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Extracellular Biosynthesis and Antimicrobial Activity of Bacillus Subtilis ATCC 6633 Zinc Oxide Nanoparticles

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Abstract

Recently, zinc oxide nanoparticles (ZnO NPs) have been used in several biological and industrial applications due to their low toxicity, high efficiency, biocompatibility, and cost-effectiveness. In the present study *Bacillus subtilis* ATCC 6633 was used in the biosynthesis of the antimicrobial ZnO NPs. The synthesized ZnO NPs were characterized using UV-Visible spectroscopy, Fourier transform-infrared (FTIR) spectroscopy, X-ray diffraction (XRD) spectroscopy, Zeta potential analysis, and transmission electron microscopy (TEM). The TEM results confirmed the spherical shape of ZnO NPs with a size range of 13.5-30.4 nm. Zeta potential analysis showed the positive charge of ZnO NPs (+16.8 mV). The ZnO NPs showed good antimicrobial activity against the tested pathogenic bacterial strains; *B. cereus* (Gram-positive bacteria) in comparison to *Escherichia coli* (Gram-negative bacteria). They also revealed a high level of antifungal activity against the tested pathogenic fungal strain; *Aspergillus niger*. Here, this cost-effective and simple biological approach for ZnO NPs synthesis has emerged with a promising capacity in biomedicine, especially in the fields of antibacterial and antifungal medicine.

Keywords: *Zinc oxide, nanoparticles, biosynthesis, characterization, antimicrobial activity.*

Introduction

Taniguchi (1994) introduced the term "nanotechnology", while Richard Feynman (1960) provided the fundamental idea of nanotechnology. Nanoparticles (NPs) are a unique class of materials that have numerous and various uses in a variety of industries such as optics, electronics, coatings, agriculture, foods, and biodiagnostics (Kołodziejczak-Radzimska & Jesionowski, 2014; El-Zahed et al., 2017; El-Dein et al., 2021). It has always piqued the curiosity of many scientists to study these characteristics. When compared to their counterparts in bulk size, NPs actually exhibit completely different characteristics (Gajjar *et al*., 2009; Baka *et al*., 2019). According to Reddy *et al*. (2007), many harmless substances start to become toxic at the nanoscale due to the change in physico-chemical characteristics of NPs including the increase in their surface area and reactivity (Adams et al., 2006; Padmavathy

& Vijayaraghavan, 2008). A higher surface area of NPs increases the possible interactions of NPs with microbial cellular biomolecules (Wahab *et al*., 2011). Metal-oxide NPs including silver oxide (AgO), zinc oxide (ZnO), titanium oxide (TiO_2), and manganese oxide (MnO) have significant antibacterial properties and are selectively hazardous to biological systems, suggesting that they may be used as antimicrobial agents, diagnostics and surgical devices (Sawai, 2003; Adams *et al*., 2006; Reddy *et al*., 2007). Among metal-oxide NPs, ZnO NPs are one of multifunctional NPs with various important characteristics, including strong catalytic behavior, physical and chemical stability, and efficient antimicrobial potential (Matei *et al*., 2008; Iqtedar *et al*., 2020).

Physical, chemical, biological, and other hybrid processes are used to synthesize the NPs. For the synthesis of monodispersed NPs, both physical and chemical approaches are particularly effective. These techniques are hazardous in one way or another because the chemicals utilised are hazardous, combustible, and difficult to dispose of in the environment (Kowshik *et al*., 2002). These techniques cause harmful compounds to become adsorbed on the surface of NPs, which could have negative effects in medicinal applications (Jain *et al*., 2010). Therefore, developing biocompatible, long-lasting, safe, non-toxic, and environmentally friendly processes for the synthesis of NPs that are found in green approaches, including biological processes (Baka & El-Zahed, 2022). Prokaryotes and eukaryotes like bacteria, fungi, and extracts from plants are used most frequently in the biosynthesis processes (Saravanan *et al*., 2021). Sabir *et al*. (2020) employed *Bacillus subtilis* to record the bacterial generation of ZnO NPs. Additionally, *B. haynesii* MG822851 and *B. cereus* MN181367 produced ZnO NPs in Rehman *et al*. (2019) and Iqtedar *et al*. (2020), respectively.

The present investigation aimed to provide a one-step and safe methodology for the biosynthesis of antimicrobial ZnO NPs against different phytopathogenic and human microbial strains using *B. subtilis* ATCC 6633.

Materials and Methods

Microbial strains

Bacterial strains: *B. subtilis* ATCC 6633, *B. cereus* ATCC 14579, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC10231 and fungal strains: *Aspergillus niger* van Tiegh, *Fusarium oxysporum* f. sp. *lycopersici* Fol4287 were obtained from the Botany and Microbiology Department, Faculty of Science, Damietta University, Egypt.

Preparation of bacterial supernatant

On nutrient agar plates, *B. subtilis* was resubcultured from its slants and incubated at 37°C. The 24-hour bacterial colonies were cultivated aerobically in nitrate broth flasks containing (g/L): peptone, 5.0; KNO_3, 1; beef extract, 3; pH 7.0±0.2. Then, flasks were incubated at 37°C for 24 hours at 150 rpm. The supernatant of *B. subtilis* was collected by centrifuging the bacterial culture for 20 minutes at 4000 rpm under sterile conditions.

Extracellular biosynthesis of ZnO NPs

The culture supernatant was mixed with an autoclaved aqueous solution of 3 mM zinc nitrate ($ZnNO_3$) (1:1 v/v). The reaction mixtures were left in a shaking incubator for five days at a temperature of 37°C and 150 rpm. Utilizing a UV-VIS spectrophotometer (Beckman DU-40) with a resolution of 1 nm, the reaction mixtures were measured against a blank flask (nitrate broth medium and $ZnNO_3$ solution) in a range of 300 to 500 nm. Triples of each experiment were done.

Categorization of ZnO NPs

The ZnO NPs X-ray diffraction (XRD) pattern was studied using the X-ray diffractometer (model LabX XRD-6000). TEM was used to analyze the ZnO NPs shape and size. Zeta analysis of ZnO NPs were studied using Malvern Zetasizer Nano-ZS90. Also, ZnO NPs were studied by Fourier Transform Infrared Spectroscopy (FTIR) to confirm the presence of bacterial capping proteins.

Antimicrobial activity of ZnO NPs

The antimicrobial activity of ZnO NPs was

studied using the agar well diffusion test at concentrations of 150 and 600 µg/ml of ZnO NPs in dimethylformamide (DMF) (CLSI, 2006). On Mueller-Hinton agar (MHA) plates, the antibacterial activity was evaluated against *B. cereus* and *S. aureus* (Gram-positive) and *E. coli* (Gram-negative bacteria). DOX agar plates were used to test the antifungal ability against *A. niger* and *F. oxysporum* as well as *C. albicans*, a pathogenic yeast, utilizing Bacto casitone agar plates. Each strain (100 µL culture) was injected separately into the flasks and then put into sterile Petri dishes at a concentration of 0.5 McFarland standard (1-2 10^8 CFU/ml). Wells (5 mm) were punched, and 100 µL of ZnO NPs colloids were added aseptically. Antibacterial and antifungal positive control standards were penicillin G and miconazole, respectively. Bacterial, yeast, and fungal agar plates were incubated at 37°C or 28°C for 24 hr and 5 days for bacteria, yeast and fungi, respectively. Inhibition zones were measured (mm).

Minimum inhibition concentration (MIC)

Minimum inhibition concentration of ZnO NPs against *S. aureus* and *C. albicans* were studied using the broth microdilution technique, in accordance with the CLSI protocols (CLSI, 2008; 2017). ZnO NPs, Penicillin G, and miconazole serial solutions (6.25–125 g/ml in water) were examined. About 0.5 McFarland standard of *S. aureus* and *C. albicans* was inoculated in Mueller-Hinton broth (MHB) and RPMI broth, respectively. *S. aureus* and *C. albicans* were incubated at 150 rpm, 35 °C and 37 °C, respectively. The microbial growth rates were measured spectrophotometrically at 600 nm to determine the MIC values.

Minimal microbicidal concentration (MBC)

MBC was defined as the dilution that fully prevented the development of microbial colonies on agar plates (Stratton *et al*., 1982). To establish the least microbicidal concentration, the tested dilutions that totally prevented the development of the tested microorganisms in the MIC assay (no visible microbial growth) were subcultured and injected into solid media. At 37°C, the agar plates were incubated for a full day.

Statistical analysis

Using the software programme SPSS version 18, the results were statistically evaluated. A one-way analysis of variance (ANOVA) was used to examine all values in the experiments at which 0.05 was chosen as the significant level.

Results

Biosynthesis and characterization of ZnO NPs

Bacillus subtilis had the ability to biosynthesize ZnO NPs within 3 days in dark conditions. The first indicator for the NPs formation was the appearance of white precipitate which increased in the production rate with the increase of the incubation period. The UV-VIS analysis displayed that the ZnO NPs had an absorption peak at 348 nm (Figure 1).

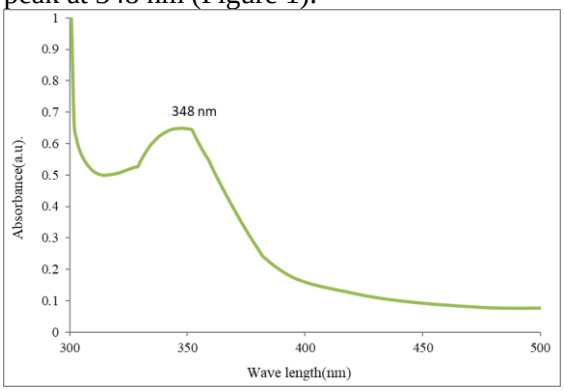

Figure 1. The UV-VIS spectra of the reaction mixture containing stabilized biogenic ZnO NPs.

The FTIR results shown in Figure 2 confirmed the presence of proteins and carbohydrates during the biogenic formation of ZnO NPs. The vibrations bands of amine have appeared at ≈3421 and 2962 cm^{-1}. The aromatic and aliphatic amines stretching vibration bands were observed at ≈1392 and 1122 cm^{-1}.

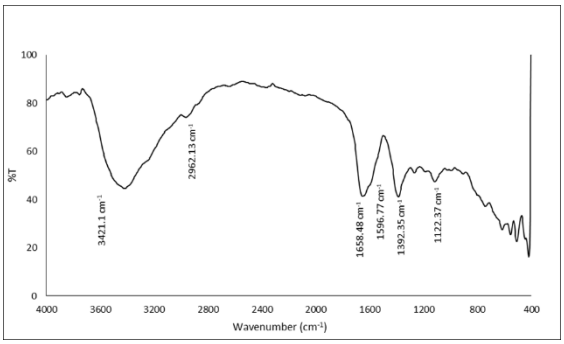

Figure 2. FTIR spectrum of ZnO NPs

The XRD pattern for ZnO NPs (Figure 3) displayed eight ZnO NPs characteristic peaks at 31.7°, 38.6°, 45.7°, 47.2°, 56°, 58°, 64.26°, and 67.4°, corresponding to respective crystal planes (100), (101), (102), (102), (110), (110), (103), and (112) (Galvez *et al*., 2021).

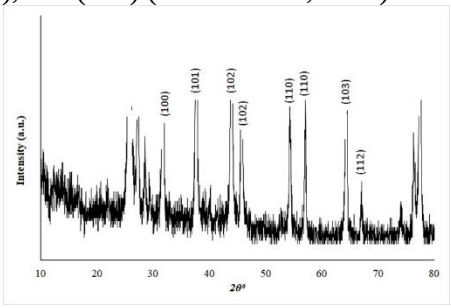

Figure 3. The XRD pattern of the produced ZnO NPs.

Figure 4 showed the TEM micrographs of ZnO NPs that appeared as well-dispersed spherical-shaped NPs with a mean size of ≈21 nm.

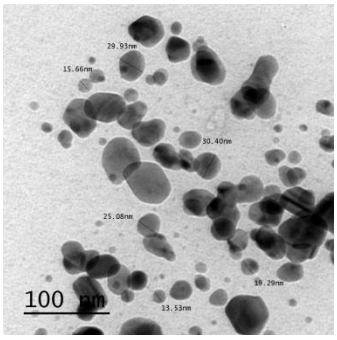

Figure 4. Transmission electron micrograph of produced ZnO NPs (scale bar = 100 nm).

The Zeta analysis confirmed the positive charge of the ZnO NPs that reach +16.8 mV (Figure 5).

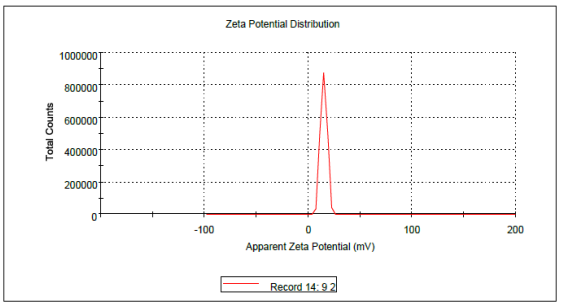

Figure 5. Zeta potential result of produced ZnO NPs.

Antimicrobial potential of ZnO NPs

Significant differences were observed between the samples treated with ZnO NPs and standard antibiotics during the antimicrobial tests (Tables 1 & 2 and Figures 6 &7), *A. niger*, *F. oxysporum* and *C. albicans* (Table 2 and Figure 7) and diameter of the inhibition zone.

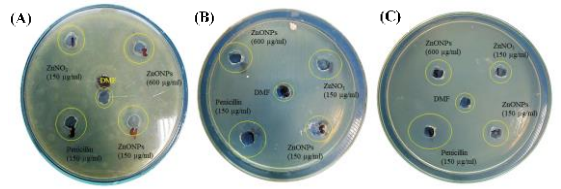

Figure 6. Antibacterial potential of ZnO NPs against pathogenic bacterial strains: (A); *B. cereus*, (B); *S. aureus* and (C); *E. coli*.

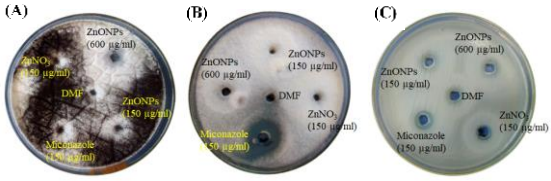

Figure 7. Antifungal potential of ZnO NPs against pathogenic fungal and yeast strains: (A); *A. niger*, (B); *F. oxysporum* and (C); *C. albicans*.

Table 1. Antibacterial potential of ZnO NPs in comparison with benzylpenicillin (Penicillin G) as a standard drug (Highly significant = **p*<0.05; *n* = 3).

Antibacterial agents	Concentration, µg/mL	Zone of inhibition (mm, mean ± SD)		
		Gram-positive bacteria		Gram-negative bacterium
		B. cereus	*S. aureus*	*E. coli*
$Zn(NO_3)_2$	150	10 ± 0.12*	13 ± 0.03*	9 ± 0.06*
ZnO NPs	150	14 ± 0.08*	11 ± 0.06*	10 ± 0.05*
	600	15 ± 0.11*	13 ± 0.14*	12 ± 0.05*
Penicillin G	150	12 ± 0.00*	16 ± 0.03*	25 ± 0*

Table 2. Antifungal potential of ZnO NPs in comparison with miconazole as a standard drug (Highly significant = **p*<0.05; *n* = 3).

Antifungal agent	Concentration, µg/mL	Zone of inhibition (mm, mean ± SD)		
		A. niger	*F. oxysporum*	*C. albicans*
$Zn(NO_3)_2$	150	9 ± 0.14*	8 ± 0.03*	8 ± 0.02*
ZnO NPs	150	23 ± 0.08*	9 ± 0.11*	10 ± 0.06*
	600	28 ± 0.03*	11 ± 0.24*	12 ± 0.08*
Miconazole	150	15 ± 0.13*	23 ± 0.16*	8 ± 0.11*

The MIC is the lowest antimicrobial agent concentration at which no detectable bacterial growth is seen. In comparison to Penicillin G, Figure 8 displayed the ZnO NPs MIC results. ZnO NPs had the greatest ability to stop *A. niger* growth. ZnO NPs had moderate activity against the Gram-positive bacterium (G+ve) *B. cereus* while it showed similar anticandidal activity to the common antibiotic miconazole against *C. albicans*. On the other hand, ZnO NPs showed lower antimicrobial activity against *E. coli*, *S. aureus*, and *F. oxysporum* than the standard antibiotics.

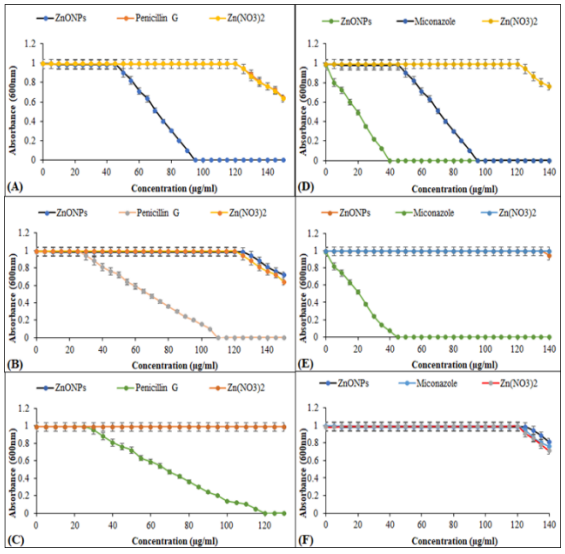

Figure 8. Minimal inhibition concentration of ZnO NPs: (A); *B. cereus*, (B); *S. aureus*, (C); *E. coli*, (D); *A. niger*, (E); *F. oxysporum* and (F); *C. albicans*.

The MBC results are shown in Figure 9. In comparison to penicillin G, ZnO NPs showed greater bactericidal activity against the G+ve than the Gram-negative bacterium (G-ve). It is obvious that ZnO NPs demonstrated strong antifungal activity against *A. niger*.

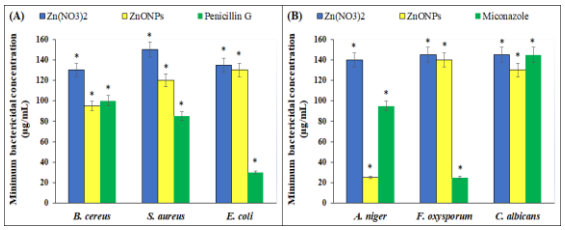

Figure 9. Minimal microbicidal concentration of ZnO NPs

Discussion

The current work used *B. subtilis* to examine the extracellular production of ZnO NPs. UV-VIS spectroscopy of biosynthesized ZnO NPs colloidal solutions revealed an adsorption peak recorded at 348 nm, which was consistent with Ahmed *et al*. (2021) results. He used *Lactobacillus plantarum* TA4 to synthesize ZnO NPs which showed an absorption peak at 351 nm. The stability of NPs is one of the most important properties For their use in medical and pharmaceutical applications. According to certain theories, The shape, size, functional bacterial groups, and surface charge of NPs play essential roles in their stability (Bian *et al*., 2011). All of these properties are easily controlled during the biosynthesis methods for NPs compared to other chemical and physical methods (Khan *et al*., 2019). *B. subtilis* biosynthesized ZnO NPs with Zeta Potential (mV): +16.8. This zeta potential value usually has a good degree of stability (Singh *et al*., 2021). The XRD and FTIR results confirmed the formation of ZnO NPs with good stability and crystallin nature. Mahdi *et al*. (2021) biosynthesized ZnO NPs using *Bacillus* sp. PTCC1538 and *L. lactis* showed identical XRD results to the current study confirming the successful formation of ZnO NPs. Jayaseelan *et al*. (2012) FTIR results were matched with the obtained FTIR spectrum which showed the presence of several functional groups including proteins and carbohydrates that Might be responsible for the long-term stability of ZnO NPs.

As a result, TEM examination allowed for the observation of the size, shape, and surface morphology of ZnO NPs. The TEM results revealed that ZnO NPs have spherical forms and a narrow size range that matched Mashrai *et al*. (2017) study which used *C. albicans* to biosynthesize spherical shaped ZnO NPs (20 nm), which supported the idea of using them in several applications.

The biosynthesized ZnO NPs showed good antimicrobial activity against the tested microorganisms. Hamk *et al*. (2022) documented the antimicrobial activity of *B. subtilis* ZBP4 ZnO NPs against different food - pathogenic G+ve and G-ve bacteria. Also, Rajabairavi *et al*. (2017) biosynthesized ZnO NPs using *Sphingobacterium thalpophilum* and confirmed their antibacterial action against *Enterobacter aerogens* and *Pseudomonas aeruginosa*. In comparison to the current results, Mohd-Yusof (2020) reported bacterial inhibition of *L. plantarum* ZnO NPs at concentrations of 2500 µg/ml for *E. coli* and 1250 µg/ml for *S. aureus*. On the other hand,

Pillai *et al*. (2020) study documented the antifungal potential of ZnO NPs against *C. albicans* and *A. niger* fungal stains. Yassin *et al*. (2022) demonstrated that the ZnO NPs produced strong antifungal potential against different strains of *Candida* sp. including *C. tropicalis*, *C. glabrata* and *C. albicans*. Also, it reported ZnO NPs MIC values reach to 10 µg/ml, and MBC values reach to 20 µg/ml against the tested strains.

From the previous results, the biosynthesized ZnO NPs using *B. subtilis* had a superior antibacterial action against G+ve compared to G-ve bacteria which might be due to the difference in their cell wall structure. G+ve bacteria contain a thick layer of peptidoglycan which has an acidic nature and strongly binds with the positively charged ZnO NPs in contrast to G-ve bacteria that have thin layers and low amounts of peptidoglycan (Sirelkhatim *et al*., 2015; El-Zahed *et al*., 2022). Moreover, G-ve bacteria have an outer lipoid layer that may make them resistant to the NPs in contrast to G+ve bacteria. The biosynthesized ZnO NPs could be utilized as pharmaceutical drugs with high antimicrobial efficiency.

Conclusion

The biosynthesis of ZnO NPs is well known to be significantly safer and more eco-friendly than chemical or physical methods. In response to this assumption, in the present study, we reported the potential antibacterial activity of biogenic ZnO NPs using *B. subtilis*. The spherical shapes of ZnO NPs with sizes ranging from 13.5 to 30 nm were recorded using TEM micrographs while the stabilization of ZnO NPs by coating proteins in the bacterial supernatant was confirmed by FTIR spectrum. The biogenic ZnO NPs revealed good antimicrobial potential against *B. cereus* (Gram-positive bacterium) and the tested pathogenic fungal strain; *A. niger*.

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الملخص العربي

عنوان البحث: التخليق الحيوي الخارجي والنشاط المضاد للميكروبات لجسيمات أكسيد الزنك النانومترية المصنعة بواسطة باسيلس ساتلس ATCC 6633

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في الآونة الأخيرة، تم استخدام جسيمات أكسيد الزنك النانومترية في العديد من التطبيقات البيولوجية والصناعية نظرًا إلى قلة سميتها، كفاءتها العالية، توافقها الحيوي، الفعالية القوية وقلة التكلفة. استخدمت الدراسة الحالية عزلة بكتيرية عالمية في التخليق الحيوي لجسيمات أكسيد الزنك النانومترية ذات القدرة المضادة للميكروبات. تم توصيف جسيمات أكسيد الزنك النانومترية باستخدام التحليل الطيفي للأشعة فوق البنفسجية المرئية، طيف الأشعة تحت الحمراء، التحليل الطيفي للأشعة السينية، تحليل زيتا وتحليل الميكروسكوب الالكتروني النافذ. أظهرت جسيمات أكسيد الزنك النانومترية نشاطًا جيدًا كمضاد للميكروبات ضد السلالات البكتيرية الممرضة: باسيلس سيريس (بكتيريا موجبة لجرام) بالمقارنة مع ايشيريشيا كولاي (بكتيريا سالبة لجرام) وكذلك نشاطا كبيرا ضد السلالة الفطرية المسببة للأمراض: أسبرجيلس نيجر. من هنا، أظهر هذا النهج البيولوجي البسيط، قليل التكلفة والفعال لتخليق جسيمات أكسيد الزنك النانومترية قدرة واعدة في مجال الطب، وخاصة في مجالات مضادات البكتيريا ومضادات الفطريات.