

Synergistic Efficacy of Biogenic ZnO-NPs and Antibiotics Against *Bacillus cereus*

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Abstract

Bacillus cereus is an important foodborne pathogen and a significant public health concern owing to its ability to produce highly resistant endospores and the increasing emergence of multidrug-resistant strains. This study evaluated the prevalence and antimicrobial susceptibility profiles of *B. cereus* isolates recovered from clinical and food sources and investigated the antibacterial activity of biogenic zinc oxide nanoparticles (ZnO-NPs) against these isolates. The forty-five *B. cereus* isolates represented a prevalence of 37.5% and exhibited high levels of resistance to β -lactam antibiotics and trimethoprim, with resistance being significantly higher among clinical isolates than food isolates. Biogenic ZnO-NPs were synthesized through an environmentally friendly method using *Moringa oleifera* leaf extract and characterized using X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), atomic force microscopy (AFM), and Fourier transform infrared spectroscopy (FTIR). The characterization confirmed the successful synthesis of semi-crystalline, spherical ZnO nanoparticles with grain sizes ranging from 37.93 to 78.39 nm. Antibacterial assays demonstrated that biogenic ZnO-NPs exhibited significant antibacterial activity against *B. cereus*, with the greatest inhibitory effect observed at a concentration of 5 mg/mL. Furthermore, the combination of ZnO-NPs with tetracycline significantly enhanced the antibacterial activity of tetracycline compared with the antibiotic alone. Previously resistant isolates became susceptible following combination treatment, an effect that may be attributed to ZnO-NP-induced alterations in the bacterial cell envelope, increased membrane permeability, and possible interference with efflux-mediated resistance mechanisms, thereby facilitating greater intracellular accumulation of tetracycline. In contrast, the combination of ciprofloxacin with ZnO-NPs did not demonstrate significant synergistic activity, likely because ciprofloxacin alone already exhibited high antibacterial efficacy against the tested isolates. Overall, these findings suggest that biogenic ZnO-NPs may serve as promising antibacterial adjuvants capable of enhancing the efficacy of conventional antibiotics against resistant *B. cereus* isolates and may contribute to future strategies aimed at combating antimicrobial resistance.

Keywords: *Bacillus cereus*, Green Synthesis, Zinc Oxide Nanoparticles, *Moringa oleifera*, Synergistic Efficacy..

Introduction

Bacillus cereus is a Gram-positive spore-forming bacterium that is found in various environments including soil, food products, and food processing plants. *Bacillus cereus* is capable of producing

extremely resistant endospores which allow it to survive under harsh conditions, e.g. high temperature, dryness and exposure to chemicals (Woh et al., 2024).

Nanotechnology is the manipulation of materials on a nanoscopic

scale (1-100 nm), where the innate properties of materials differ grossly from the properties of these large area materials. Because nanomaterials have high surface area-to-volume ratios; low molecular weights, tunable surface chemistry; and enhanced reactivity, they have become a vital component of developments in nearly every area of science and the industrial world, including medicine, biotechnology, pharmaceuticals, agriculture, and environmental science (Rao et al., 2021).

Zinc oxide nanoparticles (ZnO-NPs) are one type of several different types of nanomaterials being analysed for their antimicrobial effect; they have received a lot of interest as they have been proven to have an excellent safety record, are generally inexpensive to produce and show potency against a large variety of bacteria, including Gram-positive bacteria like *Bacillus cereus*, as documented by recent studies (Mohammed et al., 2025; Al-Mashhadani, 2025).

Material and Methods

Sample collection

A total of 120 samples were collected during the period from October 2025 to January 2026. Clinical samples were collected from patients suffering from diarrhea or vomiting whose ages ranged between 10 years to 40 years from several hospitals in Baghdad: Baghdad Educational Hospital, Specialized burns Hospital, and Ghazi Hariri Hospital, , the food isolates for this study were isolated from three different sources (rice, milk, and meat).performed by plating samples on selective agar media of *B. cereus*.

Isolation and Phenotypic Characterization

In addition to the antibacterial properties of ZnO-NPs, many researchers are investigating the use of ZnO-NPs produced from the biosynthesis of plant extracts or biological processes as they exhibit very good enzyme-inhibiting activity against Gram-positive bacteria due to enhanced production of reactive oxygen species and increased surface area to contact the bacteria (Al-Mashhadani, 2025; Mohammed et al., 2025).

The manufacture of zinc oxide nanoparticles was studied using analytical techniques such as UV-visible spectroscopy and xrd, along with imaging techniques like FE-SEM and AFM.The UV-Visible absorption scan of the supernatant infused with zinc chloride exhibited a peak at 36nm, signifying the surface plasmon resonance (SPR) of zinc oxide nanoparticles. (Jayachandran et al., 2021) (Hassan and Risan, 2024).

The initial step in isolating the samples was the use of Nutrient Agar and Blood Agar (to determine if the samples produced beta hemolysin). Selective isolation was performed using Polymyxin Pyruvate Egg Yolk Mannitol Bromothymol Blue Agar (PEMBA) to facilitate further studies on the bacterial isolates. The presence of *B. cereus* can be identified through the observation that *B. cereus* colonies are peacock blue in color they do not ferment mannitol (this is indicated by the blue color of the agar). they have a strong lecithinase activity (this manifests itself as a clear zone around the colonies on egg yolk). Isolated bacterial strains were subsequently tested to determine their metabolic and biochemical characteristics using the automated VITEK® 2 platform developed by (bioMérieux of France).

Preparation of moringa plant extract

Fresh leaves of *Moringa oleifera* were thoroughly washed with distilled water to remove surface contaminants and then shade-dried at room temperature for 24 hours until complete drying was achieved. The dried leaves (50 g, dry weight) were mechanically homogenized with 500 mL of sterile distilled water (1:10 w/v) using a high speed blender for 5 minutes following the method described by (Elumalai et al,2015).

The resulting homogenate was filtered through Whatman No. 1 filter paper, and the filtrate was further clarified by centrifugation at 8000 rpm for 15 minutes (Moodley et al., 2018) to obtain a clear, particle-free extract. The prepared extract was stored at 4 °C for the subsequent green synthesis of zinc oxide nanoparticles.

Preparation of ZnO Nanoparticles

15g of zinc chloride were gradually added to 300 mL of *Moringa oleifera* leaf extract under continuous stirring. The reaction mixture was mechanically stirred at 500–600 rpm and heated at 70 °C for 3 hours to facilitate the formation of zinc oxide nanoparticle precursors. The pH of the solution was adjusted to 10 to promote the formation of zinc hydroxide as an intermediate for ZnO nanoparticle synthesis (Jadoun et al.,2021). The reaction mixture was then allowed to stabilize for 24 hours in the dark. The

resulting precipitate was collected by centrifugation at 8000 rpm for 20 minutes at 4 °C, washed twice with absolute ethanol (Moodley et al., 2018), and dried at oven in 60°C. The dried product was further subjected to thermal treatment to obtain ZnO nanoparticles. All experiments were performed in triplicate to ensure reproducibility. Appropriate control groups were included, comprising a negative control (sterile saline), plant extract control, zinc chloride solution control, ZnO nanoparticles alone, and antibiotic-only control, in order to accurately evaluate the antibacterial activity and distinguish between individual and synergistic effects (Banoee et al., 2010; Jadoun et al., 2021).

Results and Discussion

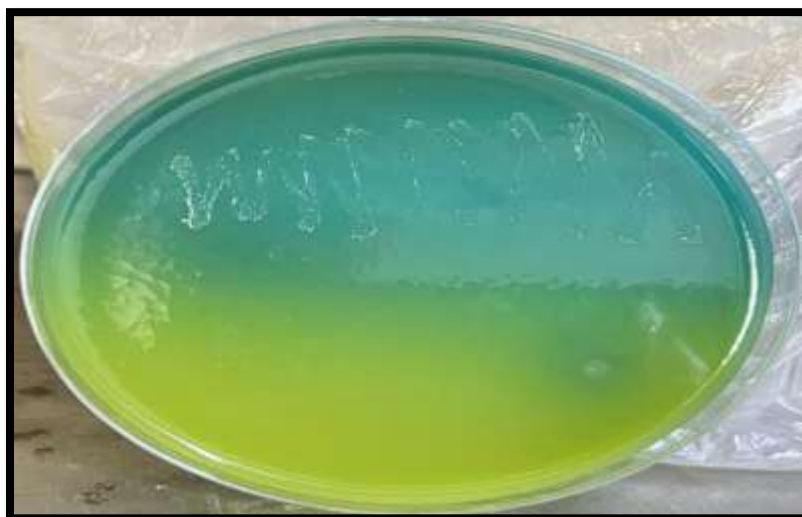
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Isolation and identification of *B.cereus*

The isolation protocol revealed that 48.4% (or 45/120 confirmed isolates) were recovered. Moreover, confirmed isolates grown on PEMBA agar had 100% specificity and inhibited the growth of any other potentially competitive microflora. The confirmed isolates had good lecithinase production and beta-hemolysis showed they had pathogenic potential. Distribution of confirmed isolates in table (1).

Table (1): distribution of positive growth among clinical and food sample.

Negative growth	positive Growth	No. of Samples	Type of isolate
19	11	30	Clinical
16	14	30	Food (rice)
22	8	30	Food (meat)
18	12	30	Food (milk)



Figure(1): Colony Morphology on PEMBA agar.

Vitek-2 System

the isolates were identified as *Bacillus cereus* with high confidence using the VITEK®2 automated identification system (Fig 2).

bioMérieux Customer:		Microbiology Chart Report		Printed December 23, 2025 11:34:37 AM AST													
Patient Name: AFRS 590		Patient ID: 15320229		Physician:													
Location:		Lab ID: 15320229		Isolate Number: 1													
Organism Quantity:		Selected Organism : <i>Bacillus cereus</i>															
Source:		Collected:															
Comments:																	
Identification Information		Analysis Time: 13.90 hours		Status: Final													
Selected Organism		94% Probability		<i>Bacillus cereus</i>													
ID Analysis Messages		Bionumber:		0326000150456671													
Biochemical Details																	
1	BXYL	-	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	-
9	BGAL	-	10	PyrA	+	11	AGAL	-	12	AlaA	(-)	13	TyrA	+	14	BNAG	+
15	APPA	-	16	CDEX	-	19	dGAL	-	21	GLYG	-	22	INO	-	24	MdG	-
25	ELLM	-	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	-	31	dMAN	-
32	dMNE	+	34	dMLZ	-	36	NAG	+	37	PLE	-	39	IRHA	+	41	BGLU	-
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	-	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	56	PSCNa	-	58	NaCl 6.5%	+	59	KAN	+
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB	+						

Figure (2): *B. cereus* identification by VITEK 2 system.

Green Synthesis of ZnO Nanoparticles using Moringa Leaves Extract

Synthesizing nanoparticles using green methods enhances the rate of inhibition as compared to conventional delivery

methods and also maintains the antimicrobial activity longer than traditional delivery methods. The presence of yellowish white precipitates in the solution combination proved the presence of the plant extract shown in figure (3).

Figure 3. Diagram of the manufacturing process for ZNO-NP .

Characterization of ZnO-AV NPs

UV/Visible Spectral Analyses

The zinc oxide nanoparticle UV-Visible absorption spectrum showed intense absorption peak at 365 nm, exhibiting an absorbance of 0.850, this confirming the successful synthesis of ZnO nanoparticles. The characteristic excitonic peak at 365 nm is the result of the electronic transition of electrons from the valence band to the conduction band over the direct band gap of ZnO nanoparticles (Talam *et al.*, 2012). The sharpness and symmetry of the absorption peak suggest a relatively uniform particle size distribution and low aggregation of the synthesized nanoparticles, whereas broad absorption bands are typically associated with particle agglomeration or non-uniform particle sizes.

the UV-Vis spectrum indicates a gradual decrease in absorbance across the visible region confirming the high transparency and purity of the ZnO nanoparticles with minimal structural defects or impurities (Senthilkumar and Sivakumar, 2014). The absorption peak at 365 nm corresponds to a photon energy of approximately 3.40 eV according to relation. Calculated Optical Bandgap is

slightly higher than that observed for ZnO due largely to a phenomenon known as blue shift that occurs from quantum confinement when particles are nanomaterials (Morkoç and Özgür, 2009). Evidence of this effect and related quantum size effects have also been reported by Zakak *et al.*, (2011) who showed how reduced particle dimensions can result in a significant increase in optical properties and increased band gap energy for nanosized ZnO.

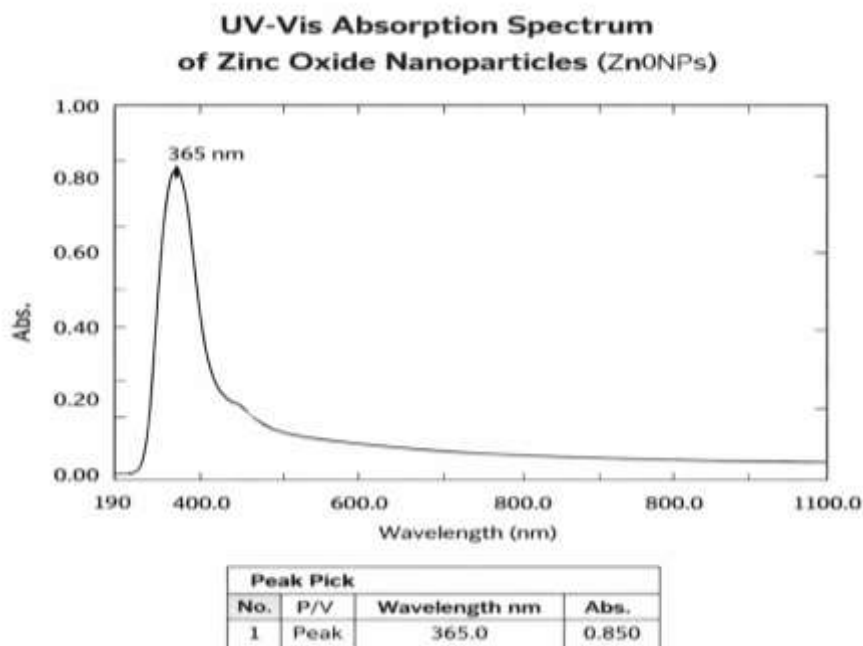


Figure 4. The absorption spectrum of ZnO NPs

Atomic Force Microscopy (AFM)

The surface topography and morphological characteristics of the prepared films were investigated using Atomic Force Microscopy (AFM). The surface exhibits a dense arrangement of self-assembled nanometric grains, the average surface roughness (S_a) was determined to be 311.11 pm, while the root mean square (RMS) roughness (S_q) was measured at 518.45 pm. The height distribution analysis indicates that the maximum peak height (S_p) reaches 781 pm, with a total vertical range (Z-axis) of approximately 1.77 nm. figure (5) illustrates the 2D and 3D AFM micrographs, which reveal a highly uniform and homogeneous distribution of nanostructures across the scanned area of (2.5 x 2.5 μm). indicating a well-controlled growth process. Quantitative analysis of the surface roughness parameters provides precise insights into the film's quality. These measurements at the picometer scale signify an ultra-smooth surface with high structural integrity and minimal surface defects.

The minimal roughness values (less than 1 nm) align with the research of (Krysova *et al.*, 2024), who demonstrated that high-density nanostructured films with low surface roughness exhibit superior structural and functional performance. This is also in agreement with (Maria and Rahman, 2025), who emphasized that such smooth morphologies and controlled grain growth are indicative of high crystallinity and a well-defined growth mechanism.

The high degree of smoothness and the organized arrangement of the nanostructures observed in this study are

consistent with findings reported in recent literature for high-quality metal oxide thin films. According to (Toma *et al.*, 2025), optimized deposition parameters lead to a uniform grain arrangement and a significant reduction in surface roughness, which is essential for enhancing functional properties. The observed topography confirms that the synthesis method successfully suppressed large-scale grain agglomeration, resulting in a more stable and uniform surface compared to conventional preparation techniques.

furthermore, the dense packing of the grains across the substrate confirms the efficiency of the synthesis process in producing high-quality nanostructured surfaces suitable for advanced applications.

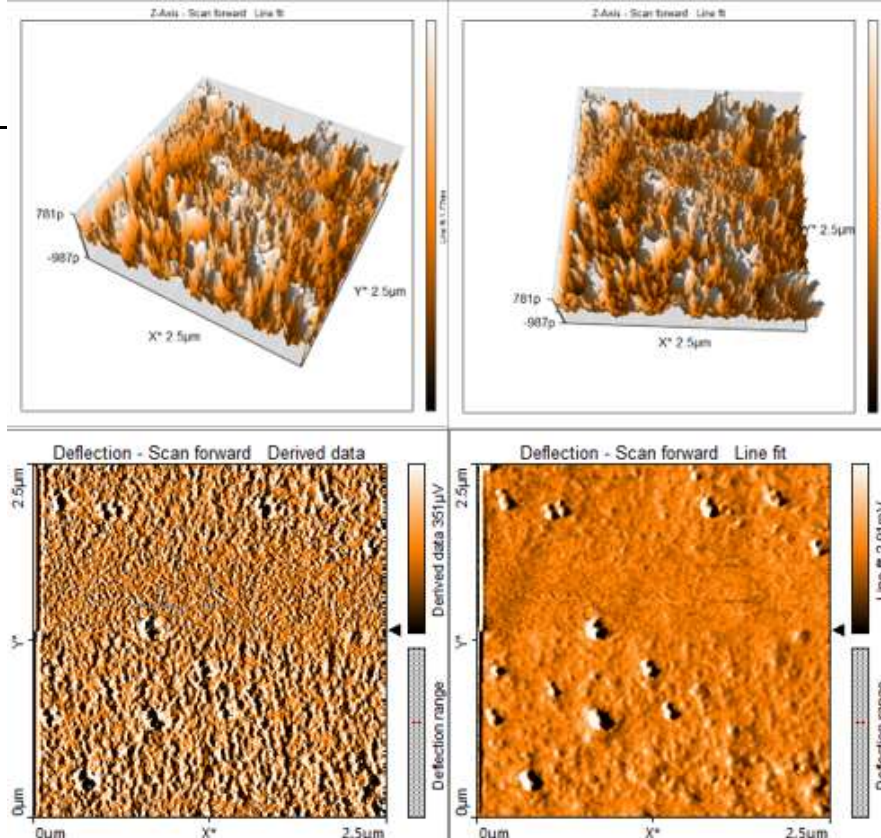


Figure 5: AFM 3-dimensional image of *Moringa* ZnO NPs

Fourier Transform Infra-Red Spectroscopy (FTIR) Test

The Fourier Transform Infrared (FTIR) spectroscopy is a powerful analytical tool for characterizing and synthesized ZNO nanoparticles and to identify the functional groups present in these compounds which help understand their molecular nature. The FT-IR spectra provide absorption peaks corresponding to the vibrational modes of the functional groups; therefore, different types of functional signature such as OH, C=O, N-H, C-H, can be detected. Thus it serves reliable evidence to help verify the presence of chemical bonds of interest within the samples (Verma *et al.*, 2022). The FTIR spectra (Figure 6) of the as-prepared sample contain several characteristic absorption bands that provide insight into its chemical structure. The broad and intense absorption peak at approximately 3283cm^{-1} attributed to O-H stretching vibrations, the is expected due

the presence of hydroxyl groups from either moisture or phenolic compounds that were involved in the stabilizing process. The peaks at 2962cm^{-1} , 2920cm^{-1} and 2851cm^{-1} are due to asymmetric and symmetric C-H stretching vibrations associated with the aliphatic groups. The absorption band at 1627cm^{-1} relates either to C=O stretching or to C=C skeletal vibrations. frequently found in proteins or flavonoids from plants used in the synthesis. Additionally, the presence of multiple peaks between $1540\text{--}1264\text{cm}^{-1}$ indicates the presence of C-C stretching and C-H bending modes. The most significant feature of the spectrum is the very strong absorption bands at lower wavenumbers (524 , 457 , and 422cm^{-1}) are indicative of metal-oxygen (ZNO). These peak confirm the successful formation of an inorganic phase of the crystalline phase of the nanoparticles. The results obtained from FTIR spectral analysis verified that several functional groups contributed to

both the reduction and stabilization of the nanoparticles. The broad spectral absorption band at 3283 cm^{-1} is attributed to the O–H stretching vibrations, which correlates with the results of Ahmed and Alias (2024). where phenolic hydroxyl functional groups were identified as the primary reducing agents during the green synthesis.

The C–H stretching vibrations (2962 and 2851 cm^{-1}) agree with (Nakamoto, 2009), who identified aliphatic chains associated with organic capping layers that limit nanoparticles agglomeration. The intense peak of $1,627\text{ cm}^{-1}$ (Amide I), consist with the work (Abdellatif *et al.*, 2023). which indicating flavonoids/proteins are responsible for the stabilization of metal oxide nanoparticles. The shift peak confirms the strong interaction between organic molecules and the nanoparticle surface. According to (Handore *et al.*, 2014) these vibrational bands in the fingerprint region ($524\text{--}422\text{ cm}^{-1}$) represent the primary characteristics of crystalline metal oxide nanostructures confirming that the materials are present in a stable inorganic solid form.

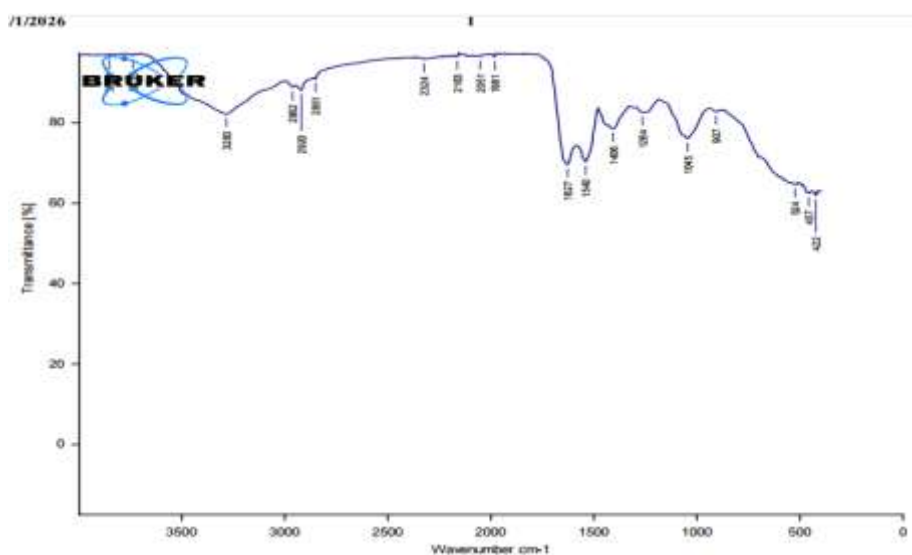


Figure (6): The FT-IR spectrum of ZnO NPs

Scanning Electron Microscopy Analysis

Field Emission Scanning Electron Microscopy (FESEM) was used to characterize the morphology and determine the particle size distribution of the manufactured nanomaterials. As compared to standard scanning electron microscopy (SEM), FESEM produces improved resolution and imaging of nanostructures at low accelerating voltages. This is important because it enables observation of the fine surface topography of either bio-derived nanoparticles or chemically manufactured nanoparticles without applying enough heat to cause damage. According to (Jagadeesh et al., 2024).

FESEM is the approved gold standard method of diagnosing nanoscale materials because of the ability to view secondary electron responses with significantly high magnifications, yielding significant information about the grain boundaries, porosity, and agglomeration state of material. In this study, FESEM was used to confirm that the transition from bulk precursors to nanometers occurred and to

identify the structural features formed from the starting materials.

The FESEM images show that the produced nanomaterials are composed primarily of densely aggregated nanoparticles with irregular shapes and a surface texture similar to that of an amorphous solid (Figure 7). The grain size analysis of the sample shows three clearly defined sizes of the nanoparticles (37.93 nm, 48.57 nm, and 78.39 nm). The morphology obtained in this study is consistent with the green synthesis methods described by (Radulescu et al., 2023). These authors point to the bio-derived stabilizers used as stabilizing agents producing these clustered structures.

Additionally, the particle sizes measured (38-79 nm) are also in accordance with data reported by (Mtavangu et al., 2024). for ZnO nanocomposites produced using leaf extracts, confirming that the method used in the present study yields materials with the appropriate nanoscale dimensions for biomedical and environmental purposes.

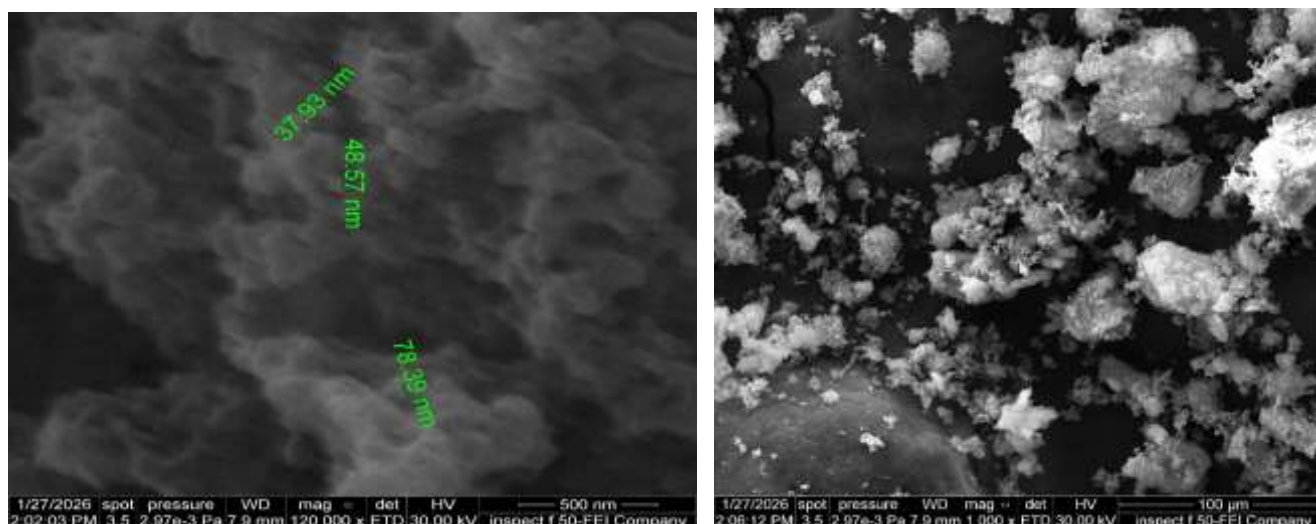


Figure (7): The SEM image of ZnO nanoparticles

X-Ray Diffraction

By using X-ray diffraction (XRD), The crystalline structure, phase purity, and crystallite size of the synthesized ZnO nanoparticles were characterized. Figure (3-12) illustrates the XRD pattern, which exhibits a series of diffraction peaks in the 2θ range of 10° – 80° . The presence of intense and sharp peaks confirms the formation of a well-defined crystalline phase.

The principal diffraction peaks observed at 2θ values of approximately 31.76° , 34.42° , and 36.25° correspond to the (100), (002), and (101) Miller indices (hkl), respectively. These peaks are in excellent agreement with the standard JCPDS card no. 36-1451. The sharpest peak observed at 33.45° and the presence of other significant peaks at 21.83° and 58.88° provide further evidence of the crystalline nature of the sample, confirming the hexagonal wurtzite structure of ZnO.

A noticeable feature of the XRD profile is the broadening of the diffraction peaks, particularly the principal peak at 33.45° with a Full Width at Half Maximum (FWHM) of 1.46° . This peak broadening is a direct physical indicator of the nanoscale dimensions of the synthesized particles. To determine the average crystallite size (D), the Debye-Scherrer equation was applied:

$$D = K \lambda / \beta \cos \theta$$

Where K is the Scherrer constant (0.9), λ is the X-ray wavelength (1.5406 for Cu-K alpha radiation), β is the FWHM in radians, and θ is the Bragg angle. The calculated size confirms that the particles are within the nanometric range. The XRD profile exhibits a broad hump at lower angles (between 20° and 30°), which indicates the presence of an amorphous organic phase. This is a typical hallmark of green synthesis, where

bioactive compounds from the plant extract (such as polyphenols and proteins) remain as a capping layer on the nanoparticle surface to provide steric stabilization and prevent agglomeration (Awwad *et al.*, 2013). The XRD results obtained in this study are consistent with recent literature on biogenic nanostructures. The observed peak broadening (FWHM $\approx 1.46^\circ$) is a characteristic phenomenon for materials with nanoscale structures, where reduced crystallite dimensions lead to incomplete destructive interference at angles slightly off the Bragg condition (Stock, 2013).

According to Suryanarayana and Norton (1998), such shifts and broadening are common in nanostructured materials due to internal micro-strain and the high surface-to-volume ratio. Furthermore, the slight peak shift observed in the main peak position (33.45°) compared to the bulk ZnO standard is attributed to lattice strain and structural imperfections induced during the rapid biogenic reduction process. These findings are in strong agreement with Toma *et al.* (2025) and Krysova *et al.* (2024), confirming that the synthesis method successfully produced stable, crystalline ZnO nanoparticles capped with a functional organic shell.

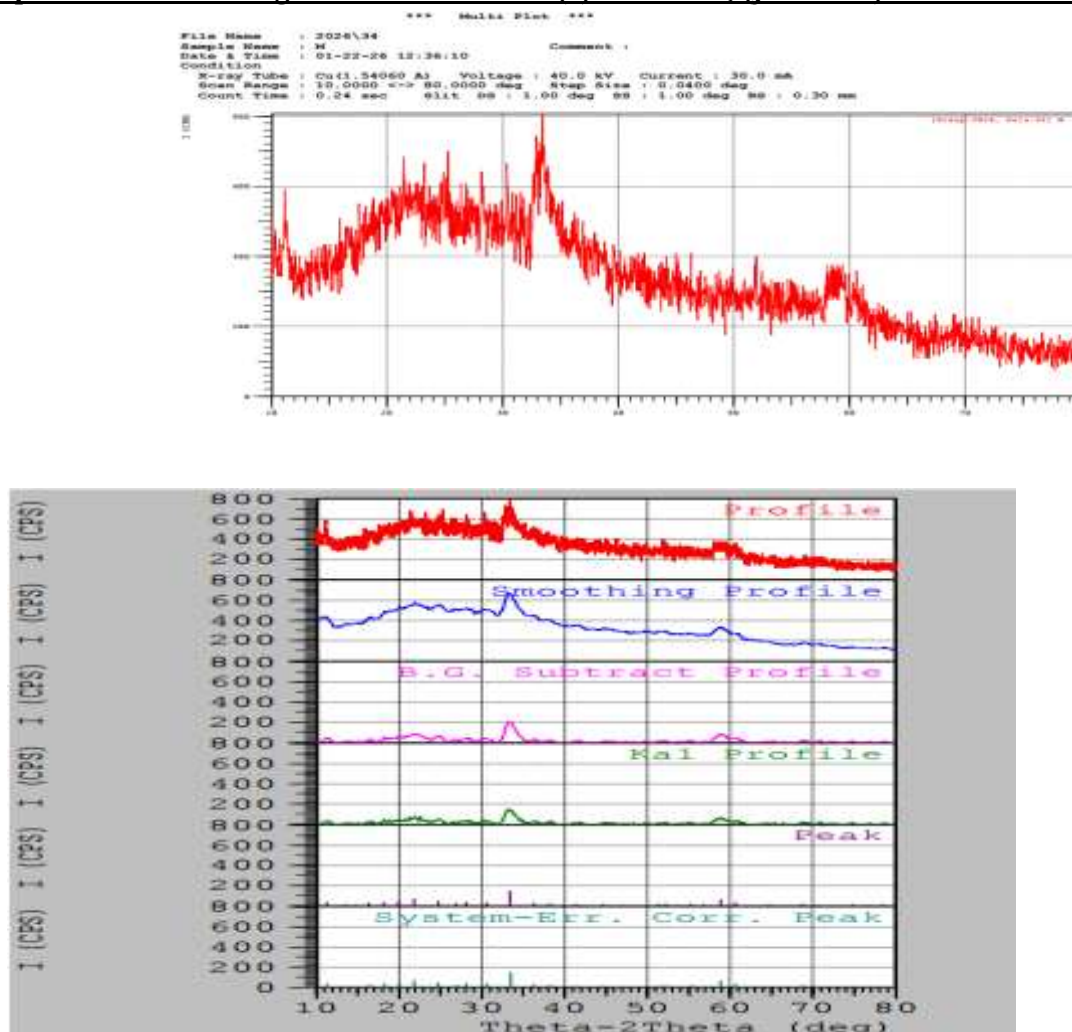


Figure (8): X-ray diffraction (XRD) patterns of ZnONP

Antibacterial activity of Moringa ZnO NPs on *Bacillus cereus*

In this study, the antibacterial synergistic efficacy of green-synthesized zinc oxide nanoparticles using *Moringa oleifera* extract, in combination with two selected antibiotics (ciprofloxacin and tetracycline), was investigated against a representative set of *Bacillus cereus* isolates isolated from food and clinical sources. Four isolates were chosen to represent the spectrum of *Bacillus cereus*, which consisted of a clinical isolate (M7), one isolated from rice (M5), one isolated from meat (M6), and one isolated from milk (M10). All four representatives were chosen for their unique colony morphologies, robust growth patterns, and corresponding antimicrobial susceptibility classifications

that were determined during an extensive screening of all isolates in the laboratory. The standard antibiotic disc potencies as recommended by the Clinical and Laboratory Standards Institute (CLSI) were used to evaluate the antibacterial activity of both the ZnO-NPs and the selected antibiotic agents; ciprofloxacin at 5 µg and tetracycline at 30 µg were the selected antibiotic concentration for this investigation to ensure clinical relevance and standardization of interpretation of the synergistic interactions.

Based on their different levels of antibacterial effectiveness against the tested *B. cereus* isolated during the preliminary susceptibility assay, ciprofloxacin and tetracycline was chosen. tetracycline exhibited comparatively weak

antibacterial activity and produced the lowest inhibition zones among the tested antibiotics whereas Ciprofloxacin demonstrated moderate inhibitory activity against most isolates, these two antibiotics provided an appropriate experimental model to investigate whether biogenic ZnO-NPs could enhance the antibacterial efficacy of agents with different intrinsic activities against *B. cereus*. The various ways that antibacterial activity occurs with ZnO-NPs include interaction with both bacterial cell wall as well as membrane which results in the disruption of the structural integrity of the bacteria, as well as the release of Zn^{2+} ions into the bacteria, where this release is disruptive to critical enzymatic systems, and, thirdly, the induction of the production of reactive oxygen species that disrupt and/or damage cellular proteins, lipids, nucleic acids, etc. (Al-Mashhadani, 2025; Mohammed et al., 2025).

As demonstrated in Tables (2 and 3) and Figure (9), clear differences were observed in the synergistic responses between the tested antibiotics and ZnO-NPs. Mechanistically, ciprofloxacin exerts its antibacterial action by inhibiting bacterial DNA gyrase and topoisomerase IV enzymes, thereby disrupting DNA replication and transcription (Hooper and Jacoby, 2015). the combination of ciprofloxacin with ZnO-NPs exhibited little to no significant synergistic enhancement in most isolates. Ciprofloxacin alone already produced relatively large inhibition zones, reaching up to 33 mm in some isolates, indicating substantial intrinsic susceptibility of *B. cereus* to this fluoroquinolone antibiotic. Consequently, the addition of ZnO-NPs resulted in only minimal increases in inhibition zone diameters. This limited enhancement may reflect saturation of antibacterial efficacy due to the already strong inhibitory activity of ciprofloxacin

alone. Since the isolates were already highly susceptible to ciprofloxacin, additional membrane permeabilization by ZnO-NPs may not have substantially increased intracellular antibiotic accumulation.

the combination of tetracycline with biogenic ZnO-NPs demonstrated a pronounced and consistent synergistic effect against tested isolates. the inhibition zone of the clinical isolate M7 increased from 35 mm with tetracycline alone to 38 mm following nanoparticle addition. Similarly, Tetracycline alone showed relatively weak antibacterial activity, particularly against the meat-derived isolate M6, where the inhibition zone diameter reached only 13 mm. upon combination with ZnO-NPs, the inhibition zone increased markedly to 17 mm. These findings indicate that ZnO-NPs significantly enhanced the antibacterial activity of tetracycline against *B. cereus* isolates with reduced susceptibility.

Synergistic between tetracyclines and ZnO NPs may be explained by the combination of their respective mechanism of actions. Tetracycline acts primarily by binding to the 30S ribosomal subunit of bacteria and inhibiting protein synthesis in those organisms (Chopra and Roberts, 2001). The instance wherein resistance to tetracycline occurs in *B. cereus* is often attributed to decreased membrane permeability, active efflux pumps, or ribosomal protection proteins, which limit the accumulation of antibiotic within the cell. ZnO NPs may overcome the above-listed resistance mechanisms through several potential mechanisms:

disruption of the bacterial cell wall and membrane integrity, generation of reactive oxygen species (ROS), release of Zn^{2+} ions, and/or increased cell membrane permeability. The combination of these events is suspected to enhance the

intracellular penetration of tetracycline into bacteria, thereby enhancing its inhibitory effects. Thus, biogenic ZnO-NPs synthesized with *Moringa oleifera* extract may act as effective nano-bioenhancers to increase the activity of conventional antibiotics against *B. cereus* (Sirelkhatim *et al.*, 2015). *B. cereus* spores possess highly resistant structural layers and reduced metabolic activity, their susceptibility to nanoparticles and antibiotics may differ significantly from

that of vegetative cells. It is important to note that the observed antibacterial activity primarily reflects the effect of ZnO-NPs and antibiotics on vegetative bacterial cells rather than dormant spores. This is because the antimicrobial susceptibility testing was performed using actively growing bacterial cultures under standard CLSI conditions. Therefore, the present findings mainly demonstrate the effectiveness of ZnO-NPs against actively proliferating *B. cereus* cells (Setlow, 2014).

Tabl 2 : Antibacterial activity of moringa ZnO-NPs in combination with Ciprofloxacin against *B. cereus* isolates from clinical and food sources.

Meat isolate (M6)	Milk isolate (M10)	Rice isolate (M5)	Clinical isolate (M7)	Treatment type	Concentration of ZNO
20mm	32mm	33mm	23mm	Antibiotic	
12mm	11mm	25mm	20mm	ZNO	
15mm	27mm	30mm	21mm	ZNO+Antibiotic	5mg
20mm	32mm	33mm	23mm	Antibiotic	2.5mg
10mm	10mm	20mm	15mm	ZNO	
14mm	19mm	32mm	20mm	ZNO+Antibiotic	
20mm	32mm	33mm	23mm	Antibiotic	1.25mg
7mm	8mm	12mm	15mm	ZNO	
13mm	17mm	31mm	17mm	ZNO+Antibiotic	

Meat (M6)	Milk (M10)	Rice (M5)	Clinical (M7)	Treatment type	Concentration of ZNO
14mm	25mm	34mm	35mm	Antibiotic	
13mm	15mm	18mm	15mm	ZNO	
17mm	26mm	36mm	38mm	ZNO+Antibiotic	5mg

14mm	25mm	34mm	35mm	Antibiotic	2.5mg
12mm	11mm	14mm	12mm	ZNO	
16mm	25mm	34mm	37mm	ZNO+Antibiotic	
14mm	25mm	34mm	35mm	Antibiotic	1.25mg
9mm	10mm	12mm	10mm	ZNO	
15mm	25mm	34mm	36mm	ZNO+Antibiotic	

Table (3): Antibacterial activity of moringa ZnO-NPs in combination with tetracycline against *B. cereus* isolates from clinical and food source

Table 4. Descriptive statistics of the antibacterial activity of ciprofloxacin, ZnO nanoparticles, and their combination against *Bacillus cereus* isolates

Treatment	n	Mean (mm)	Median (mm)	SD	CV (%)	Min (mm)	Max (mm)
Ciprofloxacin	12	25.25	23.0	5.62	22.3	20	33
ZnO nanoparticles	12	13.75	12.0	5.82	42.3	7	25
ZnO + Ciprofloxacin	12	21.33	20.5	6.82	32.0	13	32

Descriptive statistical analysis of the inhibition zone diameters demonstrated that ciprofloxacin alone produced a mean inhibition zone of 25.25 ± 5.62 mm, whereas ZnO nanoparticles alone showed a considerably lower mean value of 13.75 ± 5.82 mm. The combination of ciprofloxacin with ZnO nanoparticles

yielded a mean inhibition zone of 21.33 ± 6.82 mm, representing an overall reduction of approximately 15.5% compared with ciprofloxacin alone. These findings indicate that the addition of ZnO nanoparticles did not enhance the antibacterial activity of ciprofloxacin against the tested *B. cereus* isolates.

Table (4): Descriptive statistics of the antibacterial activity of tetracycline, ZnO nanoparticles, and their combination against *Bacillus cereus* isolates

Treatment	n	Mean (mm)	Median (mm)	SD	CV (%)	Min (mm)	Max (mm)
Tetracycline	12	27.00	29.5	8.67	32.1	14	35
ZnO nanoparticles	12	12.58	12.0	2.88	22.9	9	18
ZnO + Tetracycline	12	27.42	30.0	9.14	33.3	15	38

Descriptive statistical analysis revealed that tetracycline alone exhibited a mean inhibition zone of 27.00 ± 8.67 mm, while

ZnO nanoparticles alone produced a mean inhibition zone of 12.58 ± 2.88 mm. The combination of tetracycline with ZnO

nanoparticles resulted in a mean inhibition zone of 27.42 ± 9.14 mm, corresponding to a slight increase of approximately 1.5% compared with tetracycline alone. This

observation suggests only a marginal enhancement in antibacterial activity following the combined treatment.

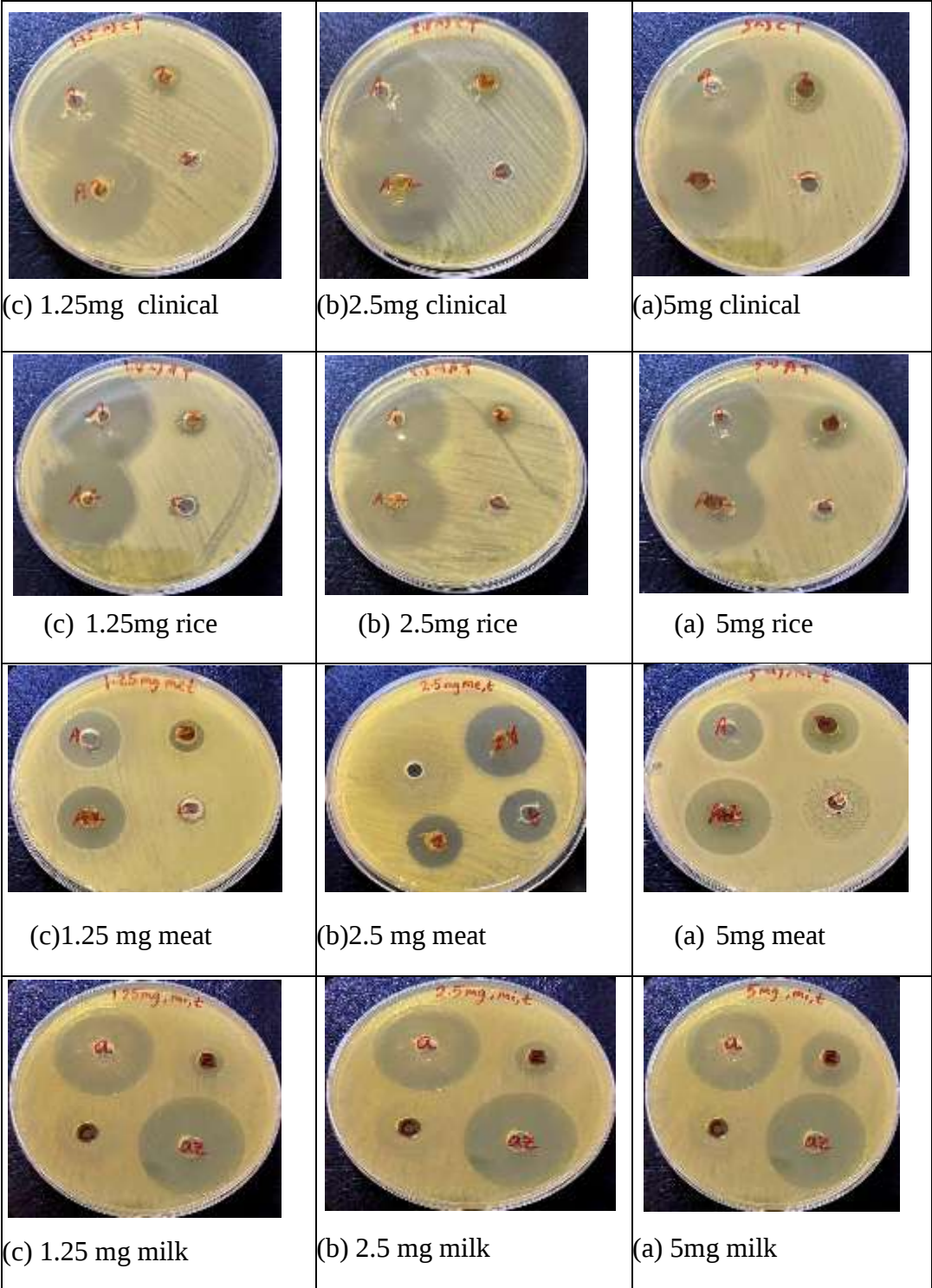


Figure (9): Effect of different concentrations (5, 2.5, 1.25 mg/ml) of ZnO nanoparticles on the inhibition of *Bacillus cereus*.

The results obtained within this experiment are consistent with those reported by Masum *et al.* (2019) and no significant synergistic effect between either the ciprofloxacin and the ZnO-NPs was observed. The strong inhibitory activity of the ciprofloxacin against the majority of the *Bacillus cereus* isolates, specifically the clinical isolate M7, indicates that these isolates are susceptible to the fluoroquinolone antibacterial (ciprofloxacin). As ciprofloxacin acts on bacterial DNA by inhibiting bacterial DNA gyrase and topoisomerase IV enzymes, there is a strong possibility that a sufficient amount of fluoroquinolone has been delivered into the bacteria, thereby minimizing any increased bactericidal action that would result from exposure to ZnO-NPs. Furthermore, due to its very small molecular size and inherent bactericidal activity, ciprofloxacin is known to penetrate bacterial cells easily. Therefore, it is unlikely that the additional disruption of the bacterial cell membrane due to exposure to ZnO-NPs had a significant effect on the accumulation of fluoroquinolone in the bacterial cells. The phenomenon commonly referred to as the ceiling effect likely accounts for the limited increase in diameter of inhibition zones observed during this study.

In contrast, this study showed strong or very high synergistic interactions between tetracycline and ZnO nanoparticles derived from *Moringa oleifera*, particularly among isolates exhibiting lower susceptibility to tetracycline, such as the meat-derived isolate M6. The synergistic mechanism was further supported by Tiwari *et al.* (2018), who demonstrated that ZnO nanoparticles induce structural damage to the bacterial cell envelope, increase membrane permeability, generate reactive oxygen species (ROS), and

facilitate enhanced intracellular antibiotic penetration. These combined effects may enable tetracycline to bypass permeability-associated resistance mechanisms and more effectively reach its ribosomal target sites within bacterial cells. The observed synergistic activity in the present study primarily reflects effects on vegetative bacterial cells rather than dormant spores, since susceptibility testing was conducted using actively growing cultures under standard CLSI conditions. These results agree with those reported by Sharmila *et al.* (2018), who observed enhanced antibacterial activity when tetracycline was combined with biogenic ZnO-NPs against several *Bacillus* species. Tetracycline acts by inhibiting bacterial protein synthesis through binding to the 30S ribosomal subunit; however, resistance mechanisms such as efflux pumps, reduced membrane permeability, and ribosomal protection proteins may limit its intracellular activity in *B. cereus*.

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