Asia-Pacific Journal of Science and Technology: Volume: 29. Issue: 03. Article ID.: APST-29-03-06. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by the Research and Graduate Studies,
Khon Kaen University, Thailand

Synthesis, characterization and antimicrobial activity of zinc oxide nanoparticles against *Escherichia coli* and *Salmonella enterica*-water borne pathogens

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Received 7 February 2023
Revised 31 March 2023
Accepted 10 May 2023

Abstract

Waterborne pathogens *viz*. *Escherichia coli* (*E. coli*) and *Salmonella enterica* (*S. enterica*) and their associated diseases are key public health threat worldwide, causing significant morbidity and mortality thereby responsible for high public health expenditure and consequent economic burden. Therefore, the research aimed to explore the facile synthesis of zinc oxide nanoparticles (ZnO NPs) using $(CH_3COO)_2Zn.2H_2O$ as precursor and further evaluation of their antimicrobial activity. Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), X-ray diffraction (XRD), Energy Dispersive X-Ray Analysis (EDX), particle size analyser (PSA) and thermogravimetric analysis (TGA) have been used to characterize the synthesized NPs. The binding of Zn by Zn-O stretching was validated by FTIR spectrum whereas the identity of the crystalline ZnO wurtzite-type material was established by XRD. SEM imaging revealed the sphere/petal shaped agglomerated particles. Furthermore, average particle size of NPs was 702.9 nm, measured by PSA. Wall zeta potential value of synthesized particles was -16.44 reflecting the agglomerating nature of the material. TGA analysis showed that the material was highly thermostable and 88% remained stable at 760°C. The qualitative well diffusion test conducted for evaluating the antimicrobial activity of synthesized material resulted in noticeable inhibitory activity against *E. coli* (20±0.2) and *S. enterica* (18±0.1). The sensitivity exhibited by both the test microbes was high @15µg of ZnO NPs whereas at lower concentrations no sensitivity was reported. Thus, the synthesized ZnO NPs played a significant role in antimicrobial activity and could be an alternative antibacterial agent in the treatment of waterborne infections.

Keywords: Antimicrobial, Nanoparticles, Pathogen, ZnO NPs

1. Introduction

Nanomaterials exhibit various improved features due to extensively large surface area. Zinc oxide (ZnO) is indeed a fascinating nanomaterial in this regard due of its wide application in coatings, sunscreens, and paints for absorbing the UV (Ultraviolet) light and plays a significant role in a variety of industries e.g., pharmaceuticals, rubber, and food. In textiles, the ZnO is incorporated as antimicrobial [1], cosmetics [2], surfaces coatings [3], and cellulose fibers [4] for inhibiting the microbial growth. ZnO is a vital ingredient and has received increased research focus recently as an antibacterial agent. ZnO has drawn attention because it displays high activity even at low concentrations, in contrast to other inorganic oxides like silver nanoparticles (AgNPs) and titanium dioxide (TiO2). ZnO is endorsed as a reliable antibacterial agent because of its stability under demanding processing

conditions and profile as a material that is safe for both animals and humans [5,6]. Compared with the other organic and inorganic materials, ZnO has higher selectivity, durability, and heat resistance [7]. Likewise, ZnO displays pretty good antibacterial activity at the nanoscale, and more stability at high pressure and temperature. ZnO nanoparticles (ZnO NPs) that can be synthesized at small costs are non-toxic, but the toxicity extent varies upon time of exposure and its agglomeration and dissolution [8]. ZnO NPs are used as a packaging material in food products which may control the occurrence of microbes in street vended food items highly prone for microbial attack [9-11].

Water is a vital resource for all living beings but because of population growth and industrialization, the water quality has become a serious concern. For improving the quality of water, traditional ways such as ozonation, chlorination, and ultraviolet therapy are commonly used. These methods have proven to be ineffective and give rise to formation of carcinogenic disinfection by-products (DBP) when used in higher disinfectant doses. Higher levels of DBP eventually arise since some water-borne bacteria have elevated their resistance to current disinfectants [12].

Similarly, UV and ozone treatment provides little protection against reinfection in the distribution network because they do not leave any deposit in the treated water [13]. As a result, it is necessary to examine new tactics to increase disinfection effectiveness. The primary problem to solve is to develop an antibacterial agent that can successfully combat water pathogens. With the given limitations of traditional disinfection techniques, nanotechnology provides novel methods of water treatment giving no formation of DBP. Milionis et al. [14] in their study observed the effective bactericidal property of ZnO nanostructured surfaces against *E. coli* to tackle with bacterial contamination of surfaces and drinking water. The efficiency of nanostructured ZnO synthesized using the precipitation method for water contaminated with *E. coli* was examined by the other group of researchers. This method was assisted with 355 nm pulsed laser irradiation [15]. Elmi et al. [16] observed the antibacterial activity of ZnO NPs against different bacterial species *viz*. *K. pneumoniae*, *E. coli, S. epidermidis, Proteus* in municipal wastewater sample. In their study they obtained significant antimicrobial activity of ZnO NPs synthesized by the sol-gel method compared to mechano-chemical method.

This research article aims to synthesize ZnO NPs using facile method and thereby evaluate their sensitivity towards water borne pathogens *viz. E. coli* and *S. enterica.* The mentioned work presents a simple, economical and less time consuming synthesis method for ZnO NPs compared to methods available. Since the sensitivity of ZnO NPs against the *E. coli* and *S. enterica* are less explored therefore the present work enlightens the behaviour of less studied microorganism towards ZnO NPs and the study may provide the evidence in support of use of ZnO NPs in reducing the microbial load.

2. Materials and methods

All the chemicals and reagents utilized were of analytical grade and procured from Hi media, SRL and Sigma Aldrich India. ZnO NPs have been synthesized employing the method as adopted by Beek et al. [17] with a little modification. Briefly in this method 3.35mmole $(CH_3COO)_2Zn.2H_2O$ used a precursor, was dissolved in 32 mL CH_3OH, and mixed well using magnetic stirrer at 60°C. Then 6.60 mmole NaOH solution (prepared in CH_3OH) was added dropwise to $(CH_3COO)_2Zn.2H_2O$ solution maintaining the temperature of magnetic stirrer at 58°C with vigorous stirring. The solution became turbid after complete addition of NaOH. The obtained solution was stirred for further three hours without any heating. The precipitate was then allowed to settle and washed three times with CH_3OH before the sample was dried at 80°C to produce powdered ZnO NPs.

Synthesized powdered material was characterized by using advanced characterization techniques including Fourier transform infrared (FTIR), PerkinElmer model-Spectrum Two; X-ray diffraction (XRD), Panalytical X'pert powder Netherlands XRD; scanning electron microscopy (SEM), Zeiss Evo; Energy Dispersive X-Ray Analysis (EDX) EDAX AMETEK USA; particle size analyser (PSA) model Zetasizer lab of Malven, UK and thermogravimetric analysis (TGA) PerkinElmer's thermogravimetric analyzer TGA 4000.

The antimicrobial susceptibility test of synthesized ZnO NPs was performed following the modified cup-plate agar diffusion method as discussed by Bennet *et al* [18]. A sterile swab was used to apply an inoculum containing 10^6 CFU/mL each of *E. coli* (ATCC 25922) and *S. enterica* (ATCC 13312) to a petri dish (90mm) containing Mueller-Hinton agar. The plates were then dried. A sterile cork borer (6.5mm) was used to create the cups in the agar plate. Using a micro-pipette, 30 µl of the ZnO nano material preparation was then added to the wells (well numbers 3, 5, and 2, 4) and left to diffuse at room temperature. In control well (no.1 for both the pathogens) 30µl of deionized water was added. The plates have been incubated for 18 hours at 37°C while standing erect. Following incubation, the zone of inhibition around each well was measured in millimetres (mm), averaged, and the mean values were noted. A duplicate of the antibacterial activity experiment was run.

3. Results and discussion

The FTIR analysis helped in identifying the surface functional groups that reveals a useful information regarding surface specification. A characteristics peak corresponding to the existence of O-H stretching obtained at 3,417 cm^{-1}. The peak found at 1,551.42 cm^{-1} and 1,339 cm^{-1} was due to the asymmetrical and symmetrical stretching respectively of the zinc carboxylate anion. Peak at 1047 cm^{-1} illustrates lattice vibration of CO_3^{-2} generated absorption peak. FTIR analysis reveals presence of C-Zn, Zn-O *etc*. which are present on the surface of powdered ZnO NPs (Figure 1.). Besides all these information regarding functional groups of synthesized ZnO NPs, peaks at 1,402 cm^{-1} and

1,340 cm^{-1} indicating the existence of $(CH_3COO)_2Zn.2H_2O$ associated with methyl ($-CH_3$) bending modes corresponding to the previous results [19].

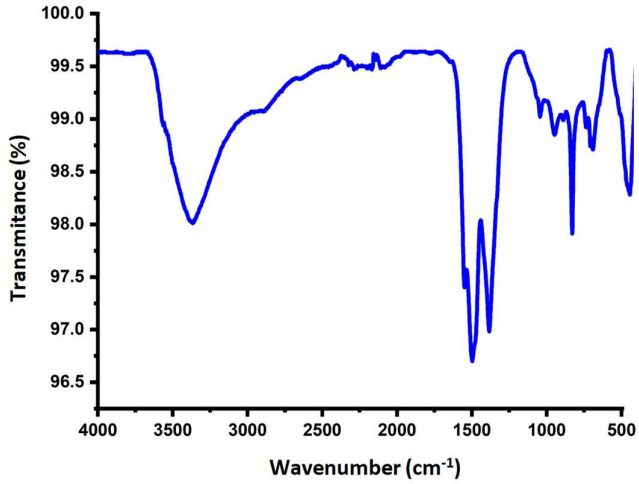

Figure 1 FTIR spectrum of ZnO.

The crystalline nature of ZnO NPs is illustrated by XRD pattern (Figure 2). The specific XRD peaks are assigned at 2 theta degree ($2\Theta^0$) = 31.7^0, 34.5^0, 36.1^0, 47.4^0, 56.5^0, 62.7^0, and 68.1^0. These peaks are related to (100), (002), (101), (102), (110), (103), and (201) planes in agreement with JPCDS card no. 36-1451 [20]. XRD pattern reveals the wurtzite crystal structure of ZnO NPs and agree with the results of Klink et al. [21]. Absence of any other additional peak within resolution limit of XRD confirms formation of pure, monophasic and petal shaped ZnO NPs.

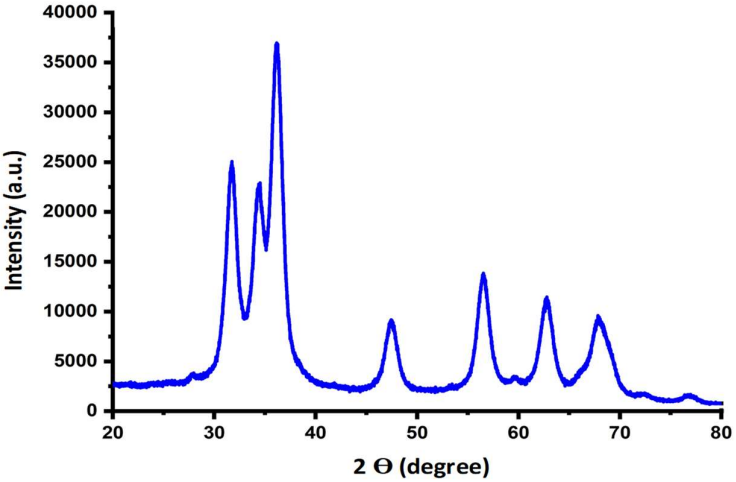

Figure 2 XRD pattern of ZnO.

The morphology of synthesized ZnO NPs was analysed through SEM analysis which is depicted in Figure 3. Obtained SEM images confirmed presence of clusters of ZnO NPs. These Clusters appeared to be petal shaped with heterogeneity and indicated agglomeration of particles.

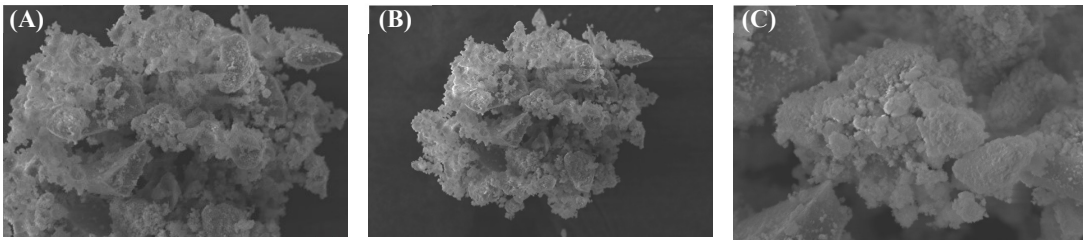

Figure 3 The SEM images of ZnONPs: (A) 10 µm, (B) 20 µm and (C) 3 µm.

The purity composition and relative percentage of elements in synthesized NPs were identified through EDX spectrum as shown in Figure 4 and Table 1. The EDX pattern showed the relative wt% of O (21.6) and Zn (78.4). The presence of two elements Zn and O verified the purity of ZnO NPs. Similar results were reported by Brintha and Ajitha and mentioned 26.1% and 73.9% for wt% of O and Zn respectively [22]. The theoretical expected mass % of O and Zn were 19.7% and 80.3% respectively [23].

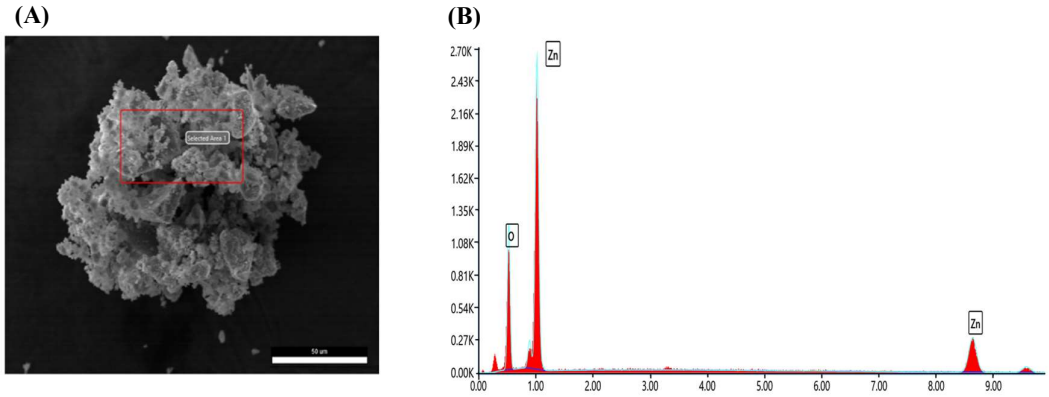

Figure 4 (A) Surface morphology and (B) EDX spectrum of ZnO NPs.

Table 1 EDX composition of ZnO.

Element	% wt	% Atomic wt
O, K	21.6	37
Zn, K	78.4	63

Thermogravimetric analysis (TGA), known to be a reliable technique in determining thermal behaviour of the substance, weight loss with temperature and, thermal degradation was conducted between 100 - 800°C under N_2 atmosphere at the flow rate value of 20 mL/min and a temperature ramp having value 10°C /min [24]. This showed major weight loss between 200 to 300°C because of removal of oxygen associated functional groups in the ZnO (Figure 5). The TGA data reveals only 12%, weight loss at 300°C temperature whereas 88% material remains stable between temperature range 300 - 700°C showing high thermostability of the synthesized material which promisingly agrees with previous report [25].

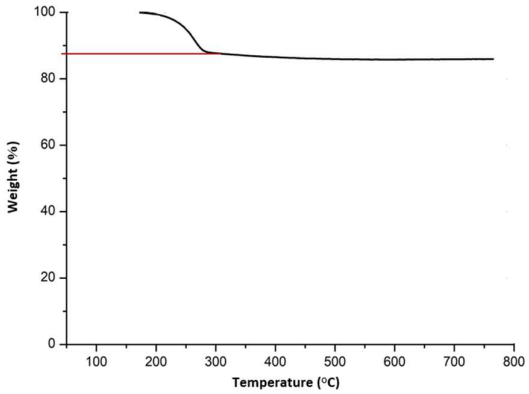

Figure 5 TGA profile of ZnO.

Particle size distribution of prepared ZnO NPs have been analyzed and Z average was obtained to be 702.9 nm and about 88 % particles lie within the range which is reflected by a single sharp peak in particle size distribution. Poly dispersity index was 1 which reveals polydisperse particle size distribution. Wall zeta potential value was -16.44 mV which showed that the prepared suspension is moderately stable and after some time the particle tends to form aggregate and settle down at the bottom (Figure 6 A, B). Since the synthesis method did not involve any additives or capping agent, the obtained particle size of ZnO NPs were found to have value of 702.9 nm which verifies the results of ZnO NPs synthesis using solvothermal synthetic method without any additive [26].

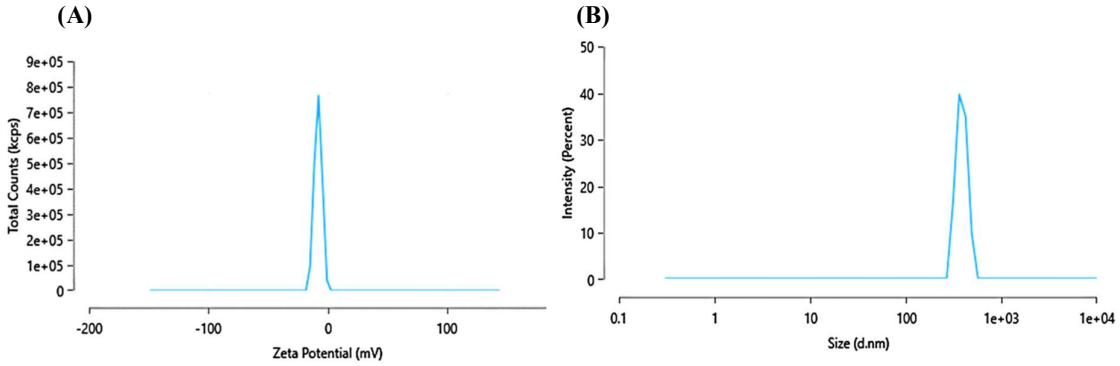

Figure 6 (A) Zeta potential of ZnO NPs and (B) Size distribution of ZnO NPs.

Furthermore, a negative zeta potential corresponding to agglomerating nature of the synthesized material in aqueous medium which may give rise to the bactericidal property of ZnO [27].

The Susceptibility of synthesized NPs against two water borne pathogens *viz*. *E. coli* and *S. enterica* was evaluated by cup-plate agar diffusion method (Figure 7 and Table 2) using two different concentrations.

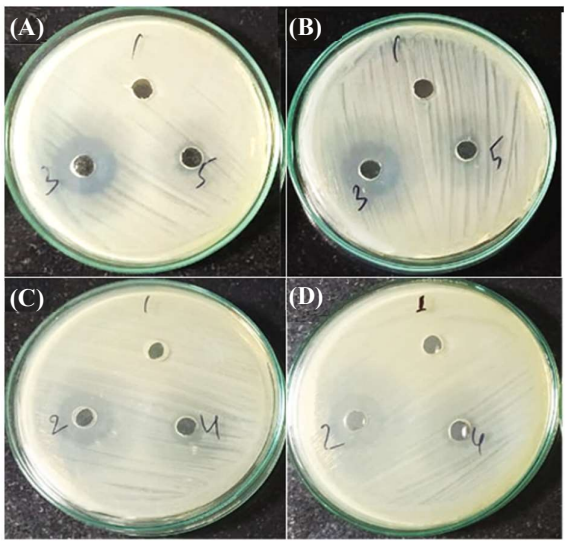

Figure 7 Zone of inhibition on MH agar plate exhibited by *E. coli* (A), (B): 1 Control, 3 and 5 sample loaded and *S. enterica* (C), (D): 1 control, 2,4 sample loaded.

Table 2 Mean zone of inhibition.

Microorganisms	Zone of Inhibition (mm) @ 500 µg/mL (well no. 3 and 2)	Zone of Inhibition (mm) @ 250 µg/mL (well no. 5 and 4)
E. coli	20±0.2	Diffused
S. enterica	18±0.1	Diffused

The obtained results reflected higher zone of inhibition *viz.* 20±0.2 mm and 18±0.1mm for *E. coli* and *S. enterica* respectively at 15 µg whereas at 7.5 µg, diffused zone of inhibition indicated less susceptibility or no

profound activity for antibacterial sensitivity of synthesized NPs. ZnO NPs showed effective susceptibility towards selected waterborne pathogens because of their large surface area and agglomerating nature which intensified the interaction of NPs with cell wall of gram-negative bacteria *viz. E. coli* and *S. enterica* and resulted in growth inhibition at higher concentration.

At higher concentration, ZnO NPs might loss the cell membrane integrity of bacteria which thereby induced oxidative stress leading to cell death [28]. Previous research has shown that aquatic ZnO NP suspensions induce high levels of reactive oxygen species (ROS), which are also thought to be the primary source of nanotoxicity [29-32]. Basically three probable hypothetical mechanisms reported [33] for antibacterial activity are (i) the internalisation and transport of metal ions into the bacterial cells, which results in reduction of intracellular ATP synthesis and interruption of DNA replication [34]; (ii) metal oxides NPs and ions lead to oxidative stress, which causes oxidative damage to biological components [35], and (iii) deviations in bacterium membrane permeability that cause a gradual release of lipopolysaccharides, other internal components, and membrane proteins as well as the decrease in proton motive force as a consequence of NP accumulation and disintegration of membrane [36]. There is a strong trend that, in addition to the aforementioned hypothetical mechanisms, consider two additional mechanisms as being particularly important in interaction of NPs with the bacteria [37]: (a) generation of excessive amount of ROS, primarily hydroxyl radicals (HO•) and singlet oxygen ($^1O_2$) [38-41], and (b) When NPs precipitate on the surface of bacteria or aggregate in the cytoplasm or periplasm, they interfere with cellular processes and cause membrane disruption and disorder [28,38]. Vidovic et al. [42] reported a considerable antimicrobial effect of ZnO NPs against *S. enterica* compared to MgO and CaO NPs. Das et al. [43] also reported the disinfection of multidrug resistant *E. coli* by the use of Fe-doped ZnO NPs. The inhibition zone obtained for *E. coli* against ZnO NPs synthesized using sol gel method was 8, 10 and 12 mm at much higher concentration than our results [16]. A latest study discussed by Michael et al. [21] found inhibition zone of 10 mm, exhibited by both *E. coli* and *S. enterica* against least agglomerating urea based synthesized ZnO NPs.

4. Conclusion

Susceptibility of selected water borne pathogens to ZnO NPs, synthesized using facile and economical method, was investigated. The investigation revealed that NPs had a pronounced inhibitory impact on both chosen pathogenic gram-negative bacterial species *viz*. *E. coli* and *S. enterica* of public health concern. One of the key findings of the current work is the effectiveness of the crystalline monophasic wurtzite petal-shaped aggregated NPs with negative zeta potential value, in restricting bacterial growth. The study may also help to elucidate the interaction of agglomerated NPs with bacteria at molecular level. The synthesized ZnO NPs had a considerable inhibitory effect on the growth of studied water borne pathogens, and hence they may serve as an alternative antibacterial agent in the treatment of contaminated water. This might be a simple and economical method to improve water safety which may play a significant role in boosting both public health and the environment.

5. Acknowledgements

The authors are grateful to the GEHU Bhimtal Campus for providing all the necessary facilities for this study.

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