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EXTRACELLULAR SYNTHESIS OF ZINC OXIDE NANOPARTICLES USING *ACINETOBACTER SCHINDLERI* SIZ7 AND ITS ANTIMICROBIAL PROPERTY AGAINST FOODBORNE PATHOGENS

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ABSTRACT

The present study focuses on the microbial synthesis of zinc oxide nanoparticles (ZnO NPs) and evaluating the antimicrobial property on foodborne pathogens. The bacterial strain, *Acinetobacter schindleri* SIZ7 was isolated from the waste filling area of Sivakasi, Tamil Nadu, India. The biogenic synthesis of ZnO NPs was carried out at room temperature and under suitable, eco-friendly environment using culture supernatant of *A. schindleri*. The physico-chemical properties exhibited by the biogenic ZnO NPs were characterised using UV-Visible Spectrophotometry, Energy dispersive X-ray spectroscopy (EDS), High Resolution Transmission Electron Microscopy (HRTEM), Fourier Transformed Infrared spectroscopy (FTIR) and Thermogravitometric Analysis (TGA). The synthesized ZnO NPs are polydispersed and spherical in shape. The antimicrobial activity of ZnO NPs was investigated against foodborne pathogens, *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844). The prepared ZnO NPs exhibited strong antimicrobial activity against *E. coli* and *S. enterica* with a minimum inhibitory concentration of 100µg ml^{-1}. Thus, the bacterial strain *Acinetobacter schindleri* SIZ7 could be used for simple, extracellular, non-hazardous and efficient synthesis of antimicrobial ZnO NPs.

Keywords: Metallic Nanoparticle, *Acinetobacter schindleri*, Antimicrobial compounds, 16s rRNA gene, HR-TEM, FTIR, Foodborne pathogens

INTRODUCTION

Metallic nanoparticles are receiving considerable attention in agriculture and medicine due to their unique physical and chemical properties. Nanoparticles (NPs) are used in the fields of drug delivery, imaging, diagnosis, development of antimicrobial compounds and anticorrosive medical devices (**Fayaz *et al.*, 2010 and Martinez-Gutierrez, *et al.*, 2012**). Metallic NPs synthesis through bacteria, yeast, fungi, plant biomass, live plants, and plant extracts offer several advantages than chemical and physical route of synthesis (**Castro-Longoria *et al.*, 2011**). Even though different biotechnological methods and various biological agents can able to synthesize the metallic nanoparticles, bacteria possess distinctive advantage over others, because of their high generation time and can be easily grown in the laboratory. Among the metallic nanoparticles, researchers reported the synthesized silver and gold nanoparticles using bacteria. The culture supernatants of bacteria like *Acetobacter xylinum*, *Aeromonas* sp., *Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Lactobacillus acidophilus*, *Rhodobacter capsulatus*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are reported for the synthesis of silver and gold nanoparticles (**Shivaji *et al.*, 2011 and Narayanan *et al.*, 2010**).

Zinc oxide nanoparticles (ZnO NPs) also have gained much interest owing to their wide applications in the preparation of personal care products, coatings and catalysts in environmental remediation, as antifungal and antibacterial agents (**Kirthi *et al.*, 2011**). **Jayaseelan *et al.*, (2012)** reported the bacterial mediated synthesis of ZnO NPs using *Aeromonas hydrophila* and its antimicrobial activity was established. Various researchers reported the antibacterial and antifungal properties of ZnO NPs (**Tayel *et al.*, 2011 and Xie *et al.*, 2011**). In the scientific literature very few reports are available on extracellular synthesis of ZnO NPs using bacteria. Our aim in the present study was to synthesize ZnO NPs using *Acinetobacter schindleri* SIZ7 and to investigate their antibacterial activity against foodborne pathogens.

MATERIAL AND METHODS

Microorganisms

Pure cultures of bacteria were isolated from soil samples, collected from Sivakasi, Tamilnadu, India by serial dilution technique. The bacteria were screened for the ability to synthesize ZnO nanoparticles. The bacterial culture, SIZ7 showed rapid synthesis and was maintained on nutrient agar plates. Morphology of the bacteria was observed under scanning electron microscope (SEM). The bacterial isolate was further identified by 16S rRNA gene amplification and sequencing. The sequence was amplified using 27F forward (AGA GTT TGA TCM TGG CTC AG) and 1492R (TAC GGY TAC CTT GTT ACG ACT T) reverse primers. Phylogenetic tree was constructed using Mega 5.05 software (**Kumar *et al.*, 2008**). Sequence of SIZ7 was submitted to the NCBI sequence database under the accession number KR135410.1.

Synthesis of ZnO NPs

The conical flasks (1 litre) containing 500ml of nutrient broth were inoculated with the bacteria SIZ7 and incubated at 37°C for 2 days. The bacterial culture was centrifuged at 1,000 rpm for 10 min and the cell free supernatant was collected. The supernatant was used with the metal ion solution for bioreduction of metals. Typically 100 ml of bacterial supernatant was brought into contact with 5 mM of zinc nitrate solution. The flasks were agitated for 30 min and kept on an orbital shaker at 37°C for 48 h.

Characterization of ZnO NPs

The reaction mixture was observed for visual colour change at different time intervals (4, 8, 12, 16, 24, 32, and 48 h). Subsequently the reaction mixture was monitored by UV–Vis spectroscopy (200-800 nm) as a function of time of reaction. The reaction mixture was subjected to centrifugation at 6,000 rpm and the resultant pellet was collected. The pellet obtained was washed with deionized water for 3 times and the obtained precipitate was dried in a hot air oven at 60°C for 6 h. Elemental composition of the NPs was observed by Energy dispersive X-ray spectroscopy (EDS). The size and shape of synthesized ZnO NPs was

determined by using high resolution transmission electron microscopy (JEOL 3010 TEM-HR) equipped with Gatan digital camera, operated at accelerating voltage of 200kV. The capping agent responsible for the stability of nanoparticles and the functional groups associated with NPs was studied by FTIR spectroscopy. FTIR spectra of the samples were measured on Thermo Scientific Nicolet iN-10, spectrophotometer at the resolution of 4 cm^{-1} in the range of 4000–500 cm^{-1} in KBr pellets. The thermal stability and degradation pattern of NPs was determined by Thermogravitometric analysis (TGA) using Thermogravitometric analyzer (Instrument SDT Q600 V20.9 Build 20 Module DSC-TGA Standard).

Antibacterial activity

The antimicrobial activity of ZnO NPs was tested against the common foodborne pathogenic bacteria, *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844) procured from Microbial type culture collection (MTCC), IMTECH, Chandigarh. Antimicrobial activity was carried by well diffusion method according to **Jaidev and Narasimha (2010)**. Briefly, 24 h active bacterial culture was seeded into nutrient agar medium. A well of 5 mm was made in the centre of the plate and filled with 150 µg ml^{-1} of ZnO NPS. The plates were incubated at 37°C for 24 h. The zone of inhibition was measured. Minimum inhibitory concentration (MIC) of ZnO NPs against the bacterial strains was determined according to **Behera *et al*., (2010)**. Briefly, test tubes containing 5 ml of Mueller Hinton broth was inoculated with 5 X 10^{-7} CFU ml^{-1} of bacterial culture and various concentrations of ZnO NPs (200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 µg ml^{-1}). The tubes were incubated at 37°C for 24 h with 180 rpm and observed for bacterial growth. Optical density was measured for the same at 600 nm using UV-Vis Spectrophotometer. All the experiments were performed in triplicate.

RESULTS

Synthesis and characterization of ZnO NPs

The bacterial isolate SIZ7 showed 99% sequence similarity with *Acinetobacter schindleri* hence named as *Acinetobacter schindleri* SIZ7 (NCBI GenBank accession no: KR135410.1) (Fig. 1). The analysis of SEM photographs revealed that *A. schindleri* SIZ7 was rod shaped bacteria (Fig. 2). Extracellular biosynthesis of ZnO NPs was observed by visual colour change, pale yellow to fluorescent yellow after 48 h of incubation (Fig. 3). UV–Visible spectra of the reaction mixture revealed a characteristic peak between 300 and 360 nm with a maximum at 310 nm, which is due to the surface plasmon resonance of ZnO NPs (Fig. 3) and the result is in good agreement with previous reports (**Bai *et al*., 2011**). Energy dispersive X-ray spectroscopy (EDS) analysis confirmed the presence of zinc and oxygen (Fig. 4). HRTEM image analysis revealed that ZnO NPs were polydispersed and spherical in shape (Fig. 5). The HRTEM micrograph suggested that particle diameter ranged from 20 to 100 nm.

FTIR spectrum analysis of ZnO NPs showed intense absorption bands at 3293, 2926, 2863, 1732, 1660, 1394 and 1058cm^{-1} (Fig. 6). The absorption band observed at 1058cm^{-1} represented the C–O–H bending vibrations. The absorption band observed at wave number 1660cm^{-1} was identified as amide I band. The absorption bands observed at 1394, 2863 and 2926cm^{-1} represents the presence of $–CH_3$ group. The absorption band at 1732cm^{-1} is characteristic of the CO-H stretching. The band around 3293cm^{-1} attributes to the $–CONH_2$ group. FT-IR spectral analysis revealed the biological components of the culture supernatant associated with the formation of ZnO NPs. Thermogravitometric analysis (TGA) measures the weight loss of ZnO NPs as a function of temperature under a controlled atmosphere (Fig. 7). The synthesized nanoparticles showed good thermal stability. Weight loss at higher temperatures 200°C and 400°C was 20.31% and 60.43 % respectively. Water soluble organic compounds secreted extracellularly by bacteria may act both as reducing and stabilizing agents in the green synthesis of ZnO NPs.

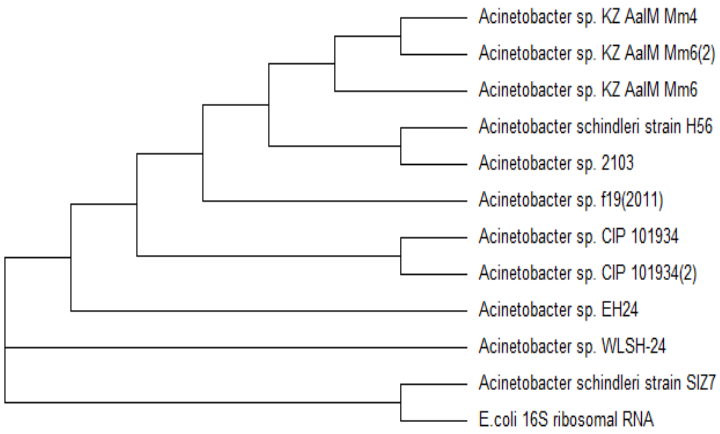

Figure 1 Phylogenetic tree showing genetic relationship between the isolate *A. schindleri SIZ7* and other closely related reference bacteria.

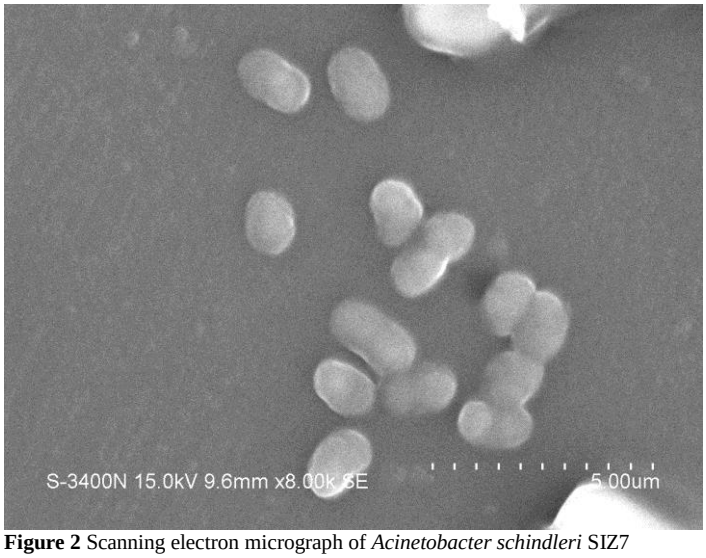

Figure 2 Scanning electron micrograph of *Acinetobacter schindleri* SIZ7

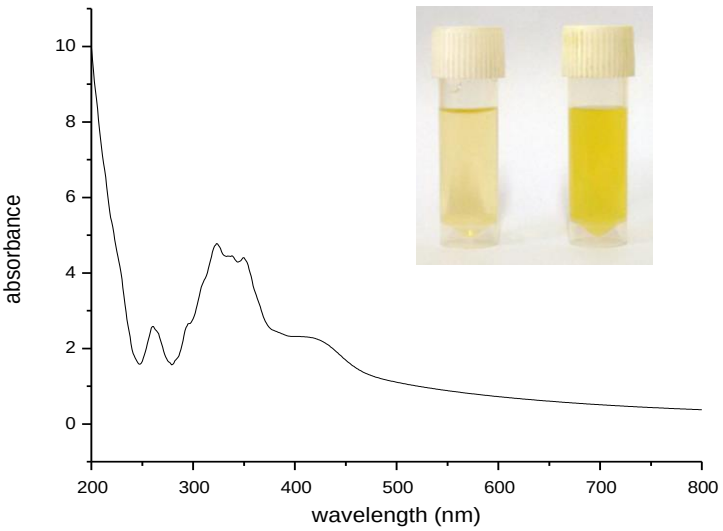

Figure 3 UV–visible spectra of bacterial filtrate as a function of time.. The peak at 310 nm corresponds to the surface plasmon resonance of

ZnO NPs. Inset shows the corresponding colour change.

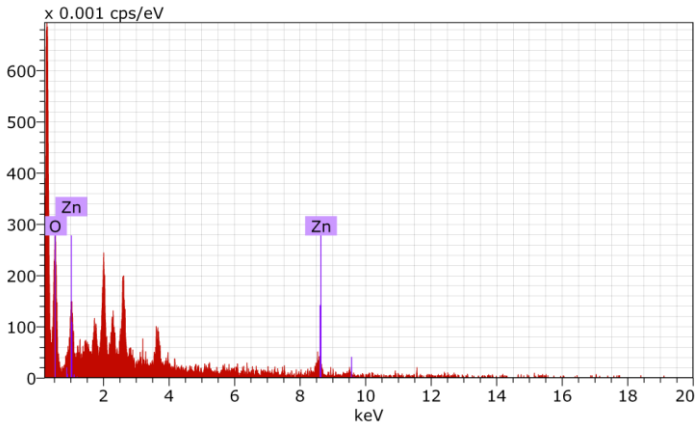

Figure 4 Energy dispersive X-ray (EDS) spectrum of ZnO NPs.

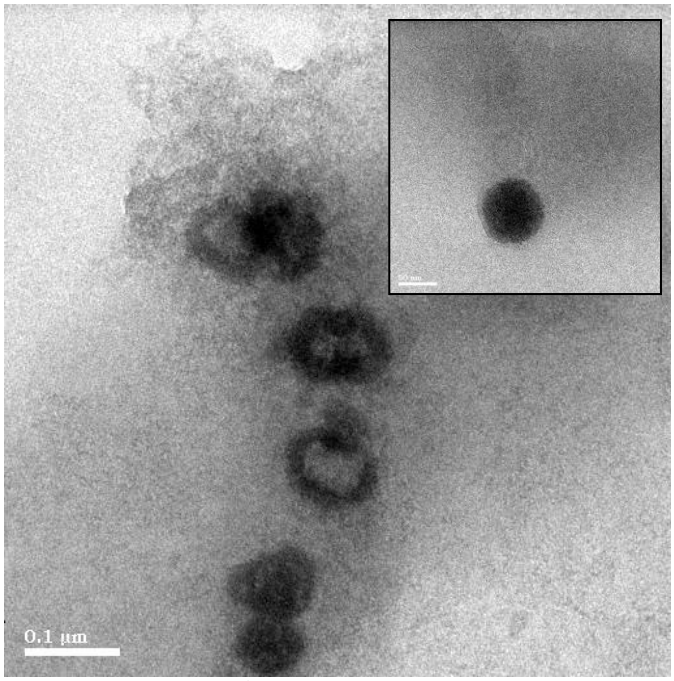

Figure 5 HR-TEM image of ZnO NPs produced by *A. schindleri* SIZ7

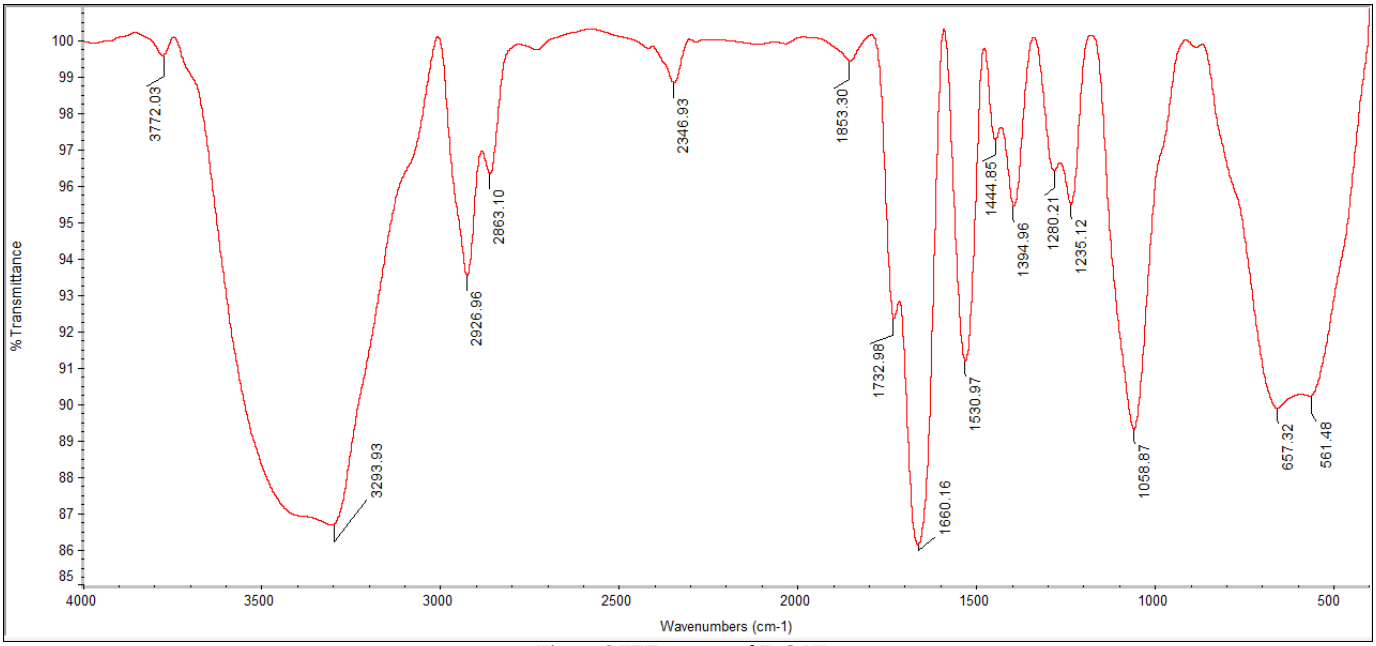

Figure 6 FTIR spectra of ZnO NPs

Antibacterial activity

The ZnO NPs exhibited the antibacterial activity against the common foodborne bacteria tested. At a concentration of 150µg ml^{-1} the zone of inhibition diameters were 16, 18, 16 and 20 mm against *Staphylococcus aureus* (MTCC 96), *Escherichia coli* (MTCC 739), *Vibrio parahaemolyticus* (MTCC 451) and *Salmonella enterica* (MTCC 9844) respectively. The MIC values of ZnO NPs against tested pathogens were in the range of 100 - 200 µg ml^{-1}. The MIC value of ZnO NPs against *E. coli* and *S. enterica* was 100 µg ml^{-1}. The bacterial strains, *S. aureus* and *V. parahaemolyticus* are less sensitive to ZnO NPs, MIC value of 200 µg ml^{-1} (Table 1).

Table 1 Antimicrobial activity of ZnO NPs synthesized by *A. schindleri* SIZ7.

Bacteria	Zone of inhibition (in mm)	MIC (µg ml^{-1})	MBC (µg ml^{-1})
S. aureus	16	200	400
E. coli	18	100	200
V. parahaemolyticus	16	200	400
S. enterica	20	100	200

DISCUSSION

Zinc oxide possesses antibacterial activity and it depends on the size of the particle (**Osamu Yamamoto, 2001**). ZnO NPs exhibits significant antimicrobial activity over a wide spectrum of bacteria and hold good biocompatibility with human cells (**Sirelkhatim *et al.*, 2015**). ZnO NPs can be used as antimicrobial agents in food or in the development of packaging materials to prevent the microbial contamination of food and to extend the shelf life of food (**Xie *et al.*, 2011**). **Sangeetha *et al.*, (2011)** reported the green synthesis of ZnO NPs by *Aloe barbadensis* miller leaf extract and its structure and optical properties were studied. **Nagarajan Sangeetha and Kumaraguru Arumugam Kuppusamy (2013)** demonstrated the extracellular synthesis of ZnO NPs using seaweed *S. myriocystum*. **Sarkar *et al.*, (2014)** reported the extracellular mycosynthesis of ZnO NPs by *Alternaria alternate*. Zinc oxide nanoparticles (ZnO NPs) were synthesized using bacteria, *Aeromonas hydrophila* and its antibacterial and antifungal activity was determined (**Jayaseelan *et al.*, 2012**). In the present study, *Acinetobacter schindleri* was employed for the extracellular synthesis of ZnO NPs.

The antibacterial potential of ZnO NPs against foodborne pathogens were evaluated and reported against *Salmonella typhimurium* and *Staphylococcus aureus* (**Tayel *et al.*, 2011**). Similarly in the present investigation antimicrobial activity of ZnO NPs against foodborne pathogens was reported. The result clearly indicates that *E. coli* and *S. enterica* are more sensitive to ZnO NPs. **Xie *et al.*,**

(2011) investigated antibacterial effect of zinc oxide (ZnO) nanoparticles on *Campylobacter jejuni* and suggested that the antibacterial mechanism of ZnO nanoparticles is most likely due to disruption of the cell membrane and oxidative stress in *Campylobacter*. Anticandidal activity of ZnO NPs against *C. albicans* is also correlated with reactive oxygen species (ROS) production (**Shoeb *et al.*, 2013**). The mechanism of the inhibitory effects of ZnO NPs on foodborne pathogens might be similar to previous reports.

Due to their potent antimicrobial activity against bacterial strains, researchers focused their investigations more on the synthesis and evaluation of mechanism of antimicrobial activity of ZnO NPs against an array of pathogenic bacteria.

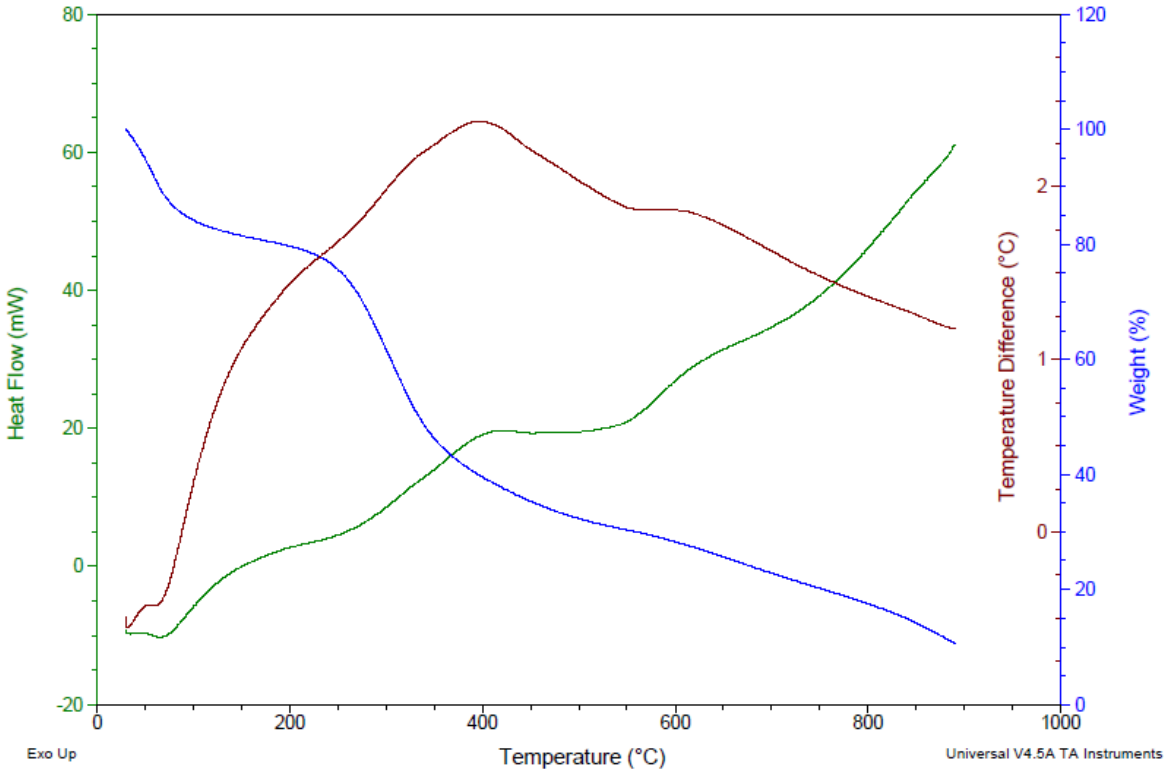

Figure 7 Thermogravitometric analysis of synthesized ZnO NPs

CONCLUSION

Antimicrobial ZnO NPs were produced with culture supernatant of *A. schindleri* SIZ7. The formation of ZnO NPs was observed, achieved after 48 h of incubation with culture supernatant. The ZnO NPs formed were spherical in shape and polydispersed with diameters of 20–100 nm. The properties of ZnO NPs were confirmed by HR-TEM, EDS, FTIR and TGA analysis. The ZnO NPs exhibited potential antimicrobial activity against foodborne pathogens, *E. coli* and *S. enterica* with a MIC of 100 µg ml^{-1}. The bacterial system, therefore, has the potential for low-cost and environmentally friendly production of antimicrobial ZnO NPs.

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