

Antibacterial Activity of Biogenic Zinc oxide Nanoparticles synthesised from *Enterococcus faecalis*

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Abstract: Zinc nanoparticles are known to be one of the multifunctional inorganic nanoparticles with effective antibacterial activity on both Gram negative and Gram positive bacteria. In the present research work, synthesis of zinc nanoparticles and its characterization was done. In this study, zinc nanoparticles were biologically synthesized from zinc sulfate solution using *Enterococcus faecalis* culture. The yellow color synthesized zinc nanoparticles were characterized by different spectroscopic and analytical techniques such as UV-Visible, Nanoparticle Tracking Analysis (NTA), Field emission Scanning Electron Microscopy (FeSEM) and Energy-dispersive X-ray (EDX). NTA and FeSEM analysis studies confirmed the formation of well-dispersed zinc nanoparticles with average particle size to be in the range of 16 to 96 nm as well as revealed their spherical structure. NTA analysis studies also confirmed the concentration of the particles in the range of 7.2×10^{10} particles/ml. These biologically synthesized nanoparticles were found to be highly toxic against different multi drug resistant human pathogens by using well diffusion method.

Key words: Zinc nanoparticles, *Enterococcus faecalis*, Zinc sulfate, Antibacterial activity.

Introduction and Experimental

Nanotechnology can be termed as the synthesis, characterization, exploration and application of nanosized (1-100nm) materials for the development of science. Research over the past few years has demonstrated that utilization of biological organisms has been emerging as a novel method for the synthesis metal nanoparticles, which can be preferred over the existing chemical and physical methods¹. Nanomaterials are being actively researched for specific functions such as microbial growth inhibition² as carriers of antibiotics and as killing agents³. Zinc oxide exhibits antibacterial activity that increases with decreasing particle size⁴. Zinc nanoparticles used as an additive for numerous materials and products including plastics, ceramics, glass, pigments, foods, etc. ZnO is nontoxic and it is a strong antibacterial agent. ZnO nanoparticles are used in industrial sectors including environmental, synthetic textiles and food packaging¹. Biosynthetic and environment friendly technology for the synthesis of ZnO nanoparticles are believed to be nontoxic, biosafe, and biocompatible and have been used as drug carriers, cosmetics, and fillings in medical materials⁵.

In the current study, zinc oxide nanoparticles were synthesized from *Enterococcus faecalis* and their antibacterial activity was checked against different multi drug resistant human pathogens.

Preparation of Bacterial free extract

Enterococcus faecalis were collected from Medical Biotechnology and Phage Therapy Laboratory (MBPT), Department of Biotechnology, Gulbarga University, Gulbarga. The culture was inoculated on Bile

esculin azide agar medium and incubated at 37°C for 24hrs for pure culture. Inoculate *Enterococcus faecalis* culture in Luria-Bertani broth, incubated at 37°C for 24hrs. After incubation time the culture were centrifuged at 10,000 rpm for 10 minute and their supernatant were used for zinc oxide nanoparticle synthesis.

Synthesis of Zinc oxide nanoparticles

Bacterial supernatant was added separately to the reaction vessel containing 100 mM Zinc sulfate solution (v/v) and control (without Zinc sulfate). The reaction was carried out in light conditions for 24 hours, at 37°C, pH: 7.4 in rotary shaker with 120 rpm.

Characterization of Zinc oxide nanoparticles:

Synthesized zinc oxide nanoparticles were characterized by visual observation and by different spectroscopic and analytical techniques. After 24 hours of incubation the reaction medium is collected and centrifuged at 10,000 rpm for 10 min, supernatant is further used for UV-Visible spectrum using T90+ UV-VIS spectrophotometer and for Nanoparticle Tracking Analyzer (NTA) pattern analysis, for UV-VIS spectrophotometer scanning the spectra between 300 to 600 nm at room temperature.

The size shape and composition of nanoparticles was analyzed using Field Emission Scanning Electron Microscopy (FeSEM), EDX and NTA analysis.

Antimicrobial activity of Zinc oxide nanoparticles by Agar well diffusion method:

The antimicrobial activity of zinc oxide nanoparticles was evaluated against *E. coli* (Clinical MDR pathogen) *Klebsiella pneumoniae* (Clinical MDR pathogen) and clinical pathogen Methicillin resistant *Staphylococcus aureus* (MRSA) by modification of the agar well diffusion method⁶. Clinically isolated pathogens were inoculated in Luria-Bertani broth and incubate at 37°C for 6hrs, 100µl of each microorganism were inoculated on Muller Hinton agar (MHA) plates; Agar wells of 5 mm diameter were prepared with the help of a sterilized stainless steel cork borer. Different concentrations (10, 20, 30, 40, 50 and 60 µl) of the nanoparticles and maintain zinc sulfate as control in another well, were loaded on marked wells with the help of micropipette under aseptic conditions and plates were incubated at 37°C for 18 and 24 hrs.

Results and Discussion:

Primary conformation of biosynthesis of Zinc oxide nanoparticles from *E. faecalis* was done by visual observation, the medium was characterized by the changes in color from yellowish white to bright yellow (shown in the Fig: 1). The characteristic surface Plasmon resonance of zinc nanoparticles due to excitation of surface Plasmon vibrations and this is responsible for the striking yellowish color of zinc oxide nanoparticles.

Spectral Analysis:

The characteristic surface plasmon resonance of zinc oxide nanoparticles ranges between 300 nm to 330 nm (shown in Fig: 2). Bright yellow color arises due to excitation of surface Plasmon vibrations in the zinc nanoparticles. The reduction of zinc was subjected to analysis by using the UV-Vis Spectrophotometer. Absorption spectra of Zn-NPs formed in the reaction media has absorbance peak at 310nm¹. Biological method of synthesis of nanoparticles exhibit strong absorption of electromagnetic waves in the visible range due to their optical resonant property, called Surface Plasmon Resonance (SPR) due to excitation of surface Plasmon vibrations. Sharp peak indicated that the particles are monodispersed in nature. Maximum absorbance is obtained at 310nm (OD: 3.87) for 24hrs of incubation of culture supernatant with 100 mM zinc sulfate in (1:1) volume.

Field Emission Scanning Electron Microscope (FeSEM):

Scanning Electron Microscope has been used to examine the surface morphology and to estimate the obtained structural rectangle, triangle, radial hexagonal, rod and spherical shapes. Typical SEM micrographs image of the ZnO nanoparticles size 96-110 nm obtained by the biosynthesis method⁷. FeSEM images of functionalized ZnO nanoparticles biosynthesized from *E. faecalis* can be seen with core shell morphology of size 16 – 96 nm and it is observed that there is a marginal variation in the particle size (Fig: 3) this result was quite similar to the result obtained from Nanoparticle Tracking Analyzer.

EDX analysis:

The EDX (energy-dispersive X-ray) shows the chemical composition or contaminants for the synthesized ZnO nanoparticles. The EDX spectrum recorded from one of the densely populated ZnO nanoparticles region. Strong signals from the Zn atoms in the nanoparticles were observed, and signals from O, K, P, S and Cl atoms were also recorded (Shown in Fig 4). The signals were likely due to X-ray emission from carbohydrates/ proteins/enzymes present in the cell wall of the biomass⁸.

Nanoparticle Tracking Analysis (NTA):

Nanoparticle Tracking Analysis is a newly developed method for the visualization and analysis of nanoparticles in liquid state. Through this analysis it's conformed that the zinc oxide nano sample is with particle size of 16 nm and monodispersed with the concentration of sample after dilution comes to 7.2×10^{10} particles/ml (Fig: 5).



Fig 1: Visual Observation

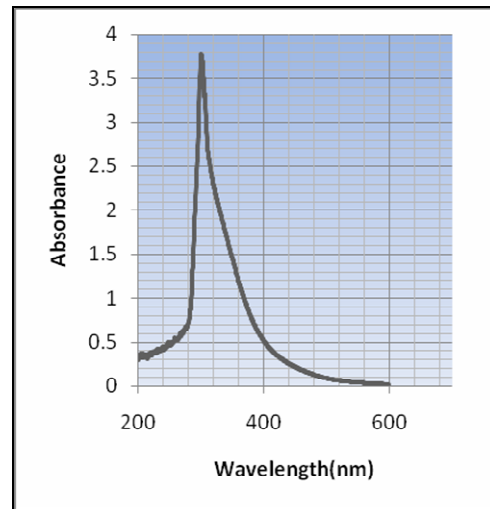


Fig 2: UV-visible spectrum (ZnO nanoparticles)

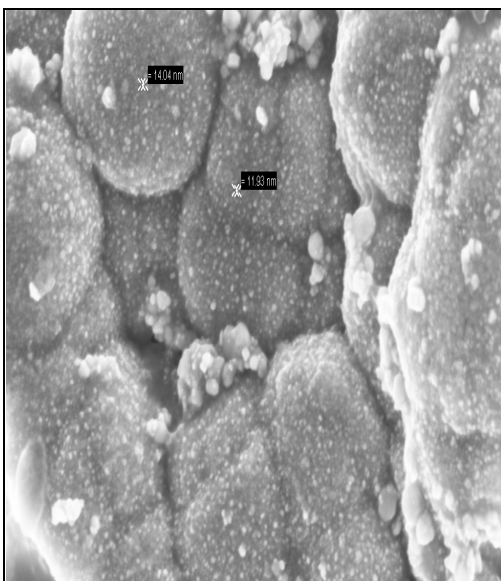


Fig: 3 FeSEM images of biosynthesized Zinc oxide nanoparticles from *Enterococcus fecalis*

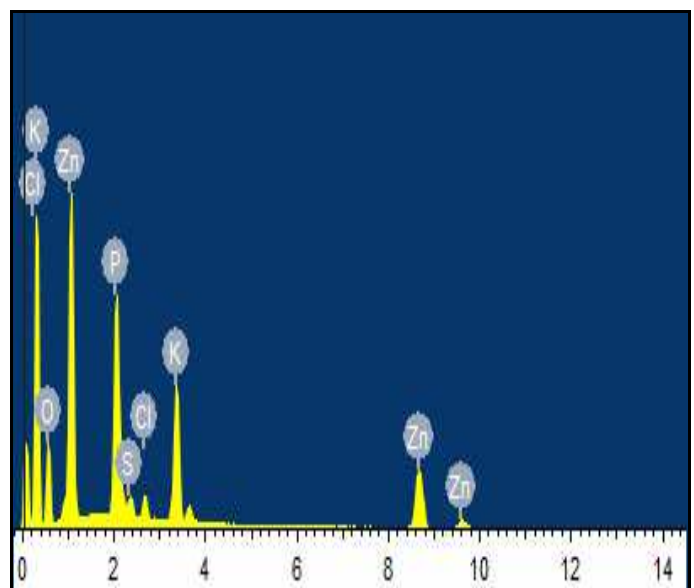


Fig: 4 EDX image of zinc oxide nanoparticle

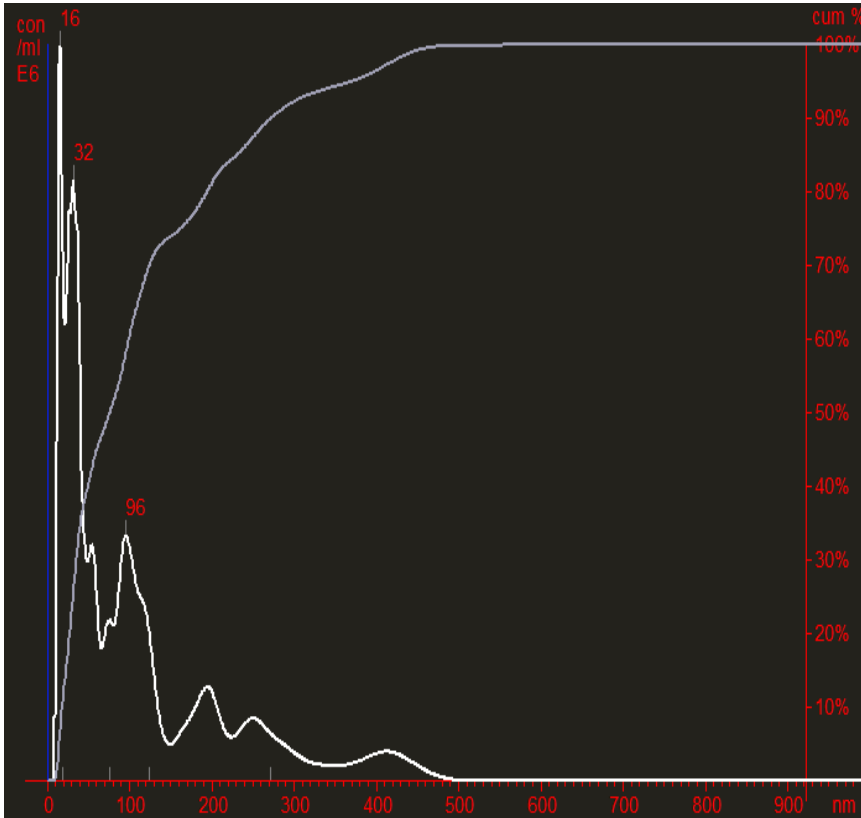


Fig 5: Particle Size / Concentration of zinc oxide nanoparticles

Antibacterial activity by Agar well diffusion method:

The antimicrobial activity of nanoparticles has been studied largely with human pathogenic bacteria, mainly *E.coli* and *S.aureus*⁹. According to Usha Rajamanickam *et al.*,2012 report Zinc nanoparticle synthesized from Actinomycetes showed greater activity against *S.aureus*. Introduction of ~13 nm ZnO NP kills gram negative *E. coli* at concentrations 3.4 mM, whereas growth of gram-positive *S. aureus* was prevented at much lower concentrations (1 mM)¹⁰. In the present work antibacterial activity of biosynthesized zinc oxide nanoparticles from *E. faecalis* also showed increased antibacterial activity against all the clinical pathogens. MDR isolates *E.coli* and *K. pneumonia* showed maximum zone of inhibition of 25mm and for *Staphylococcus aureus* 27mm at 60µl of zinc oxide nanoparticle concentrations (showed in Table: 1 and Fig: 6, 7 and 8).

In comparison study between antibiotics and zinc oxide nanoparticles on *E. coli* (with antibiotics Rifampicin, Pipracillin, Ceftazidime), *K. pneumonia* (with antibiotics Ceftazidime, Cephalexin, Ceftriaxone) and *Staphylococcus aureus* (with antibiotics Ampicillin, Methicillin, Pencillin) showed weakest antibacterial effect as compare to zinc oxide nanaoparticles. Results were shown in Table: 2.

Table 1: Zone of inhibition of Clinical MDR strains

Sl No	Clinical MDR strains	Zone of inhibition (mm)					
		10µl	20µl	30µl	40µl	50µl	60µl
1.	<i>E. coli</i>	-	-	14	16	18	25
2.	<i>K. pneumonia</i>	12	16	18	19	20	25
3.	<i>Staphylococcus aureus</i>	10	15	17	20	24	27

Control (100mM Zinc sulfate): No zone of clearance

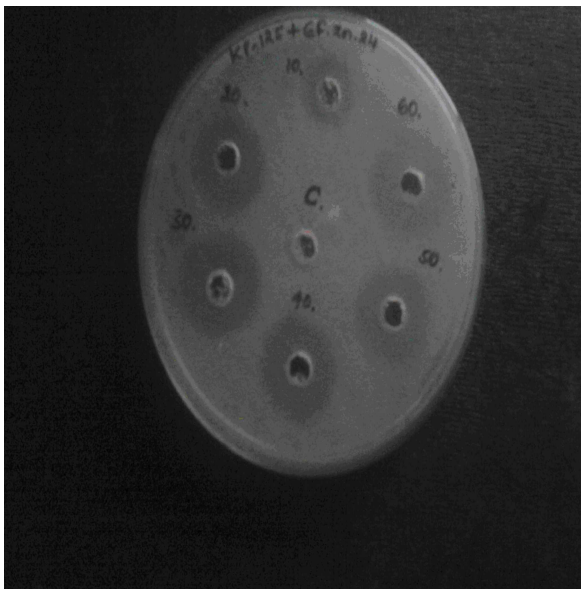


Fig 6: Zone of inhibition for *K. pneumonia*

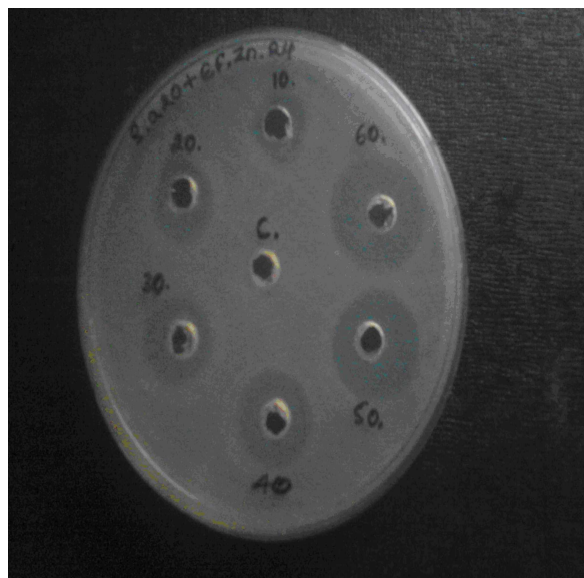


Fig 7: Zone of inhibition for MRSA



Fig 8: Zone of inhibition for *E. coli*

Table 2: Zone of inhibition of Zinc oxide nanoparticle and Antibiotics against Clinical MDR strains

Study no.	Clinical Pathogenic bacterial	Zone of Inhibition in mm		
		Antibiotics (mcg)		Zinc oxide nanoparticles (µl)
01	<i>E. coli</i>	Rifampicin	12	25 (60µl)
		Pipracillin	10	
		Ceftazidime	10	
02	<i>K. pneumonia</i>	Ceftazidime	-	25 (60µl)
		Cephalexin	-	
		Ceftriaxone	-	
03	<i>Staphylococcus aureus</i>	Ampicillin	12	27 (60µl)
		Methicillin	10	
		Pencillin	11	

Conclusion

The present biosynthesis of zinc oxide nanoparticles from *E. faecalis* is easy, ecofriendly and inexpensive method. This method has capable producing potential zinc oxide nanoparticles, have strong antibacterial activity against multidrug resistant human pathogenic bacteria's. Spectroscopic and analytical analysis confirmed the zinc oxide nanoparticles produced from biogenic route. The obtained ZnO nanoparticles had an average particle size ranges from 16 to 96nm with 7.2×10^{10} particles/ml of concentration. Thus this method proves the production of nanoparticles to be a potentially exciting tool for large-scale synthesis of nanoparticles. This clearly indicates if further research is done to evaluate the inhibitory activity of Zinc oxide nanoparticles against other pathogenic bacteria and fungi promising results could be obtained.

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