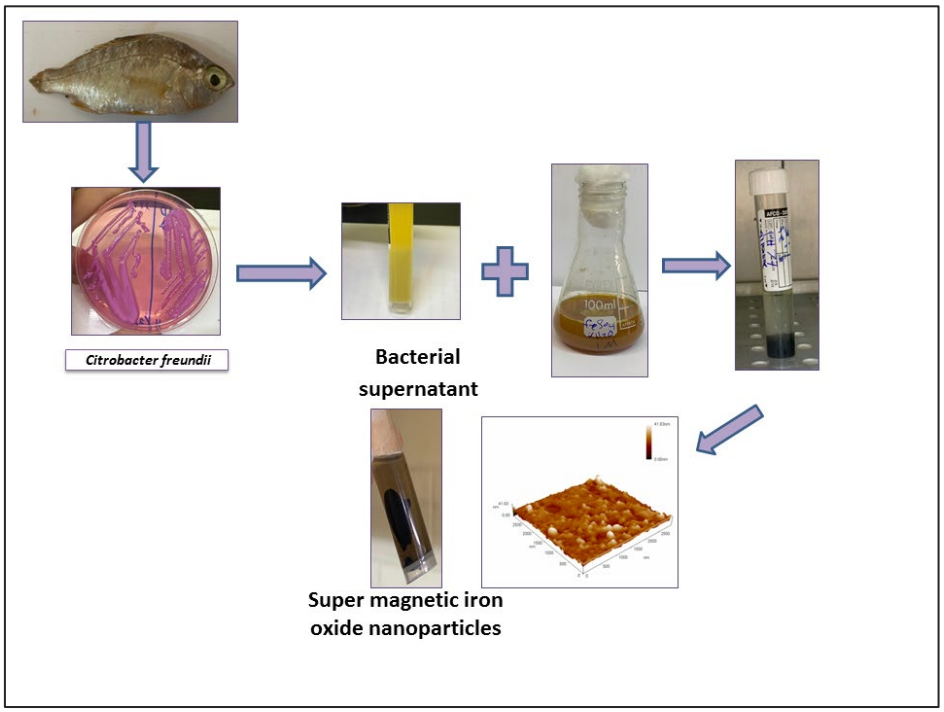

Fig. (1): Schematic representation for the Iron oxide-Nps biosynthesis in this study

Optimization of parameters for the biosynthesis of Iron oxide-Nps

In this research, three parameters: temperature, incubation period, and pH were optimized. These conditions may affect the production and quality of the nanoparticles .

Optimization of pH

The reaction mixture is composed of: ferric sulfate and *C. freundii* filtrate. pH value of this mixture was accustomed to (11, 7, and 5) and incubated for 24 hours. Negative control of bacterial filtrate without ferric sulfate was kept. Then the mixture was examined for the formation of ferric oxide nanoparticles.

Optimization of the incubation period

Different incubation periods were tested for the detection of the suitable period for the biosynthesis of IO-NPs. The reaction mixture composed of ferric sulfate and *C. freundii* filtrate was incubated for 24, 48, and 72 hours were investigated. A negative control without ferric sulfate was prepared using the bacterial filtrate. Then the mixture was examined for the formation of ferric oxide nanoparticles.

Incubation temperature optimization

The reaction mixture composed of ferric sulfate and *C. freundii* filtrate was incubated at different temperatures (50, 37, and 4 °C). Negative control of bacterial filtrate without ferric sulfate was kept. Then the mixture was examined for the formation of ferric oxide nanoparticles.

Characterization of IO-NPs

Initial identification of the biosynthesis success is the colour change, then the IO-NPs were evaluated in a 2 mL quartz cuvette with a 1 cm path length by measuring the wavelength of the reaction mixture in the UV-Vis spectrum at a resolution of 1 nm. The samples were scanned at 500 nm min-1 across a wavelength range of 300-900 nm. The spectrophotometer was calibrated against a blank reference. The UV-Vis absorption spectra of all materials were measured and displayed (Hashim & AlKhafaji, 2018).

The IO-NPs were examined using atomic force microscopy (AFM). A thin film of the nanoparticles was placed onto the glass slide's surface. The diameter, average size, and granularity of the biosynthesized nanoparticles were examined by scanning the deposited film with an AFM .

Detection of the antibacterial activity of biosynthesized IO-NPs

The antibacterial activity of IO-NPs was detected using the agar well diffusion method against clinical *C. freundii*. The overnight bacterial culture broth was homogeneously inoculated onto Mueller-Hinton agar. The wells were then done with a gel borer. The wells were filled with the biosynthesized IO-NPs nanoparticles in a concentration of 500 $\mu g.mL^{-1}$. After incubating the plates at 37°C for 24 hours, the inhibition zones around each well were measured (Logeswari *et al*., 2015).

The broth dilution method was used to evaluate the antibacterial activity of IO-NPs against clinical isolates. The minimum inhibitory concentration (MIC) of IO-NPs was determined according to CLSI standards. IO-Nps dilutions (16-1000 $\mu g.mL^{-1}$) were examined. Bacterial inoculum was adjusted to 0.5 McFarland standard (1×108 $CFU.mL^{-1}$,). Positive control composed of the bacterial isolate without IO-NPs. While the negative control consisted of the broth culture medium only. The inoculated tube was incubated at 37°C for 24 hours, and the MIC was determined as the last tube showing no visible turbidity (Gurunathan *et al*., 2014).

The biosynthesized iron oxide nanoparticles were analyzed for their antibiofilm activity against foodborne and clinical bacterial isolates. The procedure of Mohanta *et al*., (2020) was followed for this purpose. Serial dilutions of the biosynthesized IO-NPs were prepared (16-1000 $\mu g.mL^{-1}$). A 96-well TCP was used. The bacterial culture was inoculated in Tryptic Soy-glucose broth (TSB + 1% w/v glucose). Each TCP well was filled with 100 µL of broth medium and 100 µL of IO-NPs; whereas the control wells were loaded with 100 µL of medium + 100 µL D.W. Each concentration for the IO-NPs, that evaluated triplicate. The TCP was incubated for 24 hours at 37ºC. After the incubation period, a washing step was accomplished to remove the detached cells. Followed by a staining step using 0.1% (w/v) crystal-violet. Another washing step was adapted to remove any remaining stains. A drying step at room temperature was allowed before a 33% (v/v) acetic acid for 15 min. treatment. TCP was subjected to Eliza reader at 630 nm to detect the optical density of each well in the TCP. Table (1) clarified the estimation method that depended to evaluate biofilm formation.

Results & Discussion

Isolation and identification

On MacConkey agar, two types of colonies were grown, pink and pale colonies: lactose fermenters and non-lactose fermenters. The pale colonies were neglected, while the pink colonies were further investigated for the biochemical characteristics. Vitek system was used to ratify the identification to species level and the results revealed that the isolated bacterium was *Citrobacter freundii*. Out of 120 food samples (40 Fish, 40 Chicken and 40 leafy vegetables) 16 isolates were isolated and identified as *C. freundii* (Fig. 2). The isolated foodborne *C. freundii* were from different food sources: 9 (56.25 %) from fish samples, 5 (31.25 %) from chicken samples, and 2 (12.50 %) from leafy vegetables (Fig. 3 & 4).

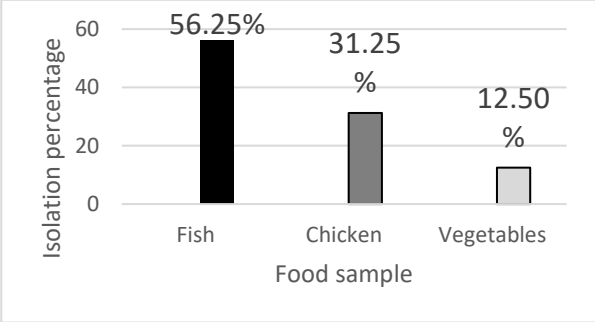

Fig. (2): The incidence of *C. freundii* from each source of food

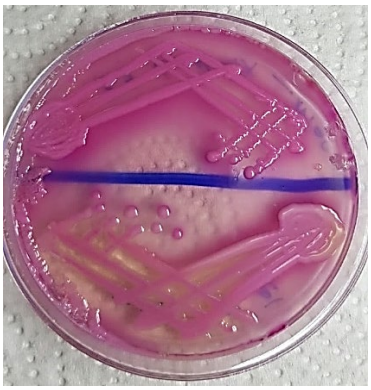

Fig. (3): Food-origin isolate of *Citrobacter freundii* on MacConkey agar (isolated from a fish sample)

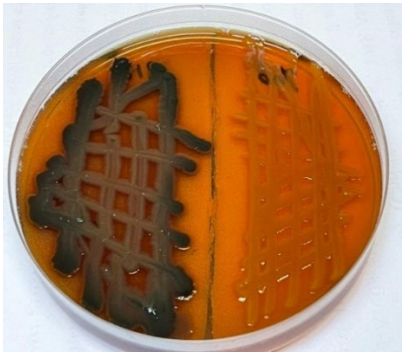

Fig. (4): H_2S Production by a food-origin isolate of *Citrobacter freundii* on S.S agar (to the left) in comparison with non-H_2S producer *K. pneumoniae* (to the right)

The results of this research showed that 16 *C. freundii* isolates were isolated from 120 samples. With higher frequency in fish rather than chicken and leafy vegetables . Gong *et al*. (2023) isolated pathogenic *C. freundii* from farmed fish in China. In a previous study, *C. freundii* was isolated from farmed catfish (Nawaz et al., 2008). According to Junior *et al*. (2018) *C. freundii* is an important pathogen causing severe economic losses in the fish farming. Liu *et al*. (2024) isolated *C. freundii* from fish and stated that this bacterium caused dysbiosis in the fish microbiome . The isolation of bacterial contaminants from chicken was in the second stage, then followed by leafy vegetables as sources for *C. freundii* isolation. This may be an indicator for poor hyigenic practices with water source that used in the irrigation of these crops. In addition to the use of organic fertilizers which represents an ideal environment for this pathogen. *C. freundii* is a member of the enterobacteriaceae family, inhabits the intestine of animals. This opinion was supported by a neighboring study in Iran (Aminharati *et al*., 2019). Previous local studies isolated foodborne *C. freundii* from domestic and imported chicken meat (Hashim & AlKhafaji 2018). Bacterial ability to form biofilm was detected using two methods: Congo red agar and Tissue culture plate.

Results conducted by this study showed the ability to form a slime layer and biofilm model of life (Fig. 5 & 6).

Fig. (5): Congo red agar cultured with foodborne *Citrobacter freundii*, black coloration indicates the strong slime layer production

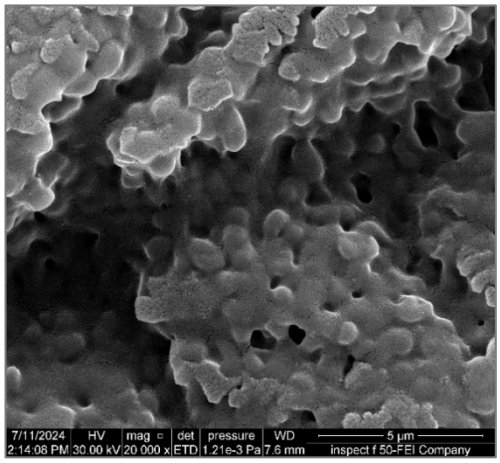

Fig. (6): Foodborne *Citrobacter freundii* Biofilm under Electron microscope

The adherence ability of *C. freundii* is still under research as compared to other members of the Enterobacteriaceae family. This was stated by Ramos-Vivas *et al*. (2020) when they studied the adherence ability of *C. freundii* isolated from the hospital environment and implants. This ability to form biofilm greatly affects antibiotic sensitivity and eventually leads to recurrent infections (Borges *et al*., 2015)

Ferric oxide nanoparticles biosynthesis

The biological method used in this research gave a positive result for a safe, rapid, and cheap method to synthesize ferric oxide nanoparticles using *C. freundii* filtrate. The biosynthesized IO-NPs were characterized by colour change, UV, AFM, and scanning electron microscope (SEM) (Fig. 7).

Fig. (7): The change in colour represents the first indicator for nanoparticle formation in the reaction vessel

The appearance of black precipitate is the first insight into the formation of Magnetic Fe_3O_4-NPs. Hamdy *et al*. (2022) reported that the first indicator for the formation of Fe_3O_4-NPs was the black precipitate. The next step for the nanoparticles characterization was the AFM analyses. The results of this work conducted for the biosynthesis of Fe_3O_4-NPs, shown an average diameter of 32.86 nm (Fig. 8, Table 2, and Fig. 9).

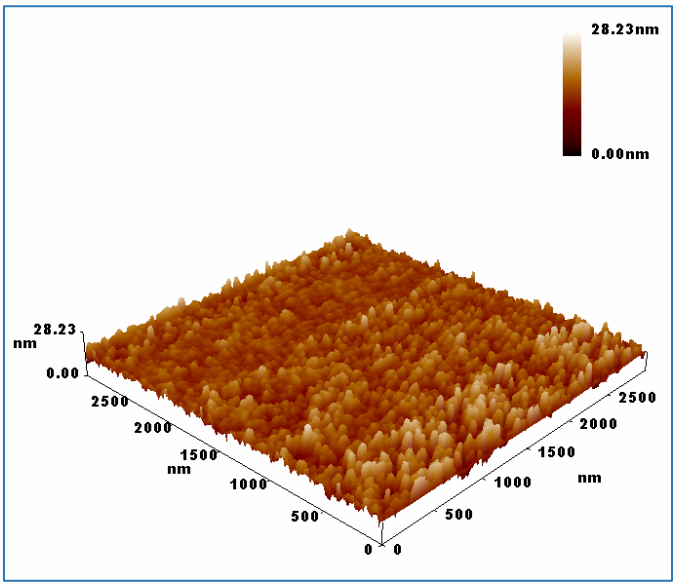

Fig. (8): Biosynthesized Fe_3O_4-NPs under atomic force microscope (Three-dimensional image)

Table 2: The report of the granularity cumulation distribution of biosynthesized Fe_3O_4-NPs

Average diameter	<=10% Diameter	<=50% Diameter	<=90% Diameter
32.86 nm	16.00 nm	30.00 nm	50.00 nm

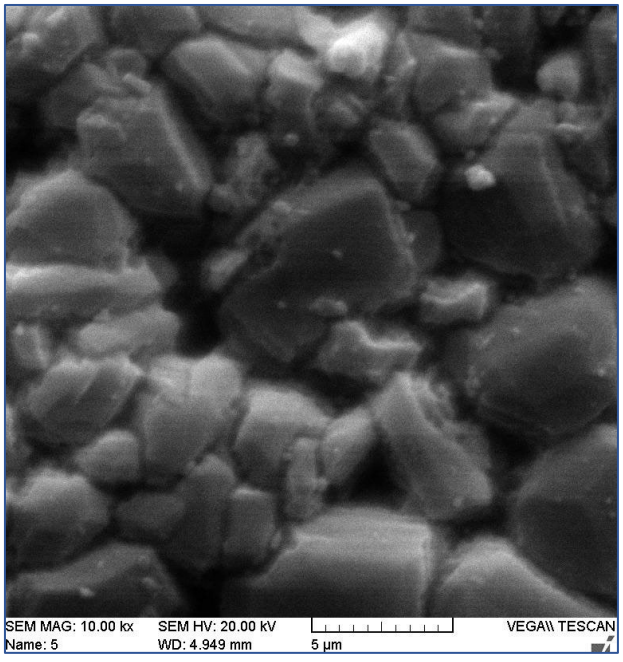

Fig. (9): Biosynthesized Fe_3O_4-NPs under Scanning Electron microscope

The conditions for the process of Fe_3O_4-NPs biosynthesis were optimized for this work. The optimized conditions included important factors. The results exhibited that: pH 11, Temp. 50 and period 24h. were the optimum

conditions for the Fe_3O_4-NPs biosynthesis process (Table 3).

Table 3: Optimization results of the parameters included in this study

pH	Diameter of the resulted Fe_3O_4-NPs	Incubation period (hours)	Diameter of the resulted Fe_3O_4-NPs	Temperature	Diameter of the resulted Fe_3O_4-NPs
5	76 nm	24	30 nm	4 °C	negative
7	negative	48	91 nm	37 °C	82 nm
11	32.86 nm	72	negative	50 °C	30 nm

Ferric oxide nanoparticles were synthesized in optimized conditions which led to the formation of Fe_3O_4-NPs in an average diameter of 32.86 nm. A previous local study conducted that the minutest nanoparticles were biosynthesized using *Pseudomonas* isolate at 30 °C at pH 6 (Abbas and Flayyih 2019). Another study used banana peels to synthesize nanoparticles using microwave irradiation. The peels served as stabilizing and capping agent in addition to playing a role in the reduction process (Tawfeeq *et al*. 2017). Almudhafar and Al-Hamdani, (2022) synthesized the nanoparticles from plants and stated the colour change which was as concentration dependent a first synthesis indicator.

Antibacterial activity of Fe_3O_4-NPs

In this study, the synthesized Fe_3O_4-NPs exhibited antimicrobial activity against *C. freundii*. Nanoparticles inhibited inhibited both life forms of bacteria: free living and biofilm-associated. Minimum inhibitory concentration of the biosynthesized Fe_3O_4-NPs was determined as 125 µg.mL^{-1}. A subinhibitory concentration of 62.5 µg.mL^{-1} along with lower concentrations was experienced for their antibiofilm activity. Antibiofilm activity was determined according to the percentage of reduction in the biofilm formation by foodborne and clinical *C. freundii* isolates. So the results of this study showed that clinical isolates required a higher concentration of Fe_3O_4-NPs to inhibit their biofilm. This concentration was higher than that needed to inhibit biofilm formation by the foodborn *C.freundii* isolates. A concentration of 125 µg.mL^{-1} of Fe_3O_4-NPs resulted in a 94 % decrease in the biofilm formation by foodborne *C. freundii*. At 62.5 µg.mL^{-1} biofilm formation was reduced by 79 %, while 31.25 µg.mL^{-1} led to a 65% reduction. Finally a concentration of 15.62 µg.mL^{-1} resulted in a 53 % decrease in biofilm formation by foodborne *C. freundii*. For the clinical isolates, 125 µg.mL^{-1} of Fe_3O_4-NPs resulted in an 80 % decrease in biofilm formation by clinical *C. freundii*. At 62.5 µg.mL^{-1}, biofilm formation was reduced by 71 %, while 31.25 µg.mL^{-1} led to a 55 % reduction. Lastly a concentration of 15.62 µg.mL^{-1} resulted in a 37 % decrease in biofilm formation by clinical *C. freundii* with significant difference ($p \leq 0.05$) compared to the control (Fig. 10).

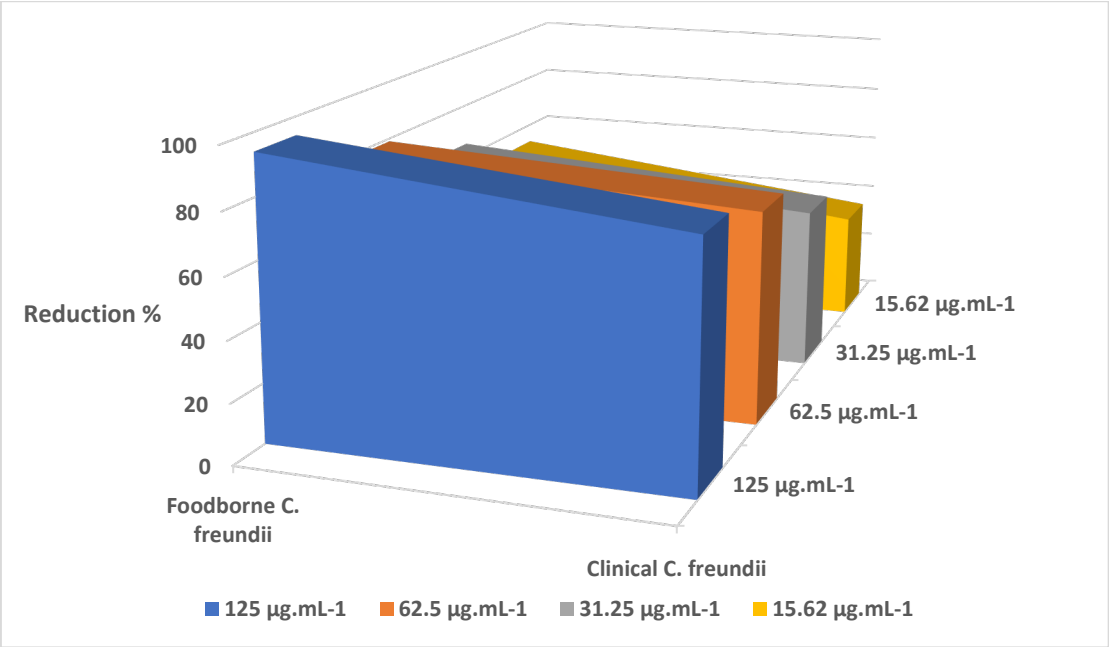

Fig. (10): The antibiofilm activity of biosynthesized Fe_3O_4-NPs against clinical and foodborne *C. freundii* as measured by ELIZA technique

Local studies in nanotechnology showed that the sub-inhibitory concentrations of nanoparticles exhibit an anti-biofilm activity against Gram-negative bacteria (Al-Khafaji, 2017). This antibiofilm effect is attributed to the ability of NPs to penetrate biofilms, which offers a practical approach for disrupting biofilm formation. Biofilms, often resistant to conventional antimicrobial treatments, pose a significant challenge in clinical settings (Saleh, 2020). The capacity of NPs to infiltrate these complex structures not only prevents the initial formation of biofilms but also increases the effectiveness of antimicrobial agents, providing a promising strategy for combating persistent bacterial infections.

Ferric-oxide nanoparticles may interact with bacterial cell membranes, but their mechanism differs slightly from silver nanoparticles (AgNPs). When IO-NPs come into contact with bacterial cells, they can generate reactive oxygen species (ROS), which induce oxidative stress within the bacteria. This oxidative stress can damage various cellular components, including lipids, proteins, and DNA.

Unlike AgNPs, which cause bacteria to aggregate as a protective mechanism for their DNA, IO-NPs primarily disrupt the bacterial cell membrane. They also interfere with metabolic processes. The ROS generated by IO-NPs can lead to bacterial cell death by impairing key cellular functions and causing DNA damage directly. Nevertheless, bacteria do not usually aggregate in response to IO-NPs as they do with AgNPs. This is because the main stressor is oxidative damage rather than direct nanoparticle-DNA interaction (Gupta *et al*., 2024).

Nanoparticles are important antimicrobial agents due to their small size with high surface area (Ansari *et al*., 2019). Nanoparticles can be synthesized by several methods, one them the oldest techniques is chemical synthesis. This technique which is the most practical and rapid simple way to synthesize nanoparticles. But on the other

hand, chemical synthesis involves the use of hazardous chemicals which can pose significant environmental and health risks (Banjara *et al*., 2024).

The green synthesis of nanoparticles is an ecofriendly cost-effective technology. It substitutes hazardous chemicals with safe biological agents such as plants and bacteria (Gupta *et al*., 2023). Green technology applications caused an increase in plant resistance to pathogenic bacteria (Abdul-Karim & Hussein 2022, Alkhafaji *et al*., 2024). As a result of the dual use of antimicrobials in farming settings, increased drug resistance raised among foodborne bacteria. (Rodríguez-Félix *et al*., 2022). Thanigaivel *et al*. (2015) documented the need for alternative therapy to overcome the economic loss in fish farming. In their study, they used neem extract to control the virulent strains of food-borne *C. freundii* (Thanigaivel *et al*., 2015). Kumar *et al*. (2022) reported the importance of using IO-NPs in the fish farming. In a concentration dependent manner, IO-NPs inhibited fish pathogens and consequently reduced the economic loss. Another important application of nanotechnology in food preservation is nano-materials addition to foods to extend their shelf-life and prevent deterioration (Karnwal & Malik, 2024).

Conclusion

The unexpected results of this work underscore the need to pay closer attention to foodborne pathogens like *C. freundii*. While they may appear less resistant, their ability to form biofilms at lower concentrations of Fe_3O_4-NPs. They still poses a significant risk, particularly in the context of food safety. The relatively lower resistance might allow these pathogens to thrive unnoticed in food production environments, leading to contamination and outbreaks. Shifting the research focus toward foodborne isolates could reveal important insights into preventing biofilm formation in the food industry. Thus safeguarding public health and preventing foodborne infections from becoming a larger issue. The clinical isolates required a higher concentration of Fe_3O_4-NPs to effectively inhibit their biofilms compared to the foodborne *C. freundii* isolates, which were inhibited at lower concentrations. This suggests that the biofilms formed by the clinical isolates are more resistant to the Fe_3O_4-NPs treatment than those from foodborne sources. The predictable application of this work lies in improving food safety, mainly in inhibiting foodborne infections. The outcomes recommend that foodborne *C. freundii* isolates, despite appearing less resistant, can form biofilms at lower concentrations of IO-NPs, posing a considerable risk in food production environments. Shifting the research application to foodborne isolates might offer valuable insights into approaches to inhibit biofilm formation, thereby defense public health and reducing the risk of contamination and outbreaks in the food manufacturing.

Limitations of the study

and Sustainability spotlight

The application of nanomaterials in different scientific and technological fields is spreading widely. However, one of the major drawbacks that has impeded their intensive application is the way they are produced, i.e., normally, their synthesis implies the use of toxic reactants and the generation of potentially dangerous by-products. Hence, green nanotechnology has recently emerged as a potential solution for the synthesis of nanomaterials. This work has the objective to produce Fe-nanoparticles using bacterial isolates as natural biofactories.

The significant limitation of this work is that the biosynthesized Ferric oxide nanoparticles were implicated in impeding the clinical and

foodborne *C. freundii* growth. While the previous studies focused on the clinical isolates only. Another important limitation point is the use of foodborne *C. freundii* as a biofactory for the green sustainable synthesis of IO-NPs.

Acknowledgment

A great appreciation goes to the University of Baghdad, and the University of Basrah for supporting this work.

Contributions of authors

M.H.A: Study design, carried out the experimental work, acquisition of data, proposal writing and drafting and revising the manuscript and submitted the final manuscript.
R.H.M: Study design, planned methodology, read, revised and approved the manuscript.
M.J.K: Study conception, analysis of data and revised the manuscript.

ORCID

M. H. Alkhafaji: https://orcid.org/0000-0002-2042-6231

R. H. Mohsin: https://orcid.org/0000-0002-4007-5774

M. J. Kadhim: https://orcid.org/my-orcid?orcid=0009-0003-8711-6996

Conflicts of interest

The authors declare that they have no conflict of interest.

References

Abbas, H. H., & Faliyyah, M. T. (2019). Biosynthesis and optimization of silver nanoparticles from Pseudomonas aeruginosa. Biochemical & Cellular Archives, 19(1). https://www.connectjournals.com/pages/articledetails/toc029489

Abdul-Karim, E. K., & Hussein, H. Z. (2022). The biosynthesis of nanoparticles by fungi and the role of nanoparticles in resisting of pathogenic fungi to plants: a review. Basrah Journal of Agricultural Sciences, 35(1), 243-256. https://doi.org/10.37077/25200860.2022.35.1.18

AlKhafaji, M. H. ., Mohsin, R. H. ., & Alshaikh Faqri, A. M. . (2024). Food Additive Mediated Biosynthesis of AgNPs with Antimicrobial Activity Against Hypermucoviscous Enterotoxigenic Foodborne Klebsiella pneumoniae. Basrah Journal of Agricultural Sciences, 37(1), 278–295. https://doi.org/10.37077/25200860.2024.37.1.21

Al-Khafaji, M. H. (2017). The Inhibition Activity of Silver Nanoparticles Compared with D-Glycin and Imipenem Effect on the Biofilm Formation by Food-origin Salmonella. Iraqi Journal of Science, 836-842. https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/6026

Almudhafar, S. M., & Al-Hamdani, M. A. (2022). Antibacterial and Anticancer Effects of Silver Nanoparticles Synthesised using Eragrostis tef and Vitellaria paradoxa Seeds Extract. Basrah Journal of Agricultural Sciences, 35(2), 132-159. https://www.bjas.bajas.edu.iq/index.php/bjas/article/view/688

Aminharati, F., Ehrampoush, M. H., Dallal, M. M. S., Yaseri, M., Tafti, A. A. D., & Rajabi, Z. (2019). Citrobacter freundii foodborne disease outbreaks related to environmental conditions in Yazd Province, Iran. Iranian journal of public health, 48(6), 1099. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6635343/

Ansari, M.T., Sami, F., Majeed, S., Hasnain, M.S. and Badgujar, V.B., 2019. Design and evaluation of topical herbal antifungal stick containing extracts of Rhinacanthus nasutus. Journal of Herbal Medicine, 17, p.100290. https://www.sciencedirect.com/science/article/abs/pii/S2210803319300375

Arias, L. S., Pessan, J. P., Vieira, A. P. M., Lima, T. M. T. D., Delbem, A. C. B., & Monteiro, D. R. (2018). Iron oxide nanoparticles for biomedical applications: a perspective on synthesis, drugs, antimicrobial activity, and toxicity. Antibiotics, 7(2), 46. https://doi.org/10.3390/antibiotics7020046

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., ... & Baloch, Z. (2018). Antibiotic resistance: a rundown of a global crisis. Infection and drug resistance, 1645-1658. https://doi.org/10.2147/IDR.S173867

Banjara, R. A., Kumar, A., Aneshwari, R., Satnami, M. L., & Sinha, S. K. (2024). A comparative analysis of chemical vs green synthesis of nanoparticles and their various applications. Environmental Nanotechnology, Monitoring & Management, 22: 100988. https://doi.org/10.1016/j.enmm.2024.100988

Benson, T. (2001) Microbiological Applications Laboratory Manual in General Microbiology. 8th Edition, The McGraw-Hill, New York. https://www.scirp.org/reference/referencespapers?referenceid=1819101

Bobo, D., Robinson, K. J., Islam, J., Thurecht, K. J., & Corrie, S. R. (2016). Nanoparticle-based medicines: a review of FDA-approved materials and clinical trials to date. Pharmaceutical research, 33, 2373-2387. https://doi.org/10.1007/s11095-016-1958-5

Borges, A., Saavedra, M. J., & Simões, M. (2015). Insights on antimicrobial resistance, biofilms and the use of phytochemicals as new antimicrobial agents. Current medicinal chemistry, 22(21), 2590–2614. https://doi.org/10.2174/092986732266615053021 0522

Chaudhari, D. S., Upadhyay, R. P., Shinde, G. Y., Gawande, M. B., Filip, J., Varma, R. S., & Zboril, R. (2024). A review on sustainable iron oxide nanoparticles: synthesis and application in organic catalysis and environmental remediation. Green Chemistry.26 (13): 7579-7655. https://doi.org/10.1039/D4GC01870B

Coffey, B. M., & Anderson, G. G. (2014). Biofilm formation in the 96-well microtiter plate. In Pseudomonas Methods and Protocols (pp. 631-641). Humana Press, New York, NY. https://europepmc.org/article/med/24818938

Feynman, R. (2018). There's plenty of room at the bottom. In Feynman and computation: 63-76. CRC Press. https://www.taylorfrancis.com/chapters/edit/10.12 01/9780429500459-7/plenty-room-bottom-richard-feynman

Freeman, D. J., Falkiner, F. R., & Keane, C. T. (1989). New method for detecting slime production by coagulase negative staphylococci. Journal of clinical pathology, 42(8): 872-874. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1 142068/

Gao, H., Yang, H. & Wang, C. (2017). Controllable preparation and mechanism of nano-silver mediated by the microemulsion system of the clove oil. Results in physics, 7, pp.3130-3136. https://www.sciencedirect.com/science/article/pii/ S2211379717309257

Gong, C., Guo, M., Lou, J., Zhang, L., An, Z., Vakharia, V. N., ... & Liu, X. (2023). Identification and characterization of a highly virulent Citrobacter freundii isolate and its activation on immune responses in largemouth bass (Micropterus salmoides). Fish & Shellfish Immunology, 143, 109224. https://doi.org/10.1016/j.fsi.2023.109224

Gudkov, S. V., Burmistrov, D. E., Serov, D. A., Rebezov, M. B., Semenova, A. A., & Lisitsyn, A. B. (2021). Do iron oxide nanoparticles have significant antibacterial properties?. Antibiotics, 10(7), 884. https://doi.org/10.3390/antibiotics10070884

Gupta, D., Boora, A., Thakur, A., & Gupta, T. K. (2023). Green and sustainable synthesis of nanomaterials: recent advancements and limitations. Environmental Research, 231, 116316. https://doi.org/10.1016/j.envres.2023.116316

Gupta, S., Kaur, R., Bhardwaj, A., & Parashar, D. (2024). Multifunctional Nanomaterials: Recent Advancements in Cancer Therapeutics and Vaccines. Indian Journal of Microbiology, 1-18. https://doi.org/10.1007/s12088-024-01274-x

Gurunathan, S., Han, J. W., Kwon, D. N., & Kim, J. H. (2014). Enhanced antibacterial and anti-biofilm activities of silver nanoparticles against Gram-negative and Gram-positive bacteria. Nanoscale research letters, 9, 1-17. https://pubmed.ncbi.nlm.nih.gov/25136281/

Hamdy, N. M., Boseila, A. A., Ramadan, A., & Basalious, E. B. (2022). Iron oxide nanoparticles-plant insignia synthesis with favorable biomedical activities and less toxicity, in the "era of the-green": a systematic review. Pharmaceutics, 14(4), 844. https://doi.org/10.3390/pharmaceutics14040844

Hashim, M. H., & AlKhafaji, M. H. (2018). Isolation and identification of Citrobacter freundii from chicken meat samples using cultural and molecular techniques. Iraqi Journal of Science, 1216-1224 . https://ijs.uobaghdad.edu.iq/index.php/eijs/article/ view/462

Hassan, A., Usman, J., Kaleem, F., Omair, M., Khalid, A., & Iqbal, M. (2011). Evaluation of different detection methods of biofilm formation in the clinical isolates. Brazilian journal of infectious diseases, 15, 305-311. https://pubmed.ncbi.nlm.nih.gov/21860999/

Jain, A. S., Pawar, P. S., Sarkar, A., Junnuthula, V., & Dyawanapelly, S. (2021). Bionanofactories for green synthesis of silver nanoparticles: Toward antimicrobial applications. International Journal of Molecular Sciences, 22(21), 11993. https://doi.org/10.3390/ijms222111993

Junior, G. B., Dos Santos, A. C., de Freitas Souza, C., Baldissera, M. D., dos Santos Moreira, K. L., da Veiga, M. L., ... & Baldisserotto, B. (2018). Citrobacter freundii infection in silver catfish (Rhamdia quelen): hematological and histological alterations. Microbial pathogenesis, 125, 276-280. https://doi.org/10.1016/j.micpath.2018.09.038

Karnwal, A., & Malik, T. (2024). Exploring the untapped potential of naturally occurring antimicrobial compounds: novel advancements in food preservation for enhanced safety and sustainability. Frontiers in Sustainable Food Systems, 8, 1307210. https://doi.org/10.3389/fsufs.2024.1307210

Kumar, M., Gupta, G., Varghese, T., Shankregowda, A. M., Srivastava, P. P., Bhushan, S., ... & Gupta, S. (2022). Synthesis and characterization of super-paramagnetic iron oxide nanoparticles (SPIONs) for minimizing Aeromonas hydrophila load from freshwater. Current Nanoscience, 18(2), 224-236. https://doi.org/10.2174/1573413717666210531153107

Liu, J., Pan, Y., Jin, S., Zheng, Y., Xu, J., Fan, H., ... & Hu, M. (2024). Effects of Citrobacter freundii on sturgeon: Insights from skin mucosal immunology and microbiota. Fish & Shellfish Immunology, 109527. https://doi.org/10.1016/j.fsi.2024.109527

Logeswari, P., Silambarasan, S., & Abraham, J. (2015). Synthesis of silver nanoparticles using plants extract and analysis of their antimicrobial property. Journal of Saudi Chemical Society, 19(3), 311-317. https://doi.org/10.1016/j.jscs.2012.04.007

Manshian, B. B., Jiménez, J., Himmelreich, U., & Soenen, S. J. (2017). Personalized medicine and follow-up of therapeutic delivery through exploitation of quantum dot toxicity. Biomaterials, 127, 1-12. https://doi.org/10.1016/j.biomaterials.2017.02.039

Mohanta YK, Biswas K, Jena SK, Hashem A, Abd_Allah EF and Mohanta TK (2020) Anti-biofilm and Antibacterial Activities of Silver Nanoparticles Synthesized by the Reducing Activity of Phytoconstituents Present in the Indian Medicinal Plants. Frontiers in Microbiology 11:1143. https://doi.org/10.3389/fmicb.2020.01143

Mussin, J., Robles-Botero, V., Casañas-Pimentel, R., Rojas, F., Angiolella, L., San Martin-Martinez, E., & Giusiano, G. (2021). Antimicrobial and cytotoxic activity of green synthesis silver nanoparticles targeting skin and soft tissue infectious agents. Scientific reports, 11(1), 14566. https://doi.org/10.1038/s41598-021-94012-y

Nahari, M.H., Al Ali, A., Asiri, A., Mahnashi, M.H., Shaikh, I.A., Shettar, A.K. and Hoskeri, J., 2022. Green synthesis and characterization of iron nanoparticles synthesized from aqueous leaf extract of vitex leucoxylon and its biomedical applications. Nanomaterials, 12(14), p.2404. https://doi.org/10.3390/nano12142404

Nawaz, M., Khan, A. A., Khan, S., Sung, K., & Steele, R. (2008). Isolation and characterization of tetracycline-resistant Citrobacter spp. from catfish. Food microbiology, 25(1), 85-91. https://doi.org/10.1016/j.fm.2007.07.008

Ramos-Vivas, J., Chapartegui-González, I., Fernández-Martínez, M., González-Rico, C., Barrett, J., Fortún, J., ... & Fariñas, M. C. (2020). Adherence to human colon cells by multidrug resistant Enterobacterales strains isolated from solid organ transplant recipients with a focus on Citrobacter freundii. Frontiers in cellular and infection microbiology, 10, 447. https://doi.org/10.3389%2Ffcimb.2020.00447

Ramsden, J. J. (2018). Global Nanotechnology. Applied Nanotechnology; Elsevier: Amsterdam, The Netherlands, 245-254. https://doi.org/10.1016/B978-0-12-813343-9.00020-2

Rodríguez-Félix, F., Graciano-Verdugo, A. Z., Moreno-Vásquez, M. J., Lagarda-Díaz, I., Barreras-Urbina, C. G., Armenta-Villegas, L., Olguín-Moreno, A. & Tapia-Hernández, J. A. (2022). Trends in sustainable green synthesis of silver nanoparticles using agri-food waste extracts and their applications in health. Journal of Nanomaterials, 2022 (1): 1-37. https://doi.org/10.1155/2022/8874003

Saleh, G. M. (2020). Green synthesis concept of nanoparticles from environmental bacteria and their effects on pathogenic bacteria. Iraqi Journal of Science, 1289-1297. https://doi.org/10.24996/ijs.2020.61.6.6

Salfinger, Y., & Tortorello, M. L. (Eds.). (2015). Compendium of methods for the microbiological examination of foods. American Public Health Association. https://ajph.aphapublications.org/doi/abs/10.2105/MBEF.0222#:~:text=10.2105/MBEF.0222.010

Socoliuc V, Peddis D, Petrenko VI, Avdeev MV, Susan-Resiga D, Szabó T, Turcu R, Tombácz E, & Vékás L. (2020). Magnetic Nanoparticle Systems for Nanomedicine—A Materials Science Perspective. Magnetochemistry.; 6(1):2. https://doi.org/10.3390/magnetochemistry6010002

Tacconelli, E., & Pezzani, M. D. (2019). Public health burden of antimicrobial resistance in Europe. The Lancet Infectious Diseases, 19(1), 4-6. https://doi.org/10.1016/S1473-3099(18)30648-0

Tang, K. W. K., Millar, B. C., & Moore, J. E. (2023). Antimicrobial resistance (AMR). British Journal of Biomedical Science, 80, 11387. https://doi.org/10.3389/bjbs.2023.11387

Taniguchi, N. (1974). On the basic concept of'nano-technology'. In Proc. Intl. Conf. Prod. Eng. Tokyo, Part II, 1974. Japan Society of Precision Engineering. https://cir.nii.ac.jp/crid/1572261550373135488

Tawfeeq, S. M. T., Maaroof, M. N., & Al-Ogaidi, I. (2017). Synergistic effect of biosynthesized silver nanoparticles with antibiotics against multi-drug resistance bacteria isolated from children with diarrhoea under five years. Iraqi Journal of Science, 14-52. https://ijs.uobaghdad.edu.iq/index.php/eijs/article/view/6189

Thanigaivel, S., Vijayakumar, S., Gopinath, S., Mukherjee, A., Chandrasekaran, N., & Thomas, J. (2015). In vivo and in vitro antimicrobial activity of Azadirachta indica (Lin) against Citrobacter freundii isolated from naturally infected Tilapia (Oreochromis mossambicus). Aquaculture, 437, 252-255. https://doi.org/10.1016/j.aquaculture.2014.12.008

التصنيع الحيوي لدقائق اوكسيد الحديد النانوية باستخدام البكتريا غذائية المنشأ Citrobacter freundii تحت الظروف المثلى

مروى حميد مطشر الخفاجي [*1], رغدان هاشم محسن [*2], مختار جواد كاظم [* 3]

[1]قسم علوم الحياة, كلية العلوم, جامعة بغداد, بغداد , العراق
[2]قسم الانتاج الحيواني, كلية الزراعة, جامعة البصرة, البصرة, العراق
[3]كلية علوم الهندسة الزراعية ,جامعة بغداد, بغداد, العراق

المستخلص: الاستدامة والتكنولوجيا الخضراء والصديقة للبيئة هي المبادئ الاساسية التي تصف عملية التصنيع الحيوي للدقائق النانوية في هذه الدراسة. كان الهدف من هذا العمل هو استخدام جسيمات أوكسيد الحديد النانوية كعوامل مضادة للميكروبات ضد مسببات الأمراض المقاومة للمضادات الحيوية وهو حل واعد لهذه المشكلة. تم تضمين 120 عينة من الأطعمة ذات الأصل الحيواني في هذه الدراسة. تم عزل وتشخيص *Citrobacter freundii* غذائية المنشأ بدقة لاستخدامها بعد ذلك في التصنيع الحيوي لجسيمات أوكسيد الحديد النانوية. تم تصنيع وتوصيف جسيمات أوكسيد الحديد النانوية باستخدام اختبارات مختلفة. حيث تم اعتماد مجهر القوة الذرية كتقنية أساسية. تم اختبار نشاطها المضاد للميكروبات ضد العزلات البكتيرية المنقولة بالغذاء والسريرية. كشفت نتائج هذه الدراسة أن جسيمات أوكسيد الحديد النانوية المصنعة كانت بقطر 32.86 نانومتر مع خصائص مغناطيسية. تمنع جسيمات أكسيد الحديد النانوية المصنعة حيويًا تكوين الأغشية الحيوية لكل من العزلات الغذائية والسريرية. يمكن الاستنتاج أن *C. freundii* غذائية المنشأ عامل اختزال ممتاز في التصنيع الحيوي لهذه المواد النانوية النشطة بيولوجيًا. هذا هو أول بحث يقوم بتصنيع جسيمات نانوية من أوكسيد الحديديك بواسطة *C. freundii* غذائية المنشأ. تطلبت العزلات السريرية جرعة اعلى من نانو الحديد اللازم لتثبيط الغشاء الحياتي للعزلات المحمولة بالغذاء, مما يستدعي الحاجة لعلاجات اقوى. وبرغم هذه المقاومة الدنيا الا ان العزلات الغذائية لازالت تمتلك خطر إحداث التلوث. وبالوقوف على مخاطر كلا المصدرين السريري و الغذائي يمكن تطوير العلاجات المضادة للميكروبات والامن الغذائي.

الكلمات المفتاحية: غذاء, تقنية النانو, حديد , صديق للبيئة, الأمن الغذائي.