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Synthesis and characterization of zinc oxide nanoparticle by using *Aspergillus niger* and their antimicrobial activity

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Abstract
Green synthesis of nanoparticle gain huge popularity in recent times. Nanoparticle synthesis using microorganisms have numerous advantages over physical and chemical methods because they don't produce toxic by-products. The present study focused on the synthesis of zinc oxide nanoparticles (ZnO NPs) using the cell filtrate of *Aspergillus niger*, their characterization, and the evaluation of their antibacterial activity against pathogenic bacteria. The synthesis of ZnO NPs was confirmed using UV-VIS spectrophotometric analysis, where ZnO NPs showed a peak at 378 nm. The nanoparticles size was within the range of 50-80 nm, confirmed by particle size analysis, and the functional groups were determined using Fourier Transmission Infrared (FTIR) analysis. The synthesized nanoparticles were also tested for their antibacterial activity against four pathogenic bacteria (*E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus entericus*). It was observed that the nanoparticles were highly effective against *E. coli* while being least effective against *S. aureus*. As a result of this research, it is concluded that zinc oxide nanoparticles play an important role in healthcare, can be synthesized at a commercial scale at a very low cost using biological methods, avoiding the formation of toxic intermediates in chemical methods, and can be used for a variety of applications.

Keywords: Zinc oxide nanoparticles, UV-VIS, antibacterial activity, FTIR

1. Introduction

Nanotechnology is a domain of science and technology that deals with nanomaterials within the range of 1-100 nm. Nanoparticles can be synthesized using chemical, biological, and physical methods. Biological methods appear to be the most effective and environmentally friendly, as the other two methods involved toxic compounds that limited their applications (Kumar *et al.*, 2017; Kalpana *et al.*, 2018) [19, 7]. The biogenic synthesis of nanoparticles includes the use of plant materials and microorganisms. Microorganisms (bacteria, fungi, and yeast) are used for nanoparticle synthesis due to their ability to remediate under stressed conditions (Bhambure *et al.*, 2009) [1]. These agents provide an organic system for nanoparticle synthesis in which different organic components reduce ions to their elemental form and produce nanoparticles. Fungus is usually preferred over bacteria for synthesis because of their better ability to accumulate metal and their higher binding capacity. Several fungi have been used for nanoparticle synthesis. *Aspergillus niger* has been used for silver nanoparticles (Sangappa, & Thiagarajan, 2012) [14] and zinc oxide nanoparticles (Kalpana *et al.*, 2018; Pavani *et al.*, 2011) [7, 12].

Zinc is present in all body tissues, including skin, brain, bone, muscle, etc., as an essential trace element, and that zinc helps in various body functions such as the body's metabolism, the synthesis of bio-molecules, and other essential functions of body cells. At the nanoscale, zinc is more readily absorbed by the body. Zinc oxide nanoparticles (ZnO NPs) are one of the most synthesized nanoparticles because of their large surface area compared to their size and high catalytic properties. Nanotechnology also requires the employment of various advanced analytical instruments for the characterization of nanoparticles. The techniques that have been exploited to characterize the synthesized ZnO NPs include UV-VIS spectroscopy, particle size analysis, FTIR, XRD, DSC, SEM, etc. (Shamhari *et al.*, 2018) [15]. UV-VIS spectroscopy is widely used to examine the optical properties of nanoparticles (Talam *et al.*, 2012) [17], particle size analyzer is generally used to analyse the size of nanoparticles, and the FTIR spectra of the sample is recorded to identify the characteristic functional groups present on the surface of ZnO NPs (Mahamuni *et al.*, 2019) [11]. ZnO NPs have some significant features like chemical and physical stability, high catalysis, and effective antibacterial activity, which make them more significant and useful than other metal oxide nanoparticles. ZnO NPs have been known to have strong inhibitory and bactericidal effects and possess broad-spectrum antimicrobial activities (Yousef and Danial, 2012) [20]. The nano-sized ZnO enters the cell and interacts with the bacterial surface, causing distinct bactericidal mechanisms to emerge (Sirelkhatim *et al.*, 2015) [16]. ZnO NPs kill cells by producing ROS species that ultimately destroy the cell wall; hence they serve as good alternative for synthetic drugs and also used in packaging. ZnO NPs are most widely used in sunscreen because they can absorb UV light while being transparent to visible light (Kessler *et al.*, 2011; Iosub *et al.*, 2017) [8, 4] and

in UV protective materials such as textiles (Wang *et al.*, 2011) [18]. It is also used in food additives, as they considered safe by the US Food and Drug Administration. In comparison to other metal oxide NPs, ZnO NPs are more cost-effective and less reactive, and they exhibit a large number of biomedical applications such as anticancer, anti-inflammation, diabetes treatment, wound healing, bio-imaging, antibacterial, and drug delivery.

2. Materials and Methods

2.1 Microorganisms

Aspergillus niger (MTCC-584), *E. coli* (MTCC-16521), *Staphylococcus aureus* (MTCC-3160), *Salmonella enterica* (MTCC-660), and *Bacillus subtilis* (MTCC-441) were obtained from the fermentation and immuno-technology lab at the Department of Bio and Nano Technology, GJUS&T, Hisar. Fungi were used for the biosynthesis of silver nanoparticles, and bacteria were used for evaluating the antibacterial activity of fungal strain-based nanoparticles.

Fungi were sub-cultured in Potato Dextrose Broth (PDB) (HIMEDIA) and incubated at 28 °C for 24 hours. In order to prevent the bacterial growth, 50 mg/L chloramphenicol (Sigma-Aldrich, Germany) was added to the cultures. Bacteria were sub-cultured on Nutrient Agar (HIMEDIA) and incubated at 37 °C for 24 hours.

2.2 Biomass production of cell filtrate

The revived fungal strain of *Aspergillus niger* (MTCC No. 584) was inoculated in the potato dextrose broth. Then the broth was incubated at 28 °C for 96 hours at 150 rpm in an incubator shaker. The fungal biomass obtained after incubation was filtered through Whatmann filters paper No. 1 and washed with sterile water to remove any remaining media. Following washing, 25 g of fungal cells were inoculated in 100 ml of distilled water and incubated for 24 hours under the conditions described above. The fungal culture thus obtained was again filtered through Whatmann filter paper No. 1, and the cell filtrate was stored to be used for the green synthesis of ZnO NPs.

2.3 Biosynthesis of Zinc Oxide Nanoparticles

Zinc nitrate solutions of different concentrations, *i.e.*, 4.8mM, 5mM, and 5.2mM, were prepared, and each concentration of zinc nitrate was mixed with cell filtrate in a 1:1 ratio and the pH was adjusted to 6.5. The mixture was then stirred with a magnetic stirrer overnight at 40 °C. The appearance of a white precipitate in the solution indicated the synthesis of nanoparticles, which was later confirmed by UV-VIS analysis. The solution containing zinc oxide nanoparticles was centrifuged at 8000 rpm for 10 minutes. Then the pellet obtained was washed twice with distilled water and finally with ethanol. The dried particles were placed in a hot air oven for 2–3 hours at 60 °C. The dried nanoparticles were scrapped and collected for further studies.

2.4 Characterization of zinc oxide nanoparticles

The colour change in the solution after a particular period of incubation indicated the formation of ZnO NPs, which was later confirmed by UV-visible spectroscopy. UV-visible spectroscopy was used to assess their optical characteristics. Further, the PSA was utilized to estimate the size of biosynthesized nanoparticles. The FTIR analysis aids in determining the various functional groups present on the synthesised nanoparticles.

2.5 Antimicrobial activity of synthesized ZnO NPs

Antibacterial activity was assessed using four pathogenic strains: *E. coli* (MTCC-16521), *Staphylococcus aureus* (MTCC-3160), *Staphylococcus entericus* (MTCC-441), and *Bacillus subtilis* (MTCC-441). The revived culture of each strain was spread on nutrient agar plates. Then three wells were bored in each plate with a sterile borer, followed by sealing. After that, in one well, streptomycin is loaded as a control, and in the second well, cell filtrate is loaded, while in the third well, ZnO NP is loaded. The plates were incubated for 24 hours at 37 °C in an incubator for the appearance of a zone of inhibition.

3. Results and discussion

3.1 Biosynthesis of zinc oxide nanoparticle

The mixture of cell filtrate and zinc nitrate in a 1:1 ratio at pH 6.5 was stirred overnight at 40 °C with a magnetic stirrer. The appearance of a white precipitate confirms the synthesis of nanoparticles as shown in Fig. 1.

Fig 1: Appearance of white precipitates in the solution

3.2 Characterization of zinc oxide nanoparticle

3.2.1 UV-Visible Spectroscopy

After preliminary confirmation of the synthesis of ZnO NPs, the nanoparticles were further analyzed by UV-Visible spectroscopy, since it is the most practical tool for detecting the reduction of metal ions based on an optical feature known as Surface Plasmon Resonance (SPR). The formation of ZnO NPs is monitored by recording the absorption spectra in the range of 200-800 nm (Kumar *et al.*, 2013) [10]. ZnO NPs show an absorption peak in the wavelength range of 350-385 nm (Jamdagni *et al.*, 2018; Kalpana *et al*., 2018) [5, 7]. The biosynthesized nanoparticles showed an absorption peak at 378 nm, as shown in Fig. 2. The presence of a strong peak confirms a good concentration of nanoparticles in solution.

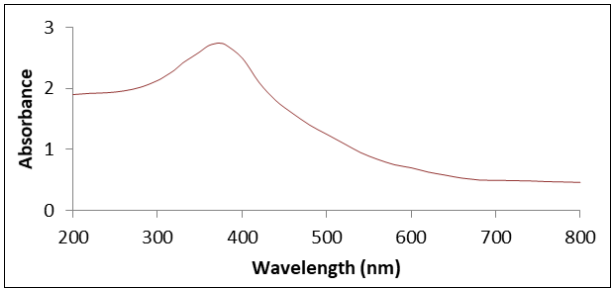

Fig 2: UV-VIS absorption spectrum of ZnO

3.2.2 Particle Size Analysis

The size of synthesized ZnO NPs was determined by PSA. The PSA analysis was done on the basis of light scattering and the size of nanoparticles analyzed was 50-80 nm. PSA analysis is done on the principle of quasi-electric light scattering. For PSA analysis, the aqueous solution of nanoparticles was diluted 100 times. For proper mixing, the samples were sonicated in a water bath sonicator for 30 minutes. The size of the particles was measured by dynamic light scattering, and a graph was recorded.

3.2.3 Zeta Potential

The assessment of zeta potential is an important characterization approach for nanoparticles for estimating surface charge, which may be used to determine the physical stability of nano-suspensions (Jiang *et al.*, 2009). Because of the electrostatic repulsion of individual particles, nanoparticles with a large positive or negative zeta potential imply good physical stability of nano-suspensions. A zeta potential value outside of -30 mV to +30 mV is generally regarded as having adequate repulsive power to achieve superior physical colloidal stability (Hunter, 2013). The zeta potential of the synthesized ZnO nanoparticles was -22 mV (Fig. 3), which indicated that their stability lie within the appropriate zeta potential range and implied that ZnO nanoparticles were negatively charged nanoparticles.

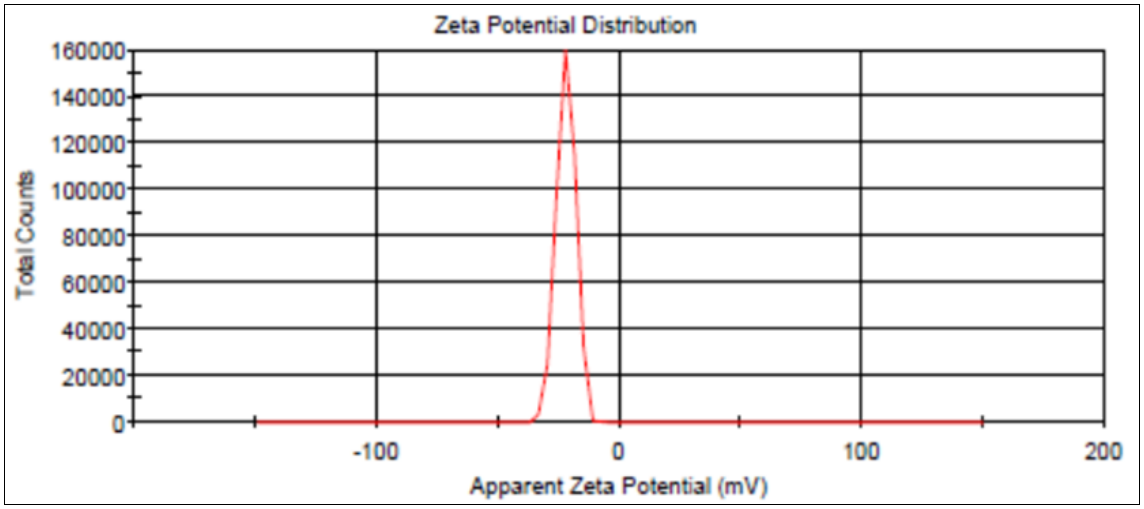

Fig 3: Zeta Potential analysis of ZnO NPs

3.3.4 FTIR Spectral Analysis

FTIR analysis was done to evaluate the functional groups' adsorption in the IR range, and it gives insight about rotational and vibrational movements for the identification and characterization of a material. In FTIR, different functional groups showed different absorption at different frequencies, which signifies the type of bonding present in a molecule (Jamdagni *et al.*, 2016) [5]. Fig. 4 depicts the FTIR spectral analysis of synthesized ZnO NPs. For the FTIR analysis of zinc oxide nanoparticles, the dried powder of nanoparticles was ground with potassium bromide to form the pellet, and the spectrum was recorded between the ranges 4000-400 cm^{-1} to examine the presence of different functional groups. The obtained data is converted into spectra using computers and algorithmic analysis.

The peaks appearing in the FTIR spectrum indicate different functional groups. The absorption peaks at 3393.60 cm^{-1}, 2928.64 cm^{-1}, 2366.36 cm^{-1}, 1647.06 cm^{-1}, 1320 cm^{-1}, and 819.58 cm^{-1}, representing the O-H stretch of alcohol, the C-H stretch of alkane, the O=C=O stretch of carbon dioxide, the C=C stretch of alkene, the C-H stretch of alkane (nitro compound), and the C-M stretch of an organo-metallic compound. The results obtained in this study were found to be very identical to the results of Kalpana *et al.* (2018) [7]. The presence of these functional groups makes the ZnO NP more effective, as described by Rajan *et al.* (2016) [13].

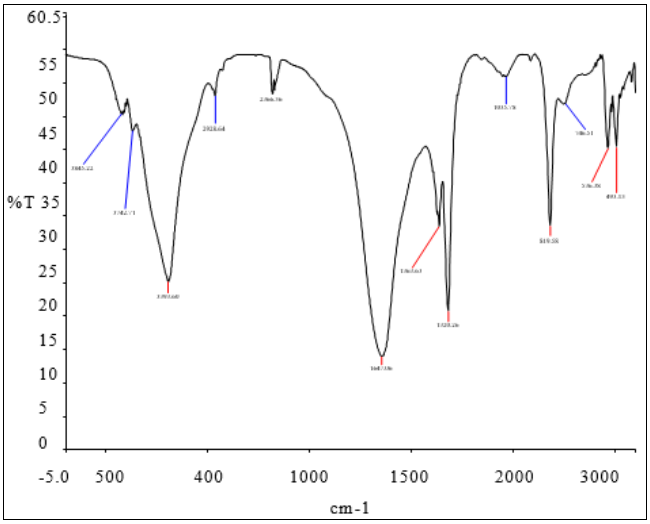

Fig 4: FTIR spectrum of zinc oxide nanoparticles

4. Evaluation of Anti-microbial activity of zinc oxide nanoparticles

ZnO-NPs have promising antibacterial capabilities due to their large surface area to particle size ratio, which leads to higher particle surface reactivity. ZnO is a biocompatible material that oxidises and catalyses chemical and biological

molecules (Sirelkhatim *et al.*, 2015) [16]. The synthesized nanoparticles were studied against different bacterial strains for antimicrobial activity using the agar well diffusion method. The zones of inhibition of nanoparticles were compared with those of a standard drug (streptomycin). The activity index and % inhibition were calculated using the following formula:

Activity index = mean zone of inhibition ÷ zone of inhibition of standard antibiotic

% Inhibition = Activity index x 100

5. Antibacterial Assay

The concentration dependent antibacterial activity of ZnO nanoparticles was tested against four pathogenic bacterial strains, *E. coli*, *Streptococcus aureus*, *Staphylococcus entericus, and Bacillus subtilis*, using the standard method of Xie *et al*. (2011) with slight modifications. The observed results of ZnO NP activity on bacterial strains are shown in Fig. 5. The zone of inhibition of ZnO NP against different strains is described in Table 1. The activity index and % inhibition are described in Table 2. The results of antibacterial activity were almost identical to the results of Ferris *et al.* (2010) [2].

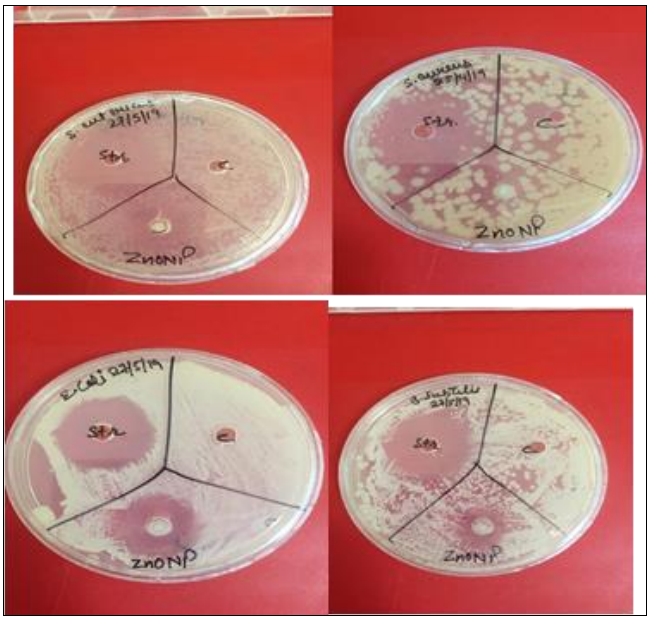

Fig 5: Antibacterial activity of zinc oxide nanoparticles against pathogenic bacterial strains

Table 1: Activity of ZnO nanoparticles against bacteria

Sr. No.	Pathogn	Strain	Zone of inhibition		
			ZnONP	Cell filterate	Drug (streptomycin)
1	*E. coli*	Gram-	7.5	0	10
2	*S. aureus*	Gram+	2.5	0	11
3	*S. entericus*	Gram+	4.5	0	10
4	*B. subtilis*	Gram+	2.7	0	10

Antibacterial activity of *Aspergillus niger* extract based ZnO nanoparticles was evaluated against four pathogenic bacterial strains namely: *E. coli*, *Staphylococcus aureus*, *Salmonella enterica*, and *Bacillus subtilis.* The maximum activity was observed against *E coli*; whereas minimum activity against *Staphylococcus aureus*.

Table 2: Activity index and % inhibition of ZnO nanoparticles against bacteria

Sr. No.	Pathogen	Activity index	%inhibition (in %)
1	*E. coli*	0.75	75
2	*S. aureus*	0.23	23
3	*S. entericus*	0.45	45
4	*B. subtilis*	0.27	27

6. Conclusion

The study of zinc oxide nanoparticles and its various aspects has increased because of their optical properties, high yield, cost-effectiveness, antimicrobial activity, and other applications. Biogenic syntheses of zinc oxide nanoparticles were preferred over chemical methods because biogenic synthesis results in the production of very few toxic substances. In this study, *Aspergillus niger* cell filtrate was used as a reducing and capping agent for the synthesis of ZnO NPs. The solution was incubated on a magnetic stirrer at 40 °C and 400-500 rpm, and the appearance of white precipitate provided primary confirmation of ZnO NPs synthesis, which was confirmed using additional analytical tools such as UV-visible spectroscopy, PSA, and FTIR analysis. The ZnO nanoparticles showed UV absorption at 378 nm, the PSA revealed that the size of the nanoparticles was in the range of 50–80 nm, and the chemical nature of the synthesized ZnO nanoparticles was studied using FTIR analysis. The synthesized ZnO NPs were tested against four bacterial strains (*E. coli*, *Staphylococcus aureus*, *Salmonella enterica*, *and Bacillus subtilis*). The maximum antibacterial activity was observed against *E. coli*, followed by *Salmonella enterica*, *Bacillus subtilis*, and the lowest activity was observed against *Staphylococcus aureus.* Hence, from this study, it is concluded that the ZnO NPs can be synthesized at a very low cost by biological method, avoiding the formation of toxic intermediates synthesized by the chemical method, and can be used in several different applications, including healthcare. Furthermore, the research can be extended towards examining the anticancer activity of synthesized ZnO NPs against cancer cell lines, which can be a major breakthrough in healthcare.

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